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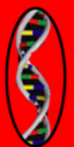
Cell Determination during Hematopoiesis

Geoffrey Brown ▪ Rhodri Ceredig
Editors



Cell Biology Research Progress Series

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CELL DETERMINATION DURING HEMATOPOIESIS

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Cell Determination during Hematopoiesis

Geoffrey Brown
and
Rhodri Ceredig
Editors

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From the Editors

We'd like to thank all authors for their tremendous efforts in producing chapters for this book which are comprehensive and state-of-the-art. For more than twenty years there was a degree of consensus about how we understand and depict the diversification of hematopoietic cells. However, the field seems to have arrived recently at an important turning point, and there is a plethora of new maps for hematopoiesis – a recent commentary said "*the latest research will necessitate revision of textbook accounts of the generation of blood cells*". As can be seen in this book, there is now a variety of subtly – and not so subtly – different views of the pathways that hematopoietic stem cells follow in order to generate each of the various types of blood cells. At present, the different maps and viewpoints can but sit alongside one other, allowing our readers to weigh up the experimental evidence for and against each one. Clearly there is still much exciting work still to be done both to arrive at a consensus and to fill in the many missing details.

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Foreword

When the Editors of this fine book kindly asked me to write a Foreword my first reaction was one of gloomy introspection. The word 'Hematopoiesis' reminded me that I have reached such an advanced age that my professional life has covered almost the entire period that is sometimes called 'hematology without the microscope', a time in the development of the field that encompasses the origins of so many of the remarkable advances that are described in this new work.

Blood was rarely mentioned when I was a medical student in the 1950's, and any hematology that was picked up along the way was largely self-taught. Since an extremely battered copy of my only teacher of the time, the 6th edition of Muir's Textbook of Pathology, published in 1951, is still on my shelves I decided to consult the section on hematopoiesis. The short and uninformative account dealt mainly with the controversy about the monophyletic and dualistic (or pluralistic) theories of the origin of blood cells, a discussion which left me no wiser then than it does today. Even as a raw medical student these arguments seemed more philosophical than physiological; surely, I thought, we all start out as a single cell.

The controversy about the origins of the cells of the blood dominated the work of the great morphologists from the end of the 19th until the middle of the 20th century. Paul Ehrlich, whose work on the morphological description of blood cells was so seminal, believed that, based on their appearances, the various cells of the blood arise from different and distinct progenitors. However, early in the 20th century other distinguished morphologists were attracted to the monophyletic theory. For the next 40 years there was a bitter schism between those who held this view and the dualists; their opposing ideas were the subject of the kind of vitriolic attacks on the work of their colleagues that would never be published in today's rather turgid scientific journals.

The scene finally changed in the late 1950's when it was found that animals can be protected against otherwise lethal doses of irradiation by grafting hematopoietic cells into irradiated recipients. It was later found that the latter develop macroscopic nodules in their spleens and that each nodule, or colony, originates from a single cell. Furthermore, using cells with marked chromosomes from donor animals it was confirmed that the same stem cell, termed a colony-forming unit in spleen, or CFU-S, could give rise to granulocytes,

monocytes, and erythrocytes. In the late 1960's the development of *in vitro* culture techniques, in which blood-cell development could be studied using conditioned media or humoral factors, made it possible to study the various phases of development of individual lineages. Work along these lines rapidly led to the current model of the existence of pluripotential stem cells and their commitment to different blood cell progenitors which follow distinct developmental pathways, and to the identification of some of the regulatory factors involved.

By the late 1970's, hematopoiesis, like many other branches of hematology, had reached a stage at which it was custom built for remarkable new developments in molecular and cell biology and cytogenetics. Over the next 30 years there was major progress in this field. Many of the genes involved in both normal and abnormal haematopoiesis were found and their regulatory regions defined. A variety of different transcription factors were purified and their genes also isolated, and at least some of their complex interactions at different stages of hematopoiesis were clarified. There were many valuable clinical consequences of this work, both for diagnosis and treatment of hematological disorders.

In the frenetic few years since the completion of the human genome project there have been further rapid developments in the technology for studying hematopoiesis, including proteomics and, of particular importance, methylomics and a variety of related techniques for analyzing the role of epigenetic factors in the complex issues of commitment and differentiation. While these reductionist approaches are continuing to throw up valuable information, like all fields of modern biology they are also uncovering layer upon layer of complexity. It may take the rest of this century, and the use of more holistic approaches arising from systems biology, before the complete story can be told.

The current model of hematopoiesis has recently been questioned again following some fascinating new discoveries. Indeed, it has been suggested that this new information may necessitate a complete revision of textbook accounts of the process of the generation of blood cells. For this reason, if no other, the editors of this fine book have done a considerable service in amassing such an excellent team of scientists to summarize some of the very recent developments in this exciting and fast moving field.

Having lived through this remarkable period in the development of hematology one cannot help wondering whether, in view of these very recent discoveries, we may be returning to a period of controversy similar to that which dogged the field for the first half of the 20th century. But at least we can rest assured that those involved will behave in a much more gentlemanly way should such a situation arise, at least in their published work.

D.J. Weatherall
Oxford
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Abbreviations

Ab	antibody
ACH	active chromatin hub
Aire	autoimmune regulator
AML	acute myeloid leukemia
AP-1	activating protein 1 (dimers of the Jun and Fos families of proteins)
5-Aza-C	5-azacytidine
B	B lymphocyte
bHLH	basic helix-loop-helix
BM	bone marrow
BMP	bone morphogenic proteins
C	cortex
cDC	conventional DC
CDP	CCAAT displacement protein
C/EBP	CCAAT/enhancer binding protein
CFU-S	colony forming unit-spleen
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CP	cryptopatch
cTEC	cortical thymic epithelial cells
DC	dendritic cell
DMR	differentially methylated regions
DN1	double negative-1 stage of thymocyte development, also called early thymocyte progenitors
DN2	double negative-2 stage of thymocyte development
DN3	double negative-3 stage of thymocyte development
DNMT	DNA methyltransferase
DP	double positive
DR	direct repeat
E	erythrocyte
E	embryonic day
EBF-1	Early B Cell Factor (also called EBF or Olf-1)
ECP	eosinophil cationic protein
EDAG	Erythroid Differentiation-Associated Gene
EDN	eosinophil-derived neurotoxin
EKLF	Erythroid Krüppel-Like Factor
ELP	early lymphoid progenitor
EMP	erythroid-myeloid progenitor
EPLM	early progenitors with lymphoid and myeloid potential
EPO	erythropoietin
Erk	extracellular-signal regulated kinase
ES	embryonic stem cells
ETP	early thymocyte progenitors, also called double negative-1 (DN1) cells

FACS fluorescence-activated cell sorting
FcεR immunoglobulin E receptor
FcγR immunoglobulin G receptor
FGF ibroblast growth factor
FL fetal liver
Flt-3 FMS-like tyrosine kinase-3 receptor
FOG-1 friend of GATTA-1

G granulocyte
G-CSF granulocyte colony-stimulating factor
GM macrophage/granulocyte restricted bipotent progenitors
GM-CSF granulocyte-macrophage colony-stimulating factor
GMP granulocyte and monocyte progenitor
GSK-3 glycogen synthase kinase-3

HAT histone acetyltransferases
HDAC histone deacetylase
HDM histone demethylase
HEV high endothelial venule
HLH helix-loop-helix
HMT histone methyltransferase
HOX homeobox
HRE hormone response element
HSC hematopoietic stem cells

ICAM-1 intercellular adhesion molecule 1
ICR imprinting control region
IFNα interferone α
IGF Insulin-like growth factor
Ikzf1 the Ikaros gene
IL interleukin
ILF isolated lymphoid follicle
IL-7R interleukin-7 receptor
iNK immature natural killer cell
iNOS inducible nitric oxide synthase
IP inverted palindrome
iPS induced pluripotent stem cell
IRF interferon regulatory factor
ITIM immunotyrosine-based inhibitory motifs

JAK Janus kinase
JNK Jun N-terminal kinase

K cytokeratins
KLF4 Kruppel-like factor 4
KSR1 kinase suppressor of Ras1

LC Langerhans cell
LCR locus control region
LMP lymphoid-myeloid progenitor
LMPP lymphoid-primed multipotent progenitor
LN lymph node
LPS lipopolysaccharide
LSK lineage^{-} sca-1^{+} kit^{+} cells
LT lymphotoxin
LTβR lymphotoxin β receptor
LTi lymphoid tissue inducer cell
LT-HSCs long-term reconstituting HSCs

M macrophage
MAdCAM-1 mucosal vascular addressin cell adhesion molecule 1
MAPK mitogen activated protein kinases
Mb mega bases
MBP major basic protein
M-CSF macrophage colony-stimulating factor
MEF murine embryonic fibroblasts
Meg megakaryocyte
Meg/E megakaryocyte/erythroid restricted bipotent progenitors
MEL murine erythroleukemia cells
MEP megakaryocyte and erythrocyte progenitor
MHC major histocompatibility complex
miRNAs microRNAs
mNK mature natural killer cell
MPO myeloperoxidase
MPP multipotent progenitor

MPS mononuclear phagocyte system
MSE monocyte specific esterase ("non-specific" esterase)
mTEC medullary thymic epithelial cell

nc-RNAs non-coding RNAs
NCC neural crest derived mesenchyme
NFI-A nuclear factor I/A
NFκB nuclear factor of κB
NIK NFκB-inducing kinase
NK natural killer cell
NKP natural killer cell precursor
NOD non-obese diabetic
NuRD nucleosome remodeling deacetylase

pDC plasmacytoid dendritic cell
piRNAs Piwi-interacting RNAs
PI3K phosphatidylinositol 3-kinase
PLZF promyelocytic leukemia zinc finger
PP Peyer's patch
pre-DC precursors of dendritic cells
pre-cDCs pre-conventional dendritic cells
pTEC progenitor of thymic epithelial cells

R receptor
RA all-*trans* retinoic acid
RANK receptor activator of nuclear factor κB
RANKL receptor activator of nuclear factor κB ligand
RAR retinoic acid receptor
Rb retinoblastoma protein
RORγt retinoic-acid-receptor-related orphan receptor-γt
RTOC reaggregate thymus organ culture

S1P1 sphingosine-1-phosphate receptor 1
SAP signaling lymphocyte associated molecule-associated protein
SCF stem-cell factor
SCID severe combined immunodeficient
SD sequential determination model of hematopiesis
Sfpi1 the gene encoding PU.1
SLAM signaling lymphocyte activation molecule
SLO secondary lymphoid organ
SP Single positive
STAT signal transducer and activator of transcription
ST-HSCs short-term reconstituting HSCs

T T lymphocyte
TCR T cell receptor
TdT terminal deoxtransferase
TEC thymic epithelial cells
TF transcription factor
TGFβ transforming growth factor β
Th2 T helper cell differentiation type 2
Tip DC inducible nitric oxide synthase producing dendritic cell
TNF tumor necrosis factor
TNFRSF tumor necrosis factor receptor super-family
TPO thrombopoietin
TRA tissue-restricted antigen
TRAF-6 tumor necrosis factor receptor associated factor 6
TRANCE tumor necrosis factor -related activation-induced cytokine
TRANCER tumor necrosis factor -related activation-induced cytokine receptor
Treg T regulatory cells
TSA trichostatin A
TSP thymus-settling progenitors

VCAM-1 vascular cell adhesion molecule 1
VDR vitamin D receptor
XLP X-linked lymphoproliferative disease

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Chapter 1

Gene Regulatory Networks Directing Cell Fates within the Hematopoietic System*

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Abstract

We review a new developmental framework for the hierarchical relationships among the various lineages of the hematopoietic system in the context of the underlying gene regulatory networks that orchestrate distinct cell fates. These regulatory networks or circuits comprise of transcription factors, chromatin modifying complexes and miRNAs. Molecular principles and mechanisms of action underlying the functions of such regulatory molecules in the activation and repression of alternate lineage gene expression programs are emphasized. Finally, we discuss mathematical models that can be used to gain an analytical appreciation of the architectures of gene regulatory networks and their developmental dynamics.

** Correspondence to: Prof. Harinder Singh, Department of Molecular Genetics and Cell Biology, Howard Hughes Medical Institute, The University of Chicago, Chicago, IL USA 60637

Introduction

The hematopoietic system represents a powerful model for exploring gene regulatory networks (molecular circuits) that enable a stem cell to generate diverse blood and immune cell types including erythrocytes, megakaryocytes, granulocytes, macrophages, dendritic cells, natural killer cells, and B and T lymphocytes. Self-renewing multipotent hematopoietic stem cells (HSC) enable the life-long production of blood and immune cells. The relatively quiescent HSC generates a series of hierarchically organized progenitors that undergo extensive proliferation as they progressively restrict their developmental potential and induce the expression of lineage-specific genes eventually culminating in the generation of mature functional progeny. The various intermediates through which multipotent hematopoietic progenitors give rise to lineage-restricted precursors have been delineated by prospective isolation using flow cytometry and determining lineage potential by transplantation in vivo or culture in vitro. Such analyses have led to the formulation of differing lineage relationships within the hematopoietic system (see below). Nevertheless, these lineage relationships can provide clues about the existence of regulatory factors that may antagonize each other's actions to direct alternative cell fates. They can be also used to probe the evolutionary origins of more recently emerged lineages such as the B and T lymphocytes of the adaptive immune system. Molecular analyses have paved the way for the assembly of gene regulatory networks that direct various cell fate choices within the hematopoietic system. These networks comprise of transcription factors, chromatin-modifying complexes, regulatory micro-RNAs (miRNAs) and developmental signaling pathways.

In this chapter, we will review our current understanding of the molecular circuits and principles that govern the specification of distinct cell fates within the hematopoietic system. We will particularly focus on increasing evidence suggesting that both innate (myeloid lineages) and adaptive (lymphoid lineages) cells of the immune system can arise from a shared lymphoid-primed multipotent progenitor (LMPP). Based on specific examples, we will attempt to highlight the architecture of the gene regulatory networks underlying cell fate determination and the molecular mechanisms used to drive lineage-specific patterns of gene expression. Finally, we will discuss the use of mathematical modeling to develop an analytical perspective of these developmental circuits.

The Classical Developmental Model of the Hematopoietic System

Historically, hematopoiesis had been viewed as a purely stochastic process by which lineage restriction of progenitors occurred randomly (1). This view began to change with the prospective isolation of intermediates based on surface markers and analysis of developmental potential using various in vitro and transplantation assays. Such analyses uncovered a hierarchical architecture underlying the hematopoietic system. Notably, during the past decade, the Weissman laboratory pursued this experimental paradigm to isolate and characterize a series of developmental intermediates including long-term and short-term HSC

(denoted LT- and ST-HSC), multipotent progenitors (MPPs) as well as a series of progenitors with more restricted differentiation potentials such as (i) the common myeloid progenitors (CMP) (2) and (ii) the common lymphoid progenitors (CLP) (3), (iii) the myelomonocytic progenitors (GMP) and (iv) the megakaryocyte/erythrocyte progenitors (MEP) (2).

Analysis of the developmental potential of these discrete developmental intermediates led to a consolidation of the classical model of hematopoiesis (Figure 1A). Importantly, this classical model places the multipotent progenitor MPP at a pivotal juncture. MPPs retain multi-lineage potential but can no longer self-renew and therefore effect long-term reconstitution of the hematopoietic system upon transplantation in lethally irradiated hosts. The classical model invokes an initial segregation of the erythro-myeloid and lymphoid developmental potentials. Supported by the isolation of CMPs and CLPs, this view is also appealing from the standpoint that B and T lymphoid cells undergo an exceptional developmental process involving assembly of their antigen receptor genes through somatic DNA rearrangements. This enables the adaptive cells of the immune system (B and T cells) to express vast repertoires of antigen receptors unlike their innate cell (myeloid) counterparts. This model was supported by molecular analysis of gene expression patterns in these progenitors at a single cell level. HSC and multipotent progenitors were shown to be "primed" for multi-lineage gene expression programs (4). The subsequent progenitor subsets revealed more restricted patterns of gene expression that appeared to be consistent with their progressively restricted developmental potentials. For example HSCs, MPPs, and CMPs have been shown to co-express granulo-monocytic and erythro-megakaryocytic genes, but this mixed lineage pattern is resolved in the downstream progenitors, GMPs and MEPs (5). Multi-lineage gene expression patterns may represent the establishment of transcriptional poised states of alternate lineage genes via chromatin modifications. Alternatively, these patterns could reflect oscillatory fluctuating states of competing genetic programs within a single progenitor.

Although the classical model has proven to be valuable in the analysis of genes that regulate hematopoiesis, it could not be easily reconciled with some of the experimental data (6). Analysis of fetal hematopoiesis in mutant embryos lacking the transcription factor PU.1 revealed a multi-lineage defect in the generation of lymphocytic (B and T), monocytic and granulocytic progenitors, while erythropoiesis and megakaryopoiesis was unaffected (7). This result, since confirmed in adult hematopoiesis (8) supported the existence of a common intermediate that generates both innate (granulocytic and macrophage) and adaptive (B and T-lymphoid) cells. Consistent with this view, several reports have highlighted the close developmental relationship between cells of the innate and adaptive immune system through the isolation of B/macrophage progenitors (9) or the analysis of mixed-lineage (myeloid/B cell) leukemias. The classical model of hematopoiesis has also been challenged from the standpoint that the CLP may not represent a physiologically relevant intermediate for the generation of T cells in the thymus. A recent study has demonstrated that T-lineage precursors can arise independently of CLPs (10) and that the earliest thymic progenitors (ETP) maintain granulocytic-macrophage potential (11, 12). Taken together, this experimental evidence challenges the central role of CLPs as key intermediates in the development of lymphocytes and instead argues for the existence a shared progenitor that gives rise to all lineages of the immune system (innate as well as adaptive).

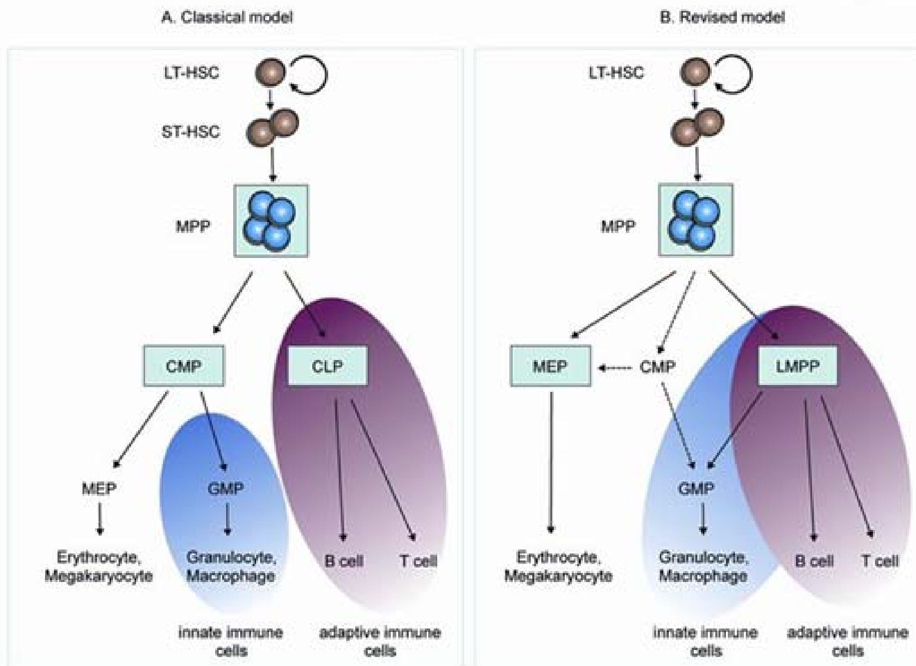

Figure 1. Classical and revised models of hematopoiesis depicting the various intermediates and lineage relationships.

Although the classical model has proven to be valuable in the analysis of genes that regulate hematopoiesis, it could not be easily reconciled with some of the experimental data (6). Analysis of fetal hematopoiesis in mutant embryos lacking the transcription factor PU.1 revealed a multi-lineage defect in the generation of lymphocytic (B and T), monocytic and granulocytic progenitors, while erythropoiesis and megakaryopoiesis was unaffected (7). This result, since confirmed in adult hematopoiesis (8) supported the existence of a common intermediate that generates both innate (granulocytic and macrophage) and adaptive (B and T-lymphoid) cells. Consistent with this view, several reports have highlighted the close developmental relationship between cells of the innate and adaptive immune system through the isolation of B/macrophage progenitors (9) or the analysis of mixed-lineage (myeloid/B cell) leukemias. The classical model of hematopoiesis has also been challenged from the standpoint that the CLP may not represent a physiologically relevant intermediate for the generation of T cells in the thymus. A recent study has demonstrated that T-lineage precursors can arise independently of CLPs (10) and that the earliest thymic progenitors (ETP) maintain granulocytic-macrophage potential (11, 12). Taken together, this experimental evidence challenges the central role of CLPs as key intermediates in the development of lymphocytes and instead argues for the existence a shared progenitor that gives rise to all lineages of the immune system (innate as well as adaptive).

Towards a Revised Model of the Hematopoietic System

Using mice carrying a GFP gene knocked into the Rag1 locus, the Kincade group demonstrated that specification of lymphoid cell fates is initiated earlier in the developmental hierarchy than proposed by the classical model of hematopoiesis (13). RAG1^{+} cells isolated in the Lin^{-} c-Kit^{hi} Sca1^{hi} (LSK) compartment (denoted ELP) displayed a strong lymphoid differentiation potential and residual granulocytic- macrophage potential. Based on Flt3 receptor expression, Jacobsen and colleagues reported that the MPP compartment can be divided into a subset of erythro-megakaryocytic progenitors (Flt3^{-}) and a subset of lympho-myeloid progenitors (Flt3^{+}, denoted LMPP) (14). Although the functional characterization of the LMPP population has been challenged (15) subsequent sub-fractioning of this population based on VCAM expression appears to confirm the existence of a progenitor exhibiting a lympho-myeloid developmental potential (16). These results have led to a revised proposal for lineage relationships in hematopoiesis (Figure 1B).

The revised model envisions an initial segregation between erythro-myeloid and lympho-myeloid lineages within the multipotent progenitor compartment (MPP). The transcription factors PU.1 and GATA1 appear to play key roles in establishing this developmental bifurcation. PU.1 and GATA1 have been shown to physically interact and functionally antagonize one another in the context of regulating erythroid or myeloid target genes (17, 18). Analysis of mice deficient for the chromatin remodeler Mi-2? β also suggests a function for this factor at this developmental juncture (19). Loss of Mi-2?β perturbs the HSC compartment and results in an increase in the generation of erythroid precursors at the expense of the other lineages. Interplay between Mi-2β? and GATA1 could explain this phenotype (20). Alternatively, Mi-2β? could interact with Ikaros transcription factors to repress erythroid developmental potential and promote lympho-myeloid fates (21, 22). Finally the bHLH transcription factor E2A also participates in this developmental bifurcation as it promotes the generation of LMPPs from HSCs (23). The revised model for hematopoiesis proposes that the innate (myeloid) and adaptive (lymphoid) lineages of the immune system arise from a common progenitor. Based on this developmental relationship, we predict that these lineages will share not only transcription factors such as PU.1 but deploy common regulatory circuits to control their differentiation.

Alternate Lineage Restriction and Cell Fate Specification

From a molecular standpoint, the restriction of developmental potentials and the induction of lineage-specific gene expression programs (cell fate specification) are two intertwined and progressive processes. These processes have been thoroughly analyzed in the context of B cell development. In this lineage, cell fate specification is initiated within the LMPP by the induction of genes such as Rag1 and Dntt (13, 24) by the transcription factors Ikaros and E2A (22, 23, 25). The subsequent induction of a B cell specific program of gene

expression is associated with a progressive repression of alternate lineage genes. This gradual process imposes a B-lymphoid "bias" rather than true cell fate commitment as these early stages of development retain residual granulocytic-macrophage potentials (26). Molecularly, this process of restriction seems to require the sequential and concerted action of several transcription factors including Ikaros, E2A, EBF and Pax5 (23, 25, 27, 28). Ultimately, this process culminates at the pro-B cell stage where the B cell fate is sealed by the self-reinforcing loop between EBF and Pax5 (27). The same progressive process appears to occur during neutrophil and macrophage cell fate specification controlled respectively by the primary determinants PU.1 and C/EBP? α (29).

Cell Cycle Regulation, Multi-Lineage Priming and Differentiation of HSC

In the HSC compartment, the decision to self-renew or differentiate is intimately coupled to the regulators of the cell cycle. Several reports have demonstrated the functional role of cell-cycle regulators such as p16/Ink4A, p21cip1/waf1, c-Myc and PTEN in controlling self-renewal of HSC versus their differentiation (30, 31). In most cases, it is assumed that molecules involved in the establishment of quiescence are required for self-renewal whereas those promoting entry into the cell cycle and division with differentiation. However, it is important to note that the molecular mechanisms underlying HSC self-renewal or differentiation remain largely obscure. Key transcription factors regulating multi-lineage "priming" can also influence the cell fate dynamics in the HSC compartment. Interestingly, disruption of this priming phenomenon induces opposite consequences within HSCs. PU.1 appears to be required for the stem cell function while C/EBP? α favors differentiation, in that loss of the former reduces HSC number whereas loss of the latter results in an expansion of HSC (8, 32). These results suggest that key transcriptional regulators dictating cell fate choices within the hematopoietic system are also important in controlling self-renewal dynamics of HSC.

Molecular Principles Underlying Cell Fate Determination

The concentration or activity states of key transcription factors appear to play an important role in cell fate determination within the hematopoietic system. Graded levels of PU.1 have been shown to regulate B versus macrophage development, as a low concentration of PU.1 induces B cell development while a 4-5 fold higher concentration drives macrophage differentiation and actively blocks B cell development (33). Similarly, in the B lineage, coordination of isotype switching with plasma cell differentiation is controlled by the kinetics of accumulation and the graded expression of the transcription factor IRF-4 (34). The molecular bases of such actions of transcription factors that involve concentration dependent

regulation of alternate cell fate choice or discrete developmental transitions remain to be elucidated.

As discussed above multi-potent cells exhibit low levels of mixed-lineage gene expression patterns (4, 5). Therefore, cell fate determination involves the resolution of mixed-lineage gene expression patterns by the concerted activation as well as repression of subsets of lineage-specific genes. This likely involves recurring use of counter-acting pairs of transcription factors that can activate as well as repress alternate lineage programs of gene expression (Figure 2).

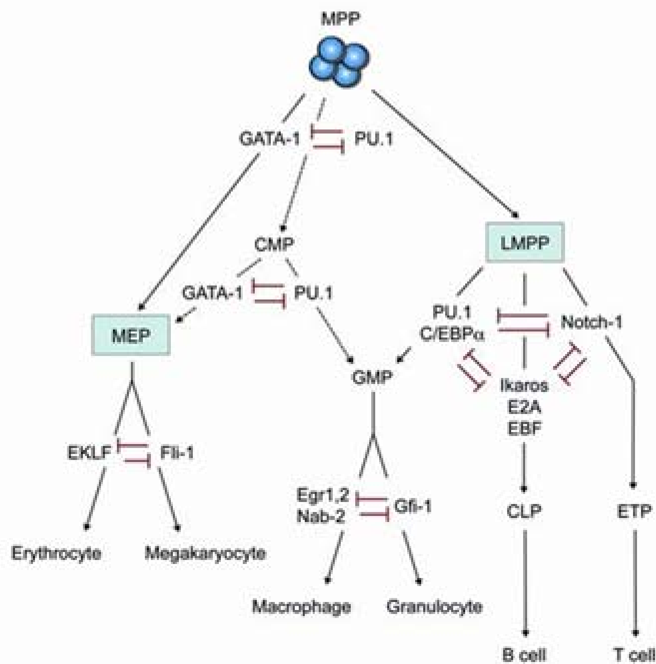

Figure 2. The revised model of hematopoiesis depicting counteracting pairs of transcription factors underlying various binary cell fate choices.

Competition between transcription factors that regulate alternate cell fates has been proposed as a molecular mechanism for initiating and resolving mixed lineage states based on studies demonstrating the antagonism between PU.1 and GATA-1 (35). This model was initially based on the physical interaction between PU.1 and GATA-1 and their ability to inhibit each other's transcriptional activation properties (17, 18). Such antagonistic interplay is thought to be responsible for the segregation between lympho-myeloid and erythro-megakaryocytic developmental potentials (see above and Figure 2). Paradoxically, we note that the PU.1 and GATA proteins can also function in a synergistic manner to regulate cell fate choice. Whereas, PU.1 and GATA-2 function in an antagonistic manner to regulate macrophage development, they function synergistically to specify the mast cell fate (36).

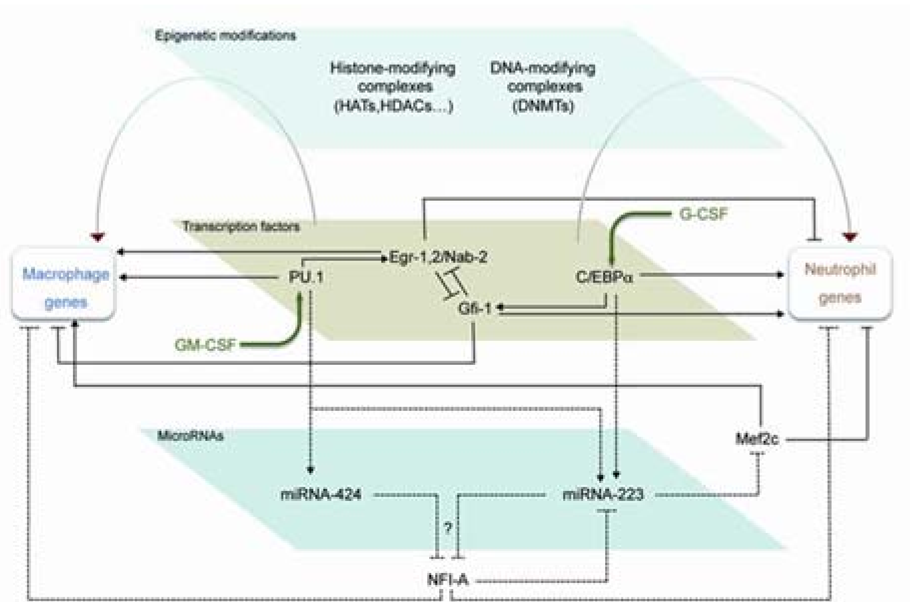

Figure 3. A gene regulatory network regulating macrophage versus neutrophil cell fate choice. The three planes represent different components of the network and comprise transcription factors, miRNAs and chromatin modifying complexes. Signaling inputs into the network by the cytokines G-CSF and GM-CSF are indicated. Positive and negative regulatory inputs in the network are indicated by arrowheads or T-junctions respectively. Solid lines depict regulatory interactions that have been experimentally demonstrated from a molecular standpoint.

The relative order of induction of shared transcription factors is another key principle underlying cell fate determination. This principle has been illustrated by the analysis of C/EBP? α and GATA-2 in the context of generation of eosinophils versus basophils (37) and C/EBP? α and PU.1 in the cell fate choice involving neutrophil versus macrophage differentiation (29). In both cases, the relative order of induction of the two lineage determining transcription factors has been shown to regulate a binary cell fate choice. Thus induction of PU.1 before C/EBP? α promotes macrophage differentiation from a bi-potential GMP whereas the induction of C/EBP? α before PU.1 promotes neutrophil development (29, 38). This analysis has been extended by uncovering a novel regulatory sub-circuit comprised of counter antagonistic repressors (secondary determinants) Egr-1,2/Nab-2 and Gfi-1 (29). In turn, this has enabled the assembly and mathematical modeling of a simple gene regulatory network whose architectural features are likely to be widely shared (Figure 3). In support of this contention the adoption of erythroid versus megakaryocytic cell fate by MEPs involves GATA-1 as a primary determinant and the transcription factors EKLF and Fli-1 as a pair of counter-acting secondary determinants (39).

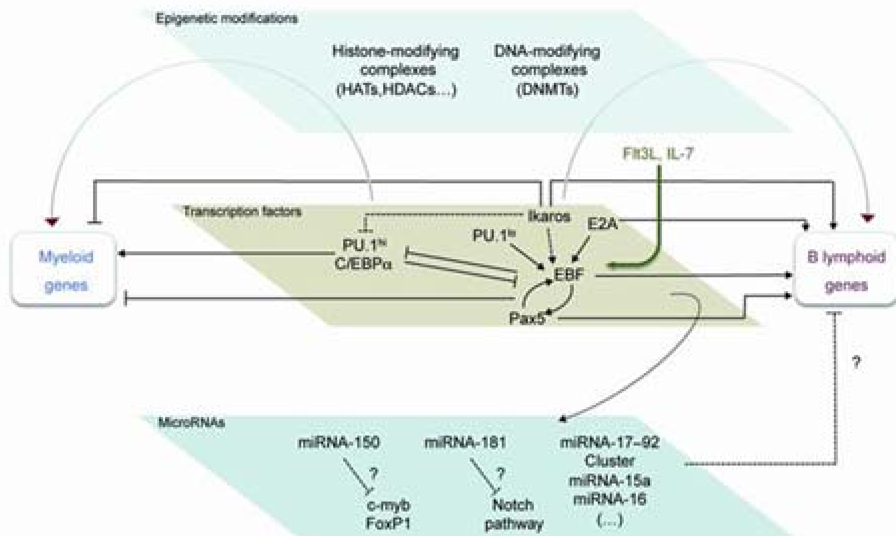

Figure 4. A gene regulatory network regulating B lymphoid versus myeloid cell fate choice. Signaling inputs into the network by the cytokines Flt3L and IL-7 are indicated. See legend to Figure 3 for additional details.

Cell fate determination involves self-reinforcing feed forward and feedback regulatory loops. Furthermore, the induction of lineage-specific programs of gene expression is brought about by the coordinated actions of multiple transcription factors. These aspects of cell fate determination have also been particularly well characterized in the context of B cell development. During B cell fate specification, multiple regulatory inputs such as PU.1^{lo}, E2A, Ikaros, Flt3L and IL-7R? are able to induce the initial expression of the transcription factor EBF that is a primary B cell fate determinant (Figure 4). EBF then induces the transcription factor Pax5, a secondary B cell fate determinant that feeds-back to sustain the expression of the EBF gene (40). This regulatory network leads to the generation of a self-reinforcing loop between EBF and Pax5, and enables commitment to the B cell fate (27). An important feature of this network is the cooperative or synergistic interplay of the fore-mentioned transcription factors in the activation of lineage-specific genes. The mb-1 gene in B-lineage cells has been used as a model system to analyze the molecular mechanisms that govern the expression of cell type specific genes. The mb-1 gene encodes for Ig? α (CD79a), a protein that is required for the assembly and signaling functions of the pre-B and B cell receptors. The developmental activation of this gene is regulated by the sequential and concerted action of multiple transcription factors. EBF and E2A collaborate with a non-B-cell specific transcription factor Runx1 to initiate mb-1 gene activation by targeting epigenetic modifications including DNA de-methylation at the promoter (41). Subsequent to these DNA and chromatin modifications Pax5 along with Ets proteins bind to the promoter to activate the mb-1 gene. Such sequential and concerted interplay of multiple transcription is thought to be a general mechanism that likely underlies the activation of additional B-lineage specific genes such as those encoding the surrogate light chains ?λ5 and VpreB (42).

Micro-RNAnas: New Players Involved in Cell Fate Determination

Micro-RNA molecules (miRNAs) are a new class of non-coding RNAs. They constitute a recently discovered group of regulators that can control gene expression programs at the level of transcription, RNA stability or translation. They provide a rapid and highly versatile mode of regulation in the context of cell fate determination (43, 44). miRNAs have been shown to play important roles in hematopoiesis involving erythroid, myeloid and B lymphocyte development (45-47). Recent reports have begun to uncover the interplay between miRNAs and lineage determining transcription factors (Figures 3 and 4). Transcription factors regulate the expression of miRNA genes during lineage specification and in turn miRNAs target mRNAs encoding lineage determining transcription factors. This leads to the generation of feedback loops between these two classes of regulatory molecules. Thus, it appears that a new type of sub-circuit composed of miRNAs can be super-imposed on the ones previously comprised of transcription factors. An important example of this type of regulation emerges from the analysis of cell fate determination involving neutrophils and macrophages. In addition to the cross-antagonism through the sub-circuit comprised of Gfi-1 and the Egr's, PU.1 and C/EBP? α appear to also compete through antagonist miRNAs (miRNA-223 and miRNA-424) with the interplay of a common transcription factor, Nuclear Factor I (NFI-A) (48, 49) (Figure 3). This mode of regulation appears to fine-tune the expression of lineage determining transcription factors in the context of cell fate determination.

Epigenetic Modifications and Changes in Nuclear Compartmentalization during Cell Fate Determination

Epigenetic modifications play a crucial role in the activation and repression of lineage-specific patterns of gene expression. They can also generate stable and heritable changes in gene expression. This mode of regulation involves DNA methylation and an extensive series of covalent modifications of histone tails (50). The latter modifications include diverse marks such as acetylation, methylation, phosphorylation, SUMOylation and ubiquitylation. Ubiquitous enzymatic complexes, including DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs) and histone demethylases (HDMs) catalyze these diverse histone modifications. Several studies have demonstrated that lineage determining transcription factors are able to recruit such complexes to specific loci during cell fate specification leading to gene activation or repression. Such mechanisms of action have been documented for transcription factors including GATA1 in erythropoiesis (51) or PU.1 in the context of myelopoiesis (52). Interestingly many transcriptional regulators appear to be bi-functional as they are able to recruit both activating and repressing chromatin-modifying complexes. Thus Ikaros has been to shown to interact with and recruit both the SWI/SNF activator as well as the NURD

repressor complexes. How a given transcription factor can recruit alternate chromatin-modifying complexes on different target genes remains an intriguing problem to be solved? .

Regulated nuclear compartmentalization has been proposed as another mechanism of gene regulation during cell fate specification (53-55). During B cell development immunoglobulin genes have been shown to re-position away from the nuclear lamina as they undergo transcriptional activation and DNA rearrangement. It will be important to explore if such a mechanism applies to larger sets of lineage-specific genes both in the context of their activation in the relevant lineage and their repression in alternate lineages.

Extrinsic Signaling Inputs and Cell Fate Determination

Regulation of hematopoiesis also involves extrinsic signaling inputs. The requirement for cytokines and their instructive potential in cell fate determination has been debated over the years. Interestingly a distinction can be made between myeloid and lymphoid cytokines. Thus the receptors of the myeloid cytokines (GM-CSF, G-CSF and M-CSF) appear not to be essential for myeloid differentiation (56) yet they have an important role in controlling the proliferation of myeloid progenitors during normal and stress-induced hematopoiesis. In contrast, a requirement for the receptors of lymphoid cytokines (Flt3 and IL-7) during lymphocyte differentiation has been documented (57). In B cell development, the nature of the Flt3 input remains to be fully elucidated. In contrast IL-7R signaling has been shown to regulate the developmental induction of the EBF gene, the primary determinant of this lineage (40). Thus IL-7 signaling drives differentiation and proliferation of pro-B cells and rearrangement of immunoglobulin heavy chain loci. Later in the developmental sequence, IL-7 signaling has to be attenuated to allow rearrangement of the immunoglobulin light chain loci (58). Interestingly modulation of IL-7 signaling at this pre-B cell stage of development appears to be regulated by a change of chemokine- receptor expression, enabling B cell precursors to move away from the IL-7 expressing stromal cells (59). The B-cell developmental pathway nicely illustrates how cell fate determination and subsequent differentiation processes are regulated by the interplay between the extrinsic components of the micro-environment such as cytokines, chemokines or adhesion molecules and the intrinsic components i.e., transcription factors. The importance of micro-environmental “niches” for the self-renewal of HSCs has been documented (60). It remains to be determined if such niches and signaling inputs are required for other multipotent or lineage-restricted progenitors.

Mathematical Modeling of Hematopoietic Gene Regulatory Networks

Although our understanding of the molecular details of how gene regulatory networks govern cell fate choices is still expanding, sufficient data are now available to begin

meaningfully modeling these developmental circuits. Such studies can provide information about whether the various pair wise interactions between transcription factors and genes give rise to the observed dynamics at the systems level. Indeed, the complex nature of responses to perturbations often makes it difficult to interpret experiments that probe hematopoietic development by perturbing it. The value of mathematical models is not that they are always right, but instead that they frame hypotheses for experimentally distinguishing between alternate mechanisms. Even predictions that are not borne out contribute to understanding and enable refinement of a unified conceptual framework that is increasingly needed as the conventional picture of hematopoietic development as a series of simple irreversible binary cell fate choices is replaced by the probabilistic one detailed above (Figure 1).

Advancing theoretical methods for faithfully representing molecular events involved in gene expression, including the statistics of fluctuations (noise), is at the forefront of research at the interface between the biological and physical sciences. However, traditional chemical kinetic methods are also proving to be useful in the analysis of experimentally assembled networks. In such an approach, a network is represented mathematically by a set of differential equations that describe how the expression levels of participating species evolve in an average sense. The advantage to such an approach is that there are well-defined means for determining the fixed points of the differential equations, which correspond to combinations of expression levels that do not change in time. Stable fixed points (or “attractors”) are robust to perturbations and correspond to well-defined (but not necessarily mature) cell types. Unstable fixed points are not readily observable in experiments but are important because they define the boundaries (in expression levels) between developmental states. Once identified, one can assess the sensitivity of the fixed points to the parameters of the model, and trends can often be connected directly with experimentally observed behaviors or exploited to make predictions that can be used to validate the model experimentally.

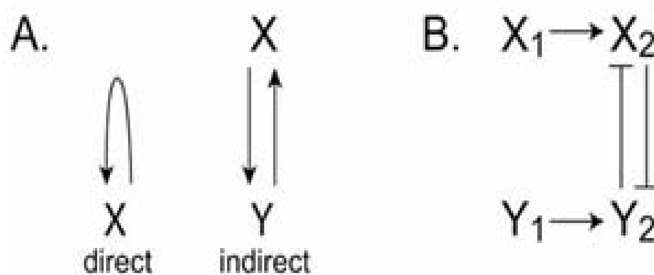

Figure 5. Common elementary network motifs giving rise to bistability. (A) Autoactivation. (B) Mutual repression (counter-antagonism).

Mathematical details are presented elsewhere (29). Here, we try to develop the reader's intuition by considering common elementary gene regulatory network motifs (see (61) for more extensive discussions). We show how these models can help elucidate many of the basic features of cell fate decisions. We then discuss how these motifs contribute, often in combination with each other, to specific hematopoietic cell fate decisions and show how they can be used to understand counterintuitive results in cellular reprogramming experiments. A key idea is that the effects of perturbations depend on both their extent and their timing.

Because most, if not all, elementary cell fate decisions are binary in nature, gene regulatory network motifs that support bistability (i.e., have two attractors under the same conditions) are ubiquitous. Such motifs typically involve some form of positive feedback. One of the simplest motifs giving rise to bistability is autoactivation, in which a protein can activate its own expression directly or indirectly through upregulating one of its activators (Figure 5A). Autoactivation ensures that high expression of the protein is self-reinforcing. Similarly, if the protein is not present, it cannot activate its own transcription so a state with low expression is stable as well. Models of this motif thus have three fixed points: two stable points in which the gene is effectively either on or off and an unstable point which separates the basins of attraction for these stable states. Functionally, this module "remembers" a past stimulus to maintain a stable cell fate in the absence of inducing signal. If the gene begins in the off state, once a signal has induced it to high expression, this state will be self-sustaining even in the absence of the signal. This form of memory is known as "hysteresis" and is a hallmark of bistable systems. Well-characterized examples of autoactivating circuits are those that determine whether E. coli and yeast transition from a (default) state in which a nutrient is not metabolized to one in which it is (62, 63).

Another network motif that can govern a decision between two alternate cell fates is that of two mutually repressing transcription factors. To model a generic bistable switch that can be employed in studying cell fate decisions, we consider a circuit where two primary determinants (X1 and Y1) each activate a single secondary regulator (X2 and Y2) (Figure 5B). It is well-known that this motif can have two stable states: each of which has one secondary gene on and the other off. The unstable fixed point separating them has balanced expression of the competing factors. This behavior derives from the fact that, if one secondary determinant is more strongly expressed, even transiently, it can overwhelm the other and progressively repress it. This ensures that lineage inappropriate genes are not misexpressed. The classic example of such a motif is the mutual repression of Cro and cI, which governs the lysis-lysogeny decision in bacteriophage ? λ (64). What is less appreciated is that, when the primary determinants are expressed at low levels, the cross-antagonism is sufficiently weak that neither secondary determinant can overwhelm the other, and a mixed lineage gene expression pattern with balanced secondary determinants becomes the only stable state. This is in agreement with experiments described above which have revealed that progenitors promiscuously transcribe genes representative of a variety of lineages at low levels ("transcriptional priming").

Interestingly, this model provides a unified means for viewing both cell fate decisions in which a population randomly partitions into two lineages (a stochastic mechanism) and ones in which populations are directed toward competing lineages by environmental cues (a deterministic or instructive mechanism). If both secondary determinants are upregulated

simultaneously to similar extents in a progenitor state with a mixed pattern of gene expression, the progenitor state will be destabilized without being biased towards either terminal cell fate and stochastic fluctuations will determine which fate is ultimately adopted. On the other hand, if X1 and X2 are increased prior to Y1 and Y2 in Figure 5B, then cells will remain in the X-associated lineage even if Y1 and Y2 are subsequently increased. Such commitment following an instructive cue is another example of hysteresis.

While the idea that the attractors of the dynamics of gene regulatory networks correspond to the stable cell fates observed in an organism was introduced many years ago, specific molecular realizations are just now beginning to be elucidated due to advances in both theory and experiment. The auto-activation model is most applicable to situations in which a cell must "remember" exposure to a stimulus after that stimulus is no longer present. This model has been used to explain how T cells maintain their cell fate choice after transient exposure to an instructive signal. In this case, the autoactivating protein is GATA-3 and the instructive signal that controls its expression is the cytokine IL-4 (65). If IL-4 is present during activation, cells upregulate GATA-3 and adopt the Th2 lineage rather than the Th1 lineage. The former is then stable even in the absence of IL-4. Autoactivation also plays a role in the decision between the erythroid and myeloid lineages. Here it helps ensure the stability of these lineages following the cell fate decision, which is governed by mutual repression (66) (discussed further below).

The mutual repression model is applicable to a wide variety of binary decisions made between mutually exclusive cell states. In the hematopoietic system, different types of myeloid cells (macrophages and neutrophils) develop from a common progenitor. The primary and secondary cell fate determinants for the macrophage fate are PU.1 and Egr-2, respectively, while those for the neutrophil lineage are C/EBP? α and Gfi-1 (Figure 6A). The main difference between this network and the basic one in Figure 5A is that the secondary determinant for the neutrophil lineage (Gfi-1) is thought to repress the primary cell fate determinant for the other (PU.1) (29, 67). This interaction breaks the symmetry in the network and complicates the analysis, but the dynamics are qualitatively similar to those described above (29).

The regulation of the expression of a macrophage-specific gene (c-fms) downstream of PU.1 and Egr-2 has been described in detail (68) and can therefore serve as a model for how the primary and secondary cell fate determinants jointly regulate genes further downstream. The c-fms gene is first induced at low levels by the presence of PU.1 and later at higher levels by Egr-2. Interestingly, C/EBP?, α, the primary cell fate determinant for the neutrophil lineage, also serves as an activator for this macrophage gene. In fact, this observation is part of a larger paradox concerning myeloid development. Counter-intuitively, differentiated cells express high levels of primary cell fate determinants from both lineages and binding sites for both of these proteins are found in the regulatory regions of lineage specific downstream genes (29). The model reveals that increasing the levels of both primary cell fate determinants destabilizes the mixed lineage progenitor and forces differentiation to one of the terminal two cell fates. Thus, increasing the expression of both primary cell fate determinants can be used as a strategy for both lineage commitment and stochastic specification (29).

A model that was similar but also included positive autoregulation for each of the secondary cell fate determinants (X and Y activate their own production) was introduced to

study an earlier developmental decision in the hematopoietic system, that between the myeloid and erythroid lineages (66). This model also has stable states corresponding to the progenitor and terminal cell fates, and the behavior in this case is qualitatively similar. However, the auto-activation alters the dynamics quantitatively during lineage commitment (66). Furthermore, the autoactivation could serve to ensure that the terminal cell fates are robust to stochastic fluctuations, which are not treated in these simple models.

Recently, there has been increased interest in the robustness and plasticity of cell fate decisions. As a cell progresses down a developmental pathway what alternatives are available and when does commitment to a given cell fate become irreversible? These questions can be explicitly probed by "reprogramming" experiments in which the expression of a transcription factor known to promote the development of an alternate lineage is enforced (69-74). These experiments suggest that common myeloid and lymphoid progenitors (as defined by cell surface proteins) and even differentiated cells that arise from them are surprisingly plastic and can be readily made to produce alternate lineages.

Although most reprogramming experiments are clearly consistent with regulatory relationships established by independent means (69, 70, 73), some yield counterintuitive results. In particular, Graf and co-workers showed that forced expression of the neutrophil promoting transcription factor (C/EBP?) α) in B and T lymphocyte precursors converted these cells into macrophages rather than neutrophils (71, 72). To understand these reprogramming experiments, we have expanded the myeloid network considered in (29) to include key cell fate determinants for the B cell lineage (75-80) (Figure 6A). A similar analysis can be performed for the T cell case by including GATA-3, Notch, and HEB, but the qualitative conclusions are the same (data not shown).

The combined lymphoid-myeloid network in Figure 6A has stable fixed points corresponding to the B cell, neutrophil, and macrophage cell fates. We simulated experiments in which C/EBP? α was induced in a progenitor cell that would have adopted the B cell fate if left unperturbed. Parameters were set to those that produce B cells (Figure 6B) and the rate of synthesis or concentration of C/EBP? α was spiked at various points along this pathway to simulate the enforced expression. When the rate of C/EBP? α synthesis was spiked very early in development, neutrophils resulted (Figure 6C). However, if the cell was allowed to proceed along the B cell pathway, suddenly increasing the C/EBP? α rate of synthesis produced macrophages (Figure 6D). In the first case, both Egr-2 and Gfi-1 were at low concentrations when the enforced expression was initiated, such that C/EBP? α promoted the neutrophil pathway at the expense of the macrophage pathway. In To understand the second case, note that PU.1 is obligate for the development of both myeloid and lymphoid lineages. Once PU.1 activates Egr-2since the Egr-2 had been activated by PU.1, the latter is obligate for development of lymphoid as well as myeloid cells, subsequent activation expression of C/EBP? α could cannot activate induce Gfi-1. However, since, in the model C/EBP? α represses several B cell specific genesantagonizes the B-lineage regulators Pax-5 and E2A, the re-programmed B-lineage cells default to a macrophage fate.

These results are summarized in Figure 6E. Early induction of C/EBP? α in sufficient quantities reprograms lymphocytes to neutrophils, and later induction reprograms them to macrophages.

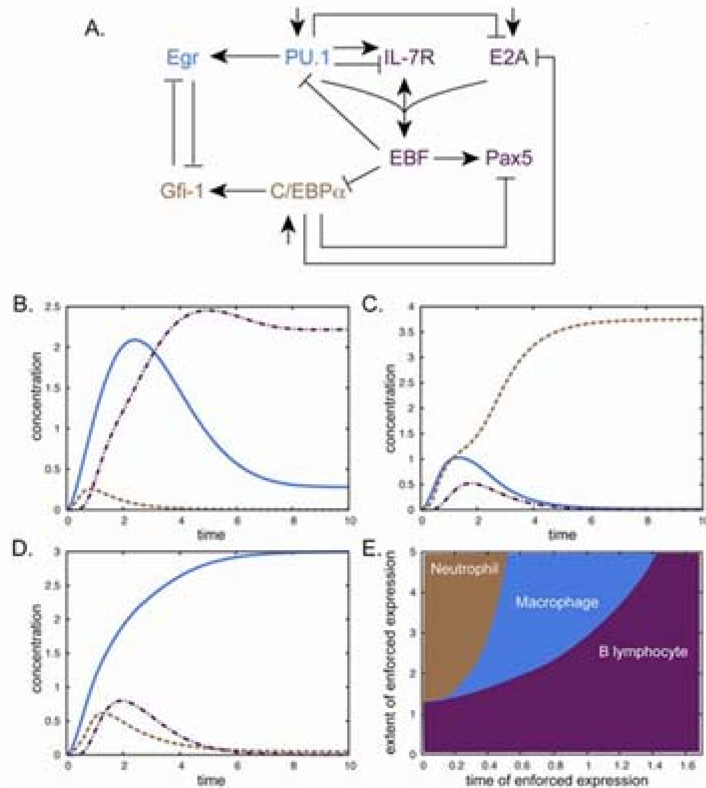

Figure 6. Developmental dynamics of a gene regulatory network that orchestrates B-lymphoid versus myeloid cell fates. (A) Schematic of the network subjected to mathematical modeling. Small arrows indicate genes with a non-zero rate of default synthesis. (B through D) Time series for key cell fate determinants. The concentrations shown are for the B lineage gene EBF (maroon, dashed-dotted lines), the macrophage gene Egr (blue, solid lines), and the neutrophil gene Gfi-1 (gold, dashed lines). (B) Time course for unperturbed development of a B lymphocyte. (C and D) The rate of C/EBP? α synthesis is artificially increased by a unit amount at time (C) t=0.3 or (D) t=0.6. (E) "Phase" diagram for reprogramming. The final cell fate is shown as a function of the timing and magnitude of the spike in C/EBPα? synthesis. The equations are of the same form and the parameters for each gene are the same as those used in (29) with the exception of the activation of EBF, which required a different form to account for the integration of signals from PU.1, IL7R, and E2A. See Warmflash and Dinner (82) for additional details.

However, as cells develop along the lymphocyte pathways, it becomes increasingly difficult to divert cells to myeloid fates. In considering these results, it is important to note that Gfi-1 is expressed in lymphocytes (81). However, the model only requires that the levels of Egr be sufficiently high that the Egr-Gfi cross-antagonism gives rise to a bistable switch biased to the macrophage promoting side immediately following the enforced C/EBP? α expression.

In summary, mathematical modeling reveals that lymphocyte development biases the myeloid bistable switch to the macrophage promoting side, so that induction of myeloid genes (even neutrophil promoting ones) can force the cell to adopt a macrophage fate. This mechanism is consistent with the finding that PU.1 is necessary to reprogram lymphocyte

progenitors to macrophages but not to downregulate lymphocyte specific genes (71, 72). C/EBP? α itself causes the downregulation of these genes, but PU.1 is necessary to bias the switch to the macrophage side. The results of the modeling could be tested by performing similar reprogramming experiments in which the levels of Egr-1,2 or Gfi-1 were perturbed either through genetic knockouts or enforced expression. For example, in the case in which Gfi-1 expression was enforced, we would expect the switch to be biased to the neutrophil side and C/EBP? α to reprogram B cell progenitors to neutrophils.

Conclusion

Hematopoiesis is a leading system in developmental biology, not only from the standpoint of analyzing stem cell function but also the elucidation of gene regulatory networks that orchestrate cell fate determination. Considerable progress is being made in experimentally assembling cell fate determination circuits comprised of transcription factors and miRNAs and in their theoretical analysis. Future work will lead to establishment of comprehensive regulatory networks in which the target genes of each transcription factor and miRNA will be determined. Such networks will provide the molecular underpinnings for the developmental lineage relationships that to date have been exclusively explored by isolating hematopoietic progenitors and examining their developmental potential by transplantation or in vitro culture. These networks can also be used to predict the outcomes of lineage reprogramming experiments and to rationally design scalable approaches for generating large numbers of specific hematopoietic cell types from embryonic or induced pluripotent stem cells (iPS) for therapeutic purposes.

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References

[1] Metcalf, D. 2007. On hematopoietic stem cell fate. *Immunity,* 26:669.
[2] Akashi, K., D. Traver, T. Miyamoto, and I. L. Weissman. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature,* 404:193.
[3] Kondo, M., I. L. Weissman, and K. Akashi. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell,* 91:661.
[4] Hu, M., D. Krause, M. Greaves, S. Sharkis, M. Dexter, C. Heyworth, and T. Enver. 1997. Multilineage gene expression precedes commitment in the hemopoietic system. *Genes Dev*. 11:774.

[5] Miyamoto, T., H. Iwasaki, B. Reizis, M. Ye, T. Graf, I. L. Weissman, and K. Akashi. 2002. Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. *Dev Cell*, 3:137.

[6] Singh, H. 1996. Gene targeting reveals a hierarchy of transcription factors regulating specification of lymphoid cell fates. *Curr Opin Immunol.* 8:160.

[7] Scott, E. W., M. C. Simon, J. Anastasi, and H. Singh. 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science*, 265:1573.

[8] Iwasaki, H., C. Somoza, H. Shigematsu, E. A. Duprez, J. Iwasaki-Arai, S. Mizuno, Y. Arinobu, K. Geary, P. Zhang, T. Dayaram, M. L. Fenyus, S. Elf, S. Chan, P. Kastner, C. S. Huettner, R. Murray, D. G. Tenen, and K. Akashi. 2005. Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood*, 106:1590.

[9] Montecino-Rodriguez, E., and K. Dorshkind. 2002. Identification of B/macrophage progenitors in adult bone marrow. *Semin Immunol.* 14:371.

[10] Allman, D., A. Sambandam, S. Kim, J. P. Miller, A. Pagan, D. Well, A. Meraz, and A. Bhandoola. 2003. Thymopoiesis independent of common lymphoid progenitors. *Nat Immunol.* 4:168.

[11] Bell, J. J., and A. Bhandoola. 2008. The earliest thymic progenitors for T cells possess myeloid lineage potential. *Nature,* 452:764.

[12] Wada, H., K. Masuda, R. Satoh, K. Kakugawa, T. Ikawa, Y. Katsura, and H. Kawamoto. 2008. Adult T-cell progenitors retain myeloid potential. *Nature,* 452:768.

[13] Igarashi, H., S. C. Gregory, T. Yokota, N. Sakaguchi, and P. W. Kincade. 2002. Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity*, 17:117.

[14] Adolfsson, J., R. Mansson, N. Buza-Vidas, A. Hultquist, K. Liuba, C. T. Jensen, D. Bryder, L. Yang, O. J. Borge, L. A. Thoren, K. Anderson, E. Sitnicka, Y. Sasaki, M. Sigvardsson, and S. E. Jacobsen. 2005. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell*, 121:295.

[15] Forsberg, E. C., T. Serwold, S. Kogan, I. L. Weissman, and E. Passegue. 2006. New evidence supporting megakaryocyte-erythrocyte potential of flk2/flt3+ multipotent hematopoietic progenitors. *Cell,* 126:415.

[16] Lai, A. Y., and M. Kondo. 2006. Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *J Exp Med*. 203:1867.

[17] Nerlov, C., E. Querfurth, H. Kulessa, and T. Graf. 2000. GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription. *Blood*, 95:2543.

[18] Zhang, P., G. Behre, J. Pan, A. Iwama, N. Wara-Aswapati, H. S. Radomska, P. E. Auron, D. G. Tenen, and Z. Sun. 1999. Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1. *Proc Natl Acad Sci U S A*, 96:8705.

[19] Yoshida, T., I. Hazan, J. Zhang, S. Y. Ng, T. Naito, H. J. Snippert, E. J. Heller, X. Qi, L. N. Lawton, C. J. Williams, and K. Georgopoulos. 2008. The role of the chromatin

remodeler Mi-2beta in hematopoietic stem cell self-renewal and multilineage differentiation. *Genes Dev*. 22:1174.

[20] Rodriguez, P., E. Bonte, J. Krijgsveld, K. E. Kolodziej, B. Guyot, A. J. Heck, P. Vyas, E. de Boer, F. Grosveld, and J. Strouboulis. 2005. GATA-1 forms distinct activating and repressive complexes in erythroid cells. *EMBO J*. 24:2354.

[21] O'Neill, D. W., S. S. Schoetz, R. A. Lopez, M. Castle, L. Rabinowitz, E. Shor, D. Krawchuk, M. G. Goll, M. Renz, H. P. Seelig, S. Han, R. H. Seong, S. D. Park, T. Agalioti, N. Munshi, D. Thanos, H. Erdjument-Bromage, P. Tempst, and A. Bank. 2000. An ikaros-containing chromatin-remodeling complex in adult-type erythroid cells. *Mol Cell Biol*. 20:7572.

[22] Yoshida, T., S. Y. Ng, J. C. Zuniga-Pflucker, and K. Georgopoulos. 2006. Early hematopoietic lineage restrictions directed by Ikaros. *Nat Immunol*. 7:382.

[23] Dias, S., R. Mansson, S. Gurbuxani, M. Sigvardsson, and B. L. Kee. 2008. E2A Proteins Promote Development of Lymphoid-Primed Multipotent Progenitors. *Immunity*, 29:217.

[24] Medina, K. L., K. P. Garrett, L. F. Thompson, M. I. Rossi, K. J. Payne, and P. W. Kincade. 2001. Identification of very early lymphoid precursors in bone marrow and their regulation by estrogen. *Nat Immunol.* 2:718.

[25] Reynaud, D., I. A. Demarco, K. L. Reddy, H. Schjerven, E. Bertolino, Z. Chen, S. T. Smale, S. Winandy, and H. Singh. 2008. Regulation of B cell fate commitment and immunoglobulin heavy-chain gene rearrangements by Ikaros. *Nat Immunol*. 9:927.

[26] Hardy, R. R., P. W. Kincade, and K. Dorshkind. 2007. The protean nature of cells in the B lymphocyte lineage. *Immunity,* 26:703.

[27] Pongubala, J. M., D. L. Northrup, D. W. Lancki, K. L. Medina, T. Treiber, E. Bertolino, M. Thomas, R. Grosschedl, D. Allman, and H. Singh. 2008. Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5. *Nat Immunol.* 9:203.

[28] Nutt, S. L., B. Heavey, A. G. Rolink, and M. Busslinger. 1999. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature*, 401:556.

[29] Laslo, P., C. J. Spooner, A. Warmflash, D. W. Lancki, H. J. Lee, R. Sciammas, B. N. Gantner, A. R. Dinner, and H. Singh. 2006. Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell,* 126:755.

[30] Huang, X., S. Cho, and G. J. Spangrude. 2007. Hematopoietic stem cells: generation and self-renewal. *Cell Death Differ*. 14:1851.

[31] Zon, L. I. 2008. Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. *Nature*, 453:306.

[32] Zhang, P., J. Iwasaki-Arai, H. Iwasaki, M. L. Fenyus, T. Dayaram, B. M. Owens, H. Shigematsu, E. Levantini, C. S. Huettner, J. A. Lekstrom-Himes, K. Akashi, and D. G. Tenen. 2004. Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. *Immunity,* 21:853.

[33] DeKoter, R. P., and H. Singh. 2000. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science,* 288:1439.

[34] Sciammas, R., A. L. Shaffer, J. H. Schatz, H. Zhao, L. M. Staudt, and H. Singh. 2006. Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity*, 25:225.

[35] Orkin, S. H. 2000. Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet.* 1:57.

[36] Walsh, J. C., R. P. DeKoter, H. J. Lee, E. D. Smith, D. W. Lancki, M. F. Gurish, D. S. Friend, R. L. Stevens, J. Anastasi, and H. Singh. 2002. Cooperative and antagonistic interplay between PU.1 and GATA-2 in the specification of myeloid cell fates. *Immunity*, 17:665.

[37] Iwasaki, H., S. Mizuno, Y. Arinobu, H. Ozawa, Y. Mori, H. Shigematsu, K. Takatsu, D. G. Tenen, and K. Akashi. 2006. The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. *Genes Dev*. 20:3010.

[38] Dahl, R., J. C. Walsh, D. Lancki, P. Laslo, S. R. Iyer, H. Singh, and M. C. Simon. 2003. Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. *Nat Immunol*. 4:1029.

[39] Starck, J., N. Cohet, C. Gonnet, S. Sarrazin, Z. Doubeikovskaia, A. Doubeikovski, A. Verger, M. Duterque-Coquillaud, and F. Morle. 2003. Functional cross-antagonism between transcription factors FLI-1 and EKLF. *Mol Cell Biol*. 23:1390.

[40] Roessler, S., I. Gyory, S. Imhof, M. Spivakov, R. R. Williams, M. Busslinger, A. G. Fisher, and R. Grosschedl. 2007. Distinct promoters mediate the regulation of Ebf1 gene expression by interleukin-7 and Pax5. *Mol Cell Biol*. 27:579.

[41] Maier, H., R. Ostraat, H. Gao, S. Fields, S. A. Shinton, K. L. Medina, T. Ikawa, C. Murre, H. Singh, R. R. Hardy, and J. Hagman. 2004. Early B cell factor cooperates with Runx1 and mediates epigenetic changes associated with mb-1 transcription. *Nat Immunol*. 5:1069.

[42] Sigvardsson, M. 2000. Overlapping expression of early B-cell factor and basic helix-loop-helix proteins as a mechanism to dictate B-lineage-specific activity of the lambda5 promoter. *Mol Cell Biol.* 20:3640.

[43] Bartel, D. P., and C. Z. Chen. 2004. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nat Rev Genet*. 5:396.

[44] Fazi, F., and C. Nervi. 2008. MicroRNA: basic mechanisms and transcriptional regulatory networks for cell fate determination. *Cardiovasc Res*. 79:553.

[45] Felli, N., L. Fontana, E. Pelosi, R. Botta, D. Bonci, F. Facchiano, F. Liuzzi, V. Lulli, O. Morsilli, S. Santoro, M. Valtieri, G. A. Calin, C. G. Liu, A. Sorrentino, C. M. Croce, and C. Peschle. 2005. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proc Natl Acad Sci U S A*, 102:18081.

[46] Fontana, L., E. Pelosi, P. Greco, S. Racanicchi, U. Testa, F. Liuzzi, C. M. Croce, E. Brunetti, F. Grignani, and C. Peschle. 2007. MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. *Nat Cell Biol*. 9:775.

[47] Ventura, A., A. G. Young, M. M. Winslow, L. Lintault, A. Meissner, S. J. Erkeland, J. Newman, R. T. Bronson, D. Crowley, J. R. Stone, R. Jaenisch, P. A. Sharp, and T.

Jacks. 2008. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell*, 132:875.
[48] Fazi, F., A. Rosa, A. Fatica, V. Gelmetti, M. L. De Marchis, C. Nervi, and I. Bozzoni. 2005. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell*, 123:819.
[49] Rosa, A., M. Ballarino, A. Sorrentino, O. Sthandier, F. G. De Angelis, M. Marchioni, B. Masella, A. Guarini, A. Fatica, C. Peschle, and I. Bozzoni. 2007. The interplay between the master transcription factor PU.1 and miR-424 regulates human monocyte/macrophage differentiation. *Proc Natl Acad Sci U S A,* 104:19849.
[50] Rice, K. L., I. Hormaeche, and J. D. Licht. 2007. Epigenetic regulation of normal and malignant hematopoiesis. *Oncogene*. 26:6697.
[51] Letting, D. L., C. Rakowski, M. J. Weiss, and G. A. Blobel. 2003. Formation of a tissue-specific histone acetylation pattern by the hematopoietic transcription factor GATA-1. *Mol Cell Biol.* 23:1334.
[52] Stopka, T., D. F. Amanatullah, M. Papetti, and A. I. Skoultchi. 2005. PU.1 inhibits the erythroid program by binding to GATA-1 on DNA and creating a repressive chromatin structure. *EMBO J*. 24:3712.
[53] Brown, K. E., S. S. Guest, S. T. Smale, K. Hahm, M. Merkenschlager, and A. G. Fisher. 1997. Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell,* 91:845.
[54] Cobb, B. S., S. Morales-Alcelay, G. Kleiger, K. E. Brown, A. G. Fisher, and S. T. Smale. 2000. Targeting of Ikaros to pericentromeric heterochromatin by direct DNA binding. *Genes Dev*. 14:2146.
[55] Reddy, K. L., J. M. Zullo, E. Bertolino, and H. Singh. 2008. Transcriptional repression mediated by repositioning of genes to the nuclear lamina. *Nature*, 452:243.
[56] Hibbs, M. L., C. Quilici, N. Kountouri, J. F. Seymour, J. E. Armes, A. W. Burgess, and A. R. Dunn. 2007. Mice lacking three myeloid colony-stimulating factors (G-CSF, GM-CSF, and M-CSF) still produce macrophages and granulocytes and mount an inflammatory response in a sterile model of peritonitis. *J Immunol*. 178:6435.
[57] Sitnicka, E., C. Brakebusch, I. L. Martensson, M. Svensson, W. W. Agace, M. Sigvardsson, N. Buza-Vidas, D. Bryder, C. M. Cilio, H. Ahlenius, E. Maraskovsky, J. J. Peschon, and S. E. Jacobsen. 2003. Complementary signaling through flt3 and interleukin-7 receptor alpha is indispensable for fetal and adult B cell genesis. *J Exp Med.* 198:1495.
[58] Johnson, K., T. Hashimshony, C. M. Sawai, J. M. Pongubala, J. A. Skok, I. Aifantis, and H. Singh. 2008. Regulation of immunoglobulin light-chain recombination by the transcription factor IRF-4 and the attenuation of interleukin-7 signaling. *Immunity,* 28:335.
[59] Nagasawa, T. 2006. Microenvironmental niches in the bone marrow required for B-cell development. *Nat Rev Immunol*. 6:107.
[60] Martinez-Agosto, J. A., H. K. Mikkola, V. Hartenstein, and U. Banerjee. 2007. The hematopoietic stem cell and its niche: a comparative view. *Genes Dev*. 21:3044.
[61] Alon, U. 2007. An introduction to systems biology; Design principles of biological circuits Chapman & Hall/CRC Press.

[62] Ozbudak, E. M., M. Thattai, H. N. Lim, B. I. Shraiman, and A. Van Oudenaarden. 2004. Multistability in the lactose utilization network of Escherichia coli. *Nature*, 427:737.

[63] Acar, M., A. Becskei, and A. van Oudenaarden. 2005. Enhancement of cellular memory by reducing stochastic transitions. Nature 435:228.

[64] Ptashne, M. 1992. A Genetic Switch. Cell Press.

[65] Hofer, T., H. Nathansen, M. Lohning, A. Radbruch, and R. Heinrich. 2002. GATA-3 transcriptional imprinting in Th2 lymphocytes: a mathematical model. . *Proc. Natl. Acad. Sci. USA,* 99:9364.

[66] Huang, S., Y.-P. Guo, G. May, and T. Enver. 2007. Bifurcation dynamics in lineage-commitment in bipotent progenitor cells. *Dev Biol.* 305:695.

[67] Dahl, R., S. R. Iyer, K. S. Owens, D. D. Cuylear, and M. C. Simon. 2007. The transcriptional repressor GFI-1 antagonizes PU.1 activity through protein-protein interaction. *J Biol Chem.* 282:6473.

[68] Krysinska, H., M. Hoogenkamp, R. Ingram, N. Wilson, H. Tagoh, P. Laslo, H. Singh, and C. Bonifer. 2007. A two-step, PU.1-dependent mechanism for developmentally regulated chromatin remodeling and transcription of the c-fms gene. *Mol Cell Biol.* 27:878.

[69] Klinken, S. P., W. S. Alexander, and J. M. Adams. 1988. Hemopoietic lineage switch: v-raf oncogene converts Emu-myc transgenic B cells into macrophages. *Cell*, 53:857.

[70] Kondo, M., D. C. Scherer, T. Miyamoto, A. G. King, K. Akashi, K. Sugamura, and I. L. Weissman. 2000. Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. *Nature,* 407:383.

[71] Xie, H., M. Ye, R. Feng, and T. Graf. 2004. Stepwise reprogramming of B cells into macrophages. *Cell*, 117:663.

[72] Laiosa, C. V., M. Stadtfeld, H. Xie, L. de Andres-Aguayo, and T. Graf. 2006. Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBPα alpha and PU.1 transcription factors. *Immunity,* 25:731.

[73] Cobaleda, C., W. Jochum, and M. Busslinger. 2007. Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors. *Nature*, 449:473.

[74] Taghon, T., M. A. Yui, and E. V. Rothenberg. 2007. Mast cell lineage diversion of T lineage precursors by the essential T cell transcription factor GATA-3. *Nat Immunol*. 8:845.

[75] Medina, K. L., J. M. Pongubala, K. L. Reddy, D. W. Lancki, R. Dekoter, M. Kieslinger, R. Grosschedl, and H. Singh. 2004. Assembling a gene regulatory network for specification of the B cell fate. *Dev Cell,* 7:607.

[76] Singh, H., K. L. Medina, and J. M. Pongubala. 2005. Contingent gene regulatory networks and B cell fate specification. *Proc Natl Acad Sci U S A*, 102:4949.

[77] Singh, H., J. M. Pongubala, and K. L. Medina. 2007. Gene regulatory networks that orchestrate the development of B lymphocyte precursors. *Adv Exp Med Biol*. 596:57.

[78] Seet, C. S., R. L. Brumbaugh, and B. L. Kee. 2004. Early B cell factor promotes B lymphopoiesis with reduced interleukin 7 responsiveness in the absence of E2A. *J Exp Med.* 199:1689.

[79] Dias, S., H. Silva, Jr., A. Cumano, and P. Vieira. 2005. Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors. *J Exp Med.* 201:971.

[80] Kikuchi, K., A. Y. Lai, C. L. Hsu, and M. Kondo. 2005. IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF. *J Exp Med*. 201:1197.

[81] Hock, H., and S. H. Orkin. 2006. Zinc-finger transcription factor Gfi-1: versatile regulator of lymphocytes, neutrophils and hematopoietic stem cells. *Curr Opin Hematol*. 13:1.

[82] Warmflash, A. & Dinner, A. R. 2009 Modeling gene regulatory networks for cell fate specification, Chapter 5 in Statistical Mechanics of Cellular Systems and Processes, M Zaman, ed. Cambridge University Press.

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Chapter 2

Lymphoid and Myeloid Lineage Commitment in Multipotent Hematopoietic Progenitors*

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Abstract

Hematopoietic stem cells (HSC) continuously replenish all types of blood cells through a series of lineage restriction steps that results in the progressive loss of differentiation potential to other cell lineages. This chapter focuses on the recent advances in understanding one of the earliest differentiation steps in HSC maturation, the diversification of the lymphoid and myeloid cell lineages, which make up the two major branches of hematopoietic cells. We will discuss the progress in identifying and characterizing progenitor populations that are downstream of HSCs. Prospective isolation of cell populations at the various maturational stages is a key in understanding the sequential biological events that take place during the course of differentiation of HSC into each hematopoietic cell type. The role of transcription factors, cytokines, and bone marrow microenvironments in lymphoid *versus* myeloid cell fate decisions will also be discussed.

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Introduction

Hematopoiesis is the process of blood cell formation. Blood cells are categorized into two lineages, lymphoid and myeloid. Lymphoid lineage cells, or lymphocytes, include T, B, and NK cells. Other cell types, such as megakaryocytes (Meg) and erythrocytes (E), as well as granulocytes (G) and macrophages (M) belong to the myeloid lineage [1,2]. The isolation and characterization of HSCs in mouse bone marrow and fetal liver provided definitive proof that lymphoid and myeloid lineage cells have a common origin [3,4]. By transplanting a single HSC into a lethally irradiated recipient mouse, long-term reconstitution of both the lymphoid and the myeloid compartment was achieved [5-7], demonstrating that the HSC is the common ancestor of all blood cell types. HSCs are highly enriched in the lineage marker $(Lin)^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}Thy\text{-}1.1^{lo}$ fraction of the bone marrow [3,8,9], and are defined by the following criteria [2]: *(i)* a long-term self-renewal ability, measured by sustained blood cell production upon transplantation into recipients, *(ii)* a high proliferative capacity, indicated by the formation of dense spleen colonies known as colony forming units-spleen (CFU-S), which consist of mature myeloid lineage cells as well as self-renewing progenitors (revealed by intravenous transfer into irradiated hosts), and *(iii)* the ability to differentiate into all hematopoietic cell types.

Maturation of Hematopoietic Stem Cells

Hematopoiesis is a tightly regulated program. HSCs must balance self-renewal and differentiation in order to sustain the production of all lymphoid and myeloid lineage cells for the entire lifespan of an animal. Characterization of progenitor populations downstream of HSCs has led to insights into the sequential events that occur during development into each cell lineage. The hierarchical relationship between hematopoietic progenitors and mature blood cells has been and continues to be an area of intense investigation. The emergence of monoclonal antibody technology in conjunction with fluorescence-activated cell sorting (FACS) has provided investigators with a means to define and purify distinct progenitor subsets on the basis of their surface markers [1,10]. The ability to isolate pure progenitor populations for use in blood cell differentiation assays has led to a more refined understanding of lineage-specific differentiation and construction of a conceptual hematopoietic tree [10-14].

The initial response of HSCs to the earliest differentiation signals is their gradual loss of self-renewal ability. This self-renewal ability sub-divides the steady state HSC pool into two populations: long-term (LT) and short-term (ST) reconstituting HSCs [9,15-17]. LT-HSCs have life-long self-renewal ability and contribute to long-term multi-lineage reconstitution of irradiated hosts upon transplantation. In contrast, ST-HSCs have limited self-renewal ability and can only support reconstitution of the hematopoietic system for about 6 weeks [9,16,18]. ST-HSCs give rise to multi-potent progenitors (MPPs) [15]. MPPs can support the generation of all the mature blood cell types, but maintain no obvious self-renewal capacity, and as a consequence can only support hematopoiesis transiently [9,16]. Furthermore, it has been demonstrated that these aforementioned progenitor populations form a sequential

developmental lineage, namely LT-HSC → ST-HSC → MPP [15,19]. All of these cells are included in the c-Kit^{hi}Lineage^{-}Sca-1^{+} (KLS) bone marrow fraction [16,19]. The lack of self-renewal activity, along with their ability to give rise to lineage-restricted oligo-potent progenitors, has led to the speculation that the first step of lineage-restriction during blood cell development occurs in MPPs (see Figure 1).

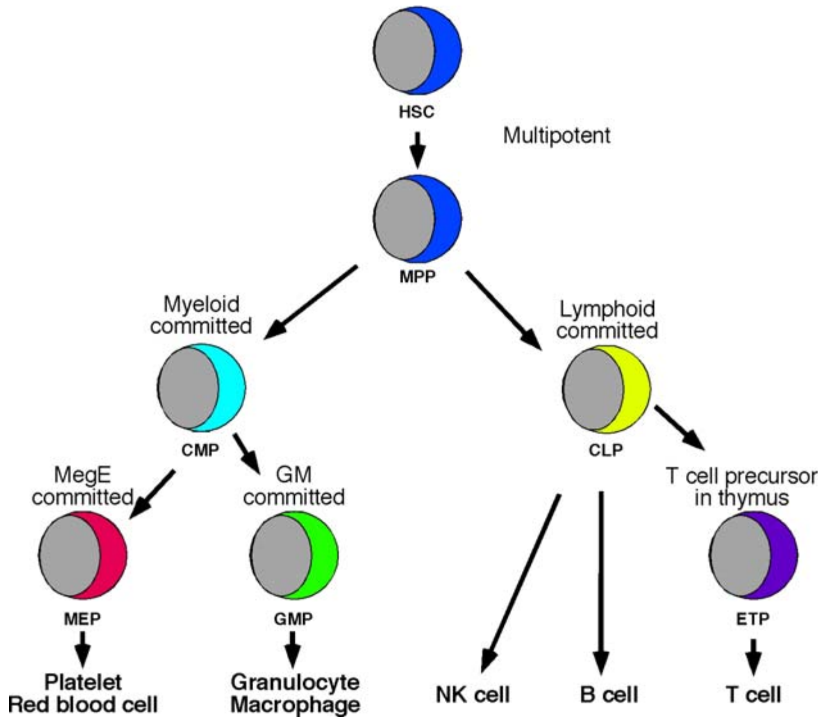

Figure 1 Classical hematopoietic tree proposed after identification of CLPs and CMPs.

Identification of Lymphoid and Myeloid Lineage Restricted Progenitors and the "Classical" Model of Hematopoiesis

Once HSCs differentiate into MPPs, which have no life-long self-renewal potential, MPPs start to lose multi-potent differentiation potential. Lymphoid and myeloid lineage segregation during early hematopoiesis was demonstrated through the discovery of common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). These cells have differentiation potentials restricted to all of the cell types within their respective lineage at a clonal level (Figure 1) [20,21]. Because of the indispensable role of IL-7/IL-7R signaling in both T and B cell development [22,23], CLPs were identified by virtue of expression of IL-7Rα on primitive hematopoietic progenitors, defined as IL-7Rα^{+}Lin^{-}Sca-1^{+}c-Kit^{+}Thy-1.1^{-} [20]. By excluding the cell surface markers that identified HSCs and CLPs, such as Sca-1 and IL-7Rα,

CMPs were similarly isolated and defined as CD34$^+$FcγRII/III$^{-/lo}$IL-7Rα^-Lin$^-$Sca-1$^-$c-Kit$^+$Thy-1.1$^-$ [21]. CMPs give rise to megakaryocyte/erythroid (MegE) restricted and macrophage/granulocyte (GM) restricted bipotent progenitors, defined as CD34$^-$FcγRII/III$^-$ IL-7Rα^-Lin$^-$Sca-1$^-$c-Kit$^+$Thy-1.1$^-$ and CD34$^+$FcγRII/III$^+$IL-7Rα^-Lin$^-$Sca-1$^-$c-Kit$^+$Thy-1.1$^-$, respectively [21]. Identification of these lineage-restricted progenitors separated the lymphoid and myeloid lineages on the progenitor level [1,2]. It has been suggested that CLPs and CMPs are generated symmetrically from a single MPP [2]. This model formed the basis of the "classical" model of hematopoiesis, where the divergence of the lymphoid and myeloid lineage represents the first step of irreversible lineage commitment from HSCs (Figure 1) [14,24]. However, careful characterization of MPPs has led to revision of the "classical" model.

Revision to the "Classical" Model: Asymmetrical Lymphoid and Myeloid Lineage Segregation at the MPP Stage

Studies of fetal hematopoiesis have revealed that the divergence of the lymphoid and myeloid lineages might not be as simplistic as first thought. Although the fetal counterpart of the adult CMP has been identified [25], the existence of lymphoid committed progenitors has yet to be demonstrated in fetal liver. Adult phenotypic CLPs are present in the fetal liver; however, these cells can give rise to macrophages in addition to T and B lymphocytes [26]. While no T/B bi-potent readout was observed for fetal liver progenitors at a clonal level, progenitors with only T/B/GM, T/GM, and B/GM differentiation potential were present [27]. Because of these findings, it is speculated that clear lymphoid and myeloid lineage segregation does not occur during fetal hematopoietic differentiation. It is unclear whether the differences observed between fetal and adult hematopoiesis are due to different intrinsic mechanisms in fetal and adult progenitors, or because fetal and adult progenitors develop in different microenvironments (fetal liver *versus* bone marrow). It is also possible that the different models proposed are simply due to the inability at the present time to isolate the adult counterpart of developmental intermediates in the fetal liver, and *vice versa*.

Further characterization of the MPP population in the bone marrow provided higher resolution to the hematopoietic tree and a revision to the "classical" model. These studies also suggested that a more similar differentiation program occurs during fetal and adult hematopoiesis. Heterogeneity of the MPP population defined as Flt3$^+$ KLS has been suggested [9,16]. Indeed, the MPP population defined as Flt3$^+$Thy-1.1$^-$ KLS can be divided into the three sub-populations based on Flt3 and VCAM-1 expression (Flt3^{lo}VCAM-1$^+$, Flt3^{hi}VCAM-1$^+$ and Flt3^{hi}VCAM-1$^-$) [28,29]. Analysis of these three MPP sub-populations indicates a lymphoid and myeloid lineage branching point and a step-wise lineage restriction process toward lymphoid lineage commitment (Figure 2) [19]. The most immature Flt3^{lo}VCAM-1$^+$ MPPs have full multi-potent differentiation potential which is not the case for the other two MPP subpopulations. Therefore, Flt3^{lo}VCAM-1$^+$ MPPs are truly "classical" MPPs by this definition. The next Flt3^{hi}VCAM-1$^+$ MPPs can give rise to GM cells and lymphocytes as efficiently as Flt3^{lo}VCAM-1$^+$ MPPs [28]. However, Flt3^{hi}VCAM-1$^+$ MPPs have

a significantly lower MegE potential, both *in vitro* and *in vivo*. In addition, $Flt3^{lo}VCAM\text{-}1^{+}$ MPPs, but not $Flt3^{hi}VCAM\text{-}1^{+}$ MPPs, can give rise to CMPs, suggesting that the $Flt3^{lo}VCAM\text{-}1^{+}$ MPP population is at the myeloid lineage branch point [28]. The third $Flt3^{hi}VCAM\text{-}1^{-}$ MPPs have significantly lower GM and almost no MegE potential with higher lymphoid potential comparable to the other MPP sub-fractions [28,29]. Although MegE potential may not be completely silenced in the $Flt3^{hi}VCAM\text{-}1^{-}$ MPP population [30], the differential potentials of the three MPP subpopulations described above suggest that MPPs first lose MegE and then GM potential before establishing lymphoid lineage commitment at the CLP stage.

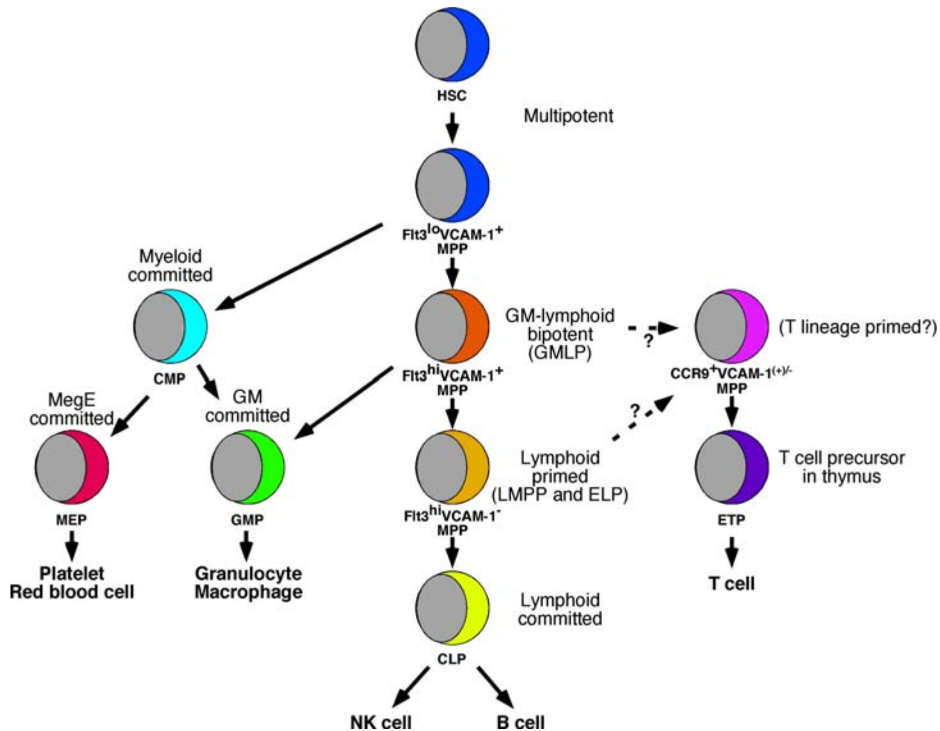

Figure 2. A revised hematopoietic tree.

This hematopoietic tree is based on the *in vivo* differentiation potential of MPP subsets.

Which Bone Marrow Cell Population Travels to the Thymus to Become T Cells?

The presence and necessity of a clear diversification of the lymphoid and myeloid lineages prior to T lineage commitment in adult thymus has been a long-standing argument (for example in [31]). In the "classical" model, progenitors first lose myeloid potential and then lose B, NK and dendritic cell differentiation potential at the onset of T lineage commitment [2]. Since multiple bone marrow populations can transit to the thymus and initiate T cell development [32], it may not be possible to draw a single developmental pathway from HSCs to

T cells [33,34]. The most immature thymocytes in the adult thymus are defined as c-Kit^{hi}Flt3^{+}CD44^{+}CD25^{-} and named early T cell progenitors (ETPs) [35,36]. These cells maintain GM (or just macrophage) potential and have significantly lower levels of B cell potential [37,38]. It has been suggested that early lymphoid progenitors (ELPs), lymphoid-primed multipotent progenitors (LMPPs) and/or CCR9^{+} MPPs are precursors of ETPs in bone marrow and the primary source of T cell progenitors under physiological conditions [32,39,40]. Presumably, B cell potential in thymic immigrants is immediately shut down by the engagement of Notch signaling, whereas NK cell, dendritic cell, and GM (or M) potential are not affected by Notch. As we will discuss in a later section, extracellular stimuli from the microenvironments where progenitors reside may influence their lineage choice. Functions of Notch in this aspect were not previously investigated very well because of the lack of appropriate experimental systems. The establishment of bone marrow stromal cells transfected with Notch ligands, such as OP9-DL1 cells, has made it possible to address the T cell potential of bone marrow and thymic progenitors by *in vitro* cultures more easily [41]. The use of the OP9-DL1 co-culture system has recently revealed a hidden Notch function during megakaryocyte development from HSCs [42]. Therefore, our current understanding of various aspect of hematopoietic differentiation may change in the future as new assay systems become available and as new genetically modified mice are generated.

For a cell to be considered a T cell precursor that has arrived from the bone marrow, three requirements need to be fulfilled. These are the cell: *(i)* can leave the bone marrow to enter the periphery (into the blood); *(ii)* can home to the thymus efficiently; and *(iii)* has a high T cell developmental potential. The original thought that CLPs were the candidate "physiological" T cell precursors was based on their intrinsic T cell differentiation potential [20]. However, the role of CLPs as the common progenitor to both T and B cells in the physiological setting became uncertain when sustained T cell development was observed in Ikaros deficient mice, even though these mice lack B cell development or CLPs in bone marrow [35]. However, since CLPs are present in the blood from wild type mice, it is still possible that CLPs can be T cell precursors under physiological conditions [32,40]. Although CLPs maintain high T cell differentiation potential both *in vitro* and *in vivo* if they are placed in the appropriate environment for T cell development, CLPs seem to have less efficient thymic homing capacity than MPPs [19,40]. Therefore, it is most likely that MPPs, rather than CLPs, are the major thymic immigrants from bone marrow. This example reminds us that it is necessary to be cautious when we interpret experimental data. The use of different experimental systems often generates different results. Furthermore, the use of different markers in defining the same cell population by different laboratories may also result in conflicting results as mentioned in [43].

Lineage Priming in Progenitors Prior to Lineage Commitment

Gene expression analyses of various progenitor types at the single cell level suggest that primitive progenitors co-express genes specific for multiple lineages prior to further lineage restrictions [44,45]. Therefore, promiscuous expression of lineage-specific genes, or lineage

priming, has been thought to be important for maintaining the differentiation plasticity of developing progenitors as well as HSCs. For example, single CLPs co-express T lineage related (pre-Tα and GATA3) and B lineage related (EBF and Pax5) genes [44]. Similarly, CMPs co-express genes for both MegE and GM lineages including β-globin and PU.1 [44]. More lineage-restricted progenitors, however, do not express genes of cell lineages for which they have lost potential [44]. The role and the importance of this "promiscuous" gene expression prior to specific lineage commitment are not clear. Perhaps, promiscuous gene expression helps progenitors maintain their multipotency. The promiscuous gene expression in progenitors is likely a result of an open chromosomal structure at lineage specific gene loci [46]. Therefore once progenitors commit to certain lineage, the cells must progressively silence the expression of genes associated with differentiation into alternate cell types, and must initiate the expression of genes that will ultimately contribute to the functionality of the mature cell.

Given the potential importance of promiscuous gene expression in maintaining multipotency in hematopoietic progenitors, it is interesting to note that HSCs express multiple myeloid affiliated genes but rarely express lymphoid related genes [21,47-51]. Therefore, identification of the cell population that initiates expression of lymphoid-affiliated genes would be a first step in understanding the molecular regulation of lymphoid lineage commitment. In general, when we first observe expression of lymphoid genes, we say that lymphocyte development is primed (lymphoid lineage priming).

Expression analyses of a set of lymphoid and myeloid genes suggest that neither $Flt3^{lo}VCAM\text{-}1^{+}$ nor $Flt3^{hi}VCAM\text{-}1^{+}$ MPPs express appreciable levels of lymphoid-related genes. If these $VCAM\text{-}1^{+}$ MPPs are cultured *in vitro*, RAG1 expression is promptly up-regulated by day one [29,52]. Expression of other lymphoid related genes, such as IL-7Rα and EBF is observed next [29]. Therefore, RAG1 is one of the earliest genes expressed by lymphoid-primed hematopoietic progenitors. Expression of all of these three genes is observed in $Flt3^{hi}VCAM\text{-}1^{-}$ MPPs [28], suggesting that lymphoid lineage priming occurs at this $Flt3^{hi}VCAM\text{-}1^{-}$ MPP stage. Jacobsen's group has demonstrated that $Flt3^{hi}$ KLS cells have no MegE potential but are fully able to differentiate along the GM and lymphoid lineages; this combination of differentiation potentials is also seen for $Flt3^{hi}VCAM\text{-}1^{-}$ MPPs [24]. Since the expression of both lymphoid and myeloid related genes is observed in $Flt3^{hi}$ KLS cells at a high frequency, $Flt3^{hi}$ KLS cells were named lymphoid-primed MPPs (LMPPs) [24]. This clearly suggests that the LMPP activity is enriched in the $Flt3^{hi}VCAM\text{-}1^{-}$ MPP population.

In addition to conventional phenotyping with cell surface markers by FACS, gene-marking methods with reporter genes, such as GFP, have been widely used to delineate cell populations. The advantage of this method is that one can prospectively isolate cells with or without expression of genes of interest in combination with other surface markers by FACS. One of the earliest studies to use gene-marking methods to identify and purify unique populations in the KLS fraction was done with RAG1-GFP knock-in mice, in which GFP cDNA is inserted into the RAG1 gene locus [53]. Therefore, RAG1 expression can be monitored by GFP on FACS. Kincade's group showed that $RAG1 (GFP)^{+}$ cells exist in the KLS subset [54]. These $RAG1^{+}$ KLS cells have high lymphoid and low myeloid differentiation potential, and are therefore named ELPs [54]. ELPs also express multiple lymphoid affiliated genes such as TdT, IL-7Rα, and EBF in addition to RAG1 [54,55], suggesting that ELPs and

$Flt3^{hi}VCAM-1^-$ MPPs (including LMPPs) may be largely overlapping. Our recent data suggest that ~50% of $Flt3^{hi}VCAM-1^-$ MPPs are positive for RAG1 (GFP) in RAG1-GFP KI mice, demonstrating that the ELP population is indeed a part of the $Flt3^{hi}VCAM-1^-$ MPP (and LMPP) population (cite blood paper?)(ref 52). Understanding the molecular mechanism that promotes the transition from $RAG1^-$ to $RAG1^+$ $Flt3^{hi}VCAM-1^-$ MPP may provide important insight into the earliest stage of lymphoid lineage differentiation.

Transcriptional Regulation in Cell Fate Determination

Transcription and/or nuclear factors are charged with the dual responsibility of activating and repressing gene expression. To activate a gene, transcription factors either recruit other co-activators which associate with chromatin remodeling molecules to make specific gene loci more accessible to the transcriptional machinery, or interfere with constitutive repressors at the site of transcription. When acting as repressors, transcription factors can recruit other co-repressors, and can associate with chromatin remodeling enzymes to make particular gene loci less accessible to the transcriptional machinery [56]. Cross antagonism between lineage-specific transcription factors has been suggested as one of the main mechanisms that directs cell fate decisions or lineage outcomes in progenitors [57]. For example, the transcription factors GATA1 and PU.1, which are involved in MegE and GM differentiation, respectively, have antagonistic effects in directing MegE *versus* GM cell fates in CMPs through direct physical interaction [58,59]. GATA1 antagonizes PU.1 by disrupting its interaction with c-jun, an essential co-activator for its function [58]. PU.1 in turn inhibits the DNA binding ability of GATA1 to prevent the transcriptional activation of downstream targets [59,60]. In addition to functional antagonism, cross-antagonistic effects of transcription factors on the transcription level have also been demonstrated. For example, C/EBPβ, which is important in activating genes involved in eosinophil differentiation, also represses expression of the gene, Friend of GATA-1 (FOG) [61]. Down-regulation of FOG expression is critical in driving loss of multipotency in progenitors and commitment to the eosinophil lineage [61].

After lineage commitment is established, lineage-specific transcription factors function to repress the activation of alternative cell lineage programs, in addition to promoting lineage specific maturation events. For example, Pax5, a B lineage specific transcription factor, promotes V to DJ rearrangement of the IgH locus [62], and up-regulates expression of genes that encode essential components of the BCR signaling complex, such as CD19 and Blnk [63]. Pax5 also inhibits the transcription of myeloid affiliated genes, such as that encoding the monocyte colony-stimulating factor (M-CSF) receptor, by directly binding to major transcription start sites [64]. In addition, Pax5 can antagonize ectopically introduced myeloid specific C/EBPα to prevent lineage conversion of lymphoid progenitors [65]. In Pax5 deficient mice, B cell developmental progression is blocked at Pro-B stage. $Pax5^{-/-}$ pro-B cells promiscuously express multiple non-B lineage affiliated genes, and can undergo de-differentiation and give rise to multiple cell types of the lymphoid and myeloid lineages as well as osteoclasts [66,67]. These studies highlight the dual role of lineage specific transcription factors in maintaining the identity of lineage-committed progenitors.

In addition to cross-transcriptional and functional antagonism, the dosage of lineage-specific transcription factors in progenitors of multiple lineages can influence the lineage outcome. PU.1, in addition to its involvement in myeloid differentiation, is also indispensable for B lineage differentiation [68]. Introduction of a low dose of PU.1 into PU.1 deficient fetal liver progenitors induced B cell differentiation. Conversely, a high dosage of PU.1 expression in fetal liver progenitors preferentially drove macrophage differentiation [69]. Similarly, dosage effects of PU.1 and C/EBPα have implications in macrophage *versus* granulocytic cell fate in the case of GM bipotent progenitors [70]. Whilst a low level of PU.1 maintains a mixed lineage gene expression pattern and bi-potency in GM progenitors, a high level of PU.1 is necessary to drive maturation towards the macrophage cell fate [71]. Expression of C/EBPa in PU.1 low GM progenitors is critical to direct a granulocytic cell fate. A threshold of expression level of ectopically introduced C/EBPα has also been shown to re-initiate the suppressed myeloid potential in immature B cell progenitors [65,72].

Recently, it has also been suggested that cell fate decisions can be influenced by the order of gene expression of transcription factors that are up-regulated in progenitors [73]. Ectopic introduction of C/EBPα into lymphoid committed CLPs induces lineage conversion and differentiation into GMPs. Subsequent introduction of GATA2 into these C/EBPα$^+$ GMPs results in further maturation into eosinophils [73]. However, if GATA2 is first introduced into CLPs followed by C/EBPα, CLPs will instead differentiate into mast cells [73]. These results illustrate the importance of the timing of lineage-specific transcription factor expression to the activation of the differentiation program for each cell lineage. The hierarchy of lineage-specific gene expression may have implications in lymphoid and myeloid lineage specification of HSCs. Early expression of lymphoid specific genes, which are normally absent in HSCs, may exert a negative effect on the self-renewal ability of HSCs or their multi-lineage differentiation capacity.

At present, it remains unclear how lineage specific transcription factors cross-interact to direct lymphoid vs. myeloid lineage cell fate within MPPs. In addition, it is unclear whether a transcription factor exists that activates the pan-lymphoid lineage differentiation program at the expense of myeloid differentiation. While transcription factors such as Ikaros and PU.1 appear to be very important regulators at the early stages of lymphocyte development [74], these factors are also critically involved in myeloid lineage differentiation [75]. Gene expression profiling of MPP subsets will facilitate the discovery of novel transcription factors and other molecular components that are involved in regulating lineage decisions during early hematopoiesis.

Insights into Lineage Commitment from Lineage Conversion

In general, cellular differentiation has been considered unidirectional. Therefore, once a cell has made a lineage-decision, this maturation process is irreversible [11,76]. It seems that this notion is still true under physiological conditions. However, introduction of exogenous genes can uncover the hidden differentiation potential(s) of progenitors. One classical example is "lineage conversion" from lymphoid to myeloid lineage in CLPs by stimulation with IL-2 or

granulocyte-macrophage colony-stimulating factor (GM-CSF) through exogenously expressed IL-2 receptor(R) or the receptor for GM-CSF [77]. This myeloid, more specifically, GM differentiation from CLPs is mediated by the transcription factor C/EBPα, which is up-regulated after exogenous cytokine stimulation [65]. Introduction of C/EBPα into CLPs is sufficient to induce GM differentiation. Ectopic IL-2 stimulation cannot initiate lineage conversion from T and B lineage committed progenitors, such as DN3/4 cells in the thymus and pro-B cells in the bone marrow, respectively [65,77]. However, ectopic C/EBPα can initiate the reprogramming of these committed progenitors to macrophages [72,78]. Similarly, ectopically expressed GATA-1, a MegE lineage-affiliated transcription factor, can induce erythroid lineage cell differentiation in CLPs [79]. Therefore, coordinated expression of cytokine receptors and transcription factors needs to be tightly regulated prior to the onset of oligo-potent lymphoid lineage and subsequent mono-potent T and B lineage commitment during lymphocyte development.

In contrast to lineage conversion from lymphoid to myeloid fates, very few examples of reprogramming from myeloid to lymphoid fates have been reported. The mouse pre-B cell line 70Z/3 that can spontaneously change to a macrophage-like cell type can reverse back to a pre-B phenotype upon introduction of E2A [80]. Another example is the initiation of $CD19^{+}$ pro-B cell development from CMPs (and from GMPs at a much lower efficiency) by exogenous introduction of EBF, although the conversion efficiency is very low (1 in 800) [81]. It is possible that other lymphoid-related transcription factors can efficiently reprogram myeloid progenitors to lymphocytes. But one may also assume that myeloid progenitors might be resistant to reprogramming to the lymphoid lineage once they have committed to the myeloid lineage. The step-wise and progressive loss of myeloid differentiation potential during lymphoid differntiation described above (Figure 2) suggest that the choice of lymphoid lineage by HSCs may be possible only when HSCs/MPPs do not receive any myeloid differentiation cue prior to lymphoid lineage commitment. Much more intense studies are necessary to clarify this issue.

What is the possible explanation for the relative ease of inducing lineage conversion from lymphoid to myeloid but not *vice versa*? It may be said that myeloid cell development needs fewer transcriptional inputs for their terminal differentiation output. This, in turn, may mean that the myeloid differentiation program is the default pathway in hematopoietic development. Then, what are the transcription factors that actually initiate lymphoid lineage differentiation or promote lymphoid lineage commitment? One possibility is that some T or B lymphocyte specific, but not pan-lymphoid, transcription factors play a major role in lymphoid lineage commitment. Pro-B cells have already committed to the B lineage. However, pro-B cells in which the Pax5 gene has been conditionally deleted reacquire multi-lineage differentiation and give rise to myeloid cells (macrophages) in *in vitro* cultures [82]. CLPs also express Pax5 at a low level, which is immediately down-regulated if lineage conversion is initiated by ectopic IL-2 stimulation [65]. Forced Pax5 expression can block IL-2-mediated lineage conversion in CLPs [65], suggesting that Pax5 has a role in blocking myeloid lineage potential in addition to B lineage specification [83] as mentioned above and in other chapters (see chapters 5 and 10). EBF, another B lineage specific transcription factor, also antagonizes myeloid lineage differentiation [81,84]. T cell counterparts of EBF and Pax5, however, have not been documented.

GATA-3 is indispensable for T cell development [85]. However, ectopic GATA-3 expression in HSCs results in one wave of MegE development and inhibition of the self-renewal potential of HSCs and other lineage development *in vivo* [86]. Signaling through Notch1 is essential for intrathymic T cell development [87]. It seems that Notch signaling may not be necessary for the stage transition from DN1 (or early T-cell progenitors, ETPs) to DN2 [88]. Rather, Notch signaling is indispensable for the maintenance of $CD25^+$ DN2 and DN3 cells [41,89]. $Pax5^{-/-}$ pro-B cells give rise to T cells with Notch stimulation. Upon engagement of Notch signaling, $Pax5^{-/-}$ pro-B cells promptly down-regulate expression of B cell specific genes and up-regulate T cell related genes including GATA-3 [90]. However, it was shown recently that even after the engagement of Notch, thymocytes such as DN2 cells still possess macrophage differentiation potential [37,38]. Therefore, the mechanisms that silence myeloid potential in T cell progenitors and B cell progenitors might be different. This issue is discussed in more detail in other chapters (see chapters 5 and 6).

A requirement for multiple transcription factors for cell reprogramming has been reported. As mentioned in the previous section, Akashi's group demonstrated that ordered expression of C/EBPα and GATA-2 determines the cell types upon reprogramming of CLPs [73]. A more drastic example is the induction of macrophage-like cells from fibroblasts by the ectopic expression of PU.1 and C/EBPα [91]. Recently, it was shown that pluripotent cells, namely inducible pluripotent stem (iPS) cells, can be derived from fibroblasts and other terminally differentiated cells by ectopic expression of a set of transcription factors, Oct4, Sox2, Klf4 and c-Myc [92]. The combination of transcription factors necessary for the generation of iPS cells may vary with different target cells. Interestingly, the set of four transcription factors described above is sufficient for the generation of iPS cells from immature B cells but not from terminally differentiated mature B cells [93]. Reprogramming of mature B cells required the introduction of an additional transcription factor, C/EBPα. Since C/EBPα antagonizes Pax5 function and deletion of Pax5 from mature B cells leads to loss of B lineage commitment status [72,94], immature cells may respond well to the four transcription factors and give rise to iPS cells. Since iPS cells have pluripotent differentiation potential, all types of blood cells can be generated from iPS cells derived from mature B cells. Therefore future studies may provide more clear evidence of when and what genes are necessary for lymphoid and myeloid lineage specification and commitment, possibly by searching for combinations of transcription factors that can change the phenotype of cells from one lineage to another.

Initiation of the Lymphocyte Developmental Program in the MPP Population

In the previous section, we discussed the regulation of lymphoid and myeloid lineage commitment. Prior to commitment of HSCs and MPPs to a certain lineage, promiscuous expression of genes affiliated with multiple cell lineages is observed [44]. As multiple myeloid-affiliated genes are expressed by HSCs, myeloid lineage priming has already occurred at the HSC stage. However, expression of lymphoid-related genes is first evident at the

$Flt3^{hi}VCAM\text{-}1^{-}$ MPPs (or LMPPs), after MegE potential is extinguished [28]. Then what are the genes that play a role in initiating lymphoid lineage priming?

The requirement for several lineage-specific transcription factors in lymphopoiesis was determined using conventional and conditional knockout studies. B cell development, for example, requires the expression of PU.1, Ikaros, EBF, E2A, Pax5, and Bcl11. Ablation of any of these factors, by targeted gene knockout, results in a severe defect in B cell development [83]. Advances in understanding the molecular mechanisms of lymphoid lineage specification and commitment are largely dependent on the phenotypic characterizations of bone marrow populations that are at different maturational stages. Recent progress in sub-fractionation and precise analyses of the differentiation potentials of hematopoietic and lymphoid progenitors makes it possible to re-define the true nature of the phenotypes of gene knock-out mice that were previously established [95].

The first line of Ikaros knockout mice was established by deleting a part of exon 3 and exon 4. This resulted in the generation of a dominant negative form of Ikaros ($Ikaros^{DN/DN}$) [96,97]. No mature lymphocyte populations were observed in $Ikaros^{DN/DN}$ mice, suggesting a critical role of Ikaros (and Ikaros family proteins) in lymphocyte development [97]. Since Ikaros family proteins including Ikaros and Aiolos share a common C-terminal DNA-binding domain, the dominant negative form of Ikaros may have interfered with the functions of other Ikaros family proteins. In fact, the next line of Ikaros knockout mice, in which exon 7 was deleted (Ikaros null mice), had a milder phenotype than $Ikaros^{DN/DN}$ mice in terms of lymphocyte development [96]. The KLS bone marrow fraction of cells from GFP reporter transgenic mice driven by the promoter and regulatory elements in the Ikaros gene [98] was subdivided into two populations, namely $GFP^{neg\text{-}lo}$ and GFP^{hi} [99]. $GFP^{neg\text{-}lo}$ KLS and GFP^{hi} KLS cells express Flt3 at negative to low and high levels, respectively. This suggests that in Ikaros reporter mice the $GFP^{neg\text{-}lo}$ KLS population is composed of HSCs and $Flt3^{lo}VCAM\text{-}1^{+}$ MPPs that have multi-potent differentiation potential, whereas GFP^{hi} KLS cells include $Flt3^{hi}VCAM\text{-}1^{+}$ and $Flt3^{hi}VCAM\text{-}1^{-}$ MPPs [28], which overlap significantly with LMPPs described by Adolfsson and colleagues [24]. More importantly, although the number of GFP $(Ikaros)^{hi}$ KLS cells in the absence of Ikaros (on the Ikaros null background) was not significantly changed, expression of lymphoid affiliated genes such as RAG1 and IL-7Rα was diminished, suggesting that Ikaros plays a role in lymphoid lineage priming [99].

Another transcription factor that plays a role in lymphoid lineage priming and/or commitment is PU.1. PU.1 is a member of the ETS transcription factor family and is the product of the *Spi1* oncogene [100,101]. PU.1 is necessary for GM cell (especially macrophage) and lymphocyte development, but not for MegE cell development [68,102,103]. Since PU.1 deficient mice die within 48 hrs after birth, the generation of a conditional PU.1 deficient mouse was necessary to analyze the requirement of PU.1 in adult hematopoiesis. Since PU.1 is also necessary for the self-renewal potential of HSCs [104], a precise role for PU.1 in lymphoid lineage priming is unclear. Loss of CLPs, CMPs and GMPs, but not MEPs was observed after the deletion of PU.1 in all hematopoietic cells of adult mice [104], suggesting that PU.1 is required for the generation and/or maintenance of GM and lymphoid progenitors. As mentioned above, high and low levels of PU.1 expression induce macrophage and B cell development, respectively, from MPPs in *in vitro* cultures [69]. In accordance with this functional requirement of PU.1 in different lineages, GM cells express higher levels of PU.1

than B cells judging by GFP expression levels in PU.1-GFP reporter mice [105]. PU.1 expression is up-regulated at the LMPP stage, presumably in the $Flt3^{hi}VCAM\text{-}1^{-}$ population, suggesting a functional requirement for PU.1 in lymphoid lineage priming [106]. Much higher PU.1 expression is observed in CMPs. PU.1 expression is further up-regulated in GMPs but is almost completely shut off in MEPs [105,106]. Although PU.1 regulates IL-7Rα gene expression in lymphoid progenitors [107], myeloid progenitors that express higher levels of PU.1 do not express IL-7Rα [21]. Therefore, other transcription/nuclear factors must cooperate with PU.1 in lymphoid lineage priming. Since PU.1 and GATA-1 functionally antagonize each other [58-60,108], the loss of MegE potential at the $Flt3^{hi}VCAM\text{-}1^{+}$ or LMPP population may rely on PU.1 up-regulation.

Recently, an involvement of E2A in lymphoid lineage priming has been reported [95]. E2A is a member of E-box family proteins which play various roles at multiple stages of T and B cell development [109]. Kee and colleagues found that the number of $VCAM\text{-}1^{-}$ MPPs (or $Flt3^{hi}$ LMPPs) is significantly reduced in the absence of E2A [95]. Importantly, LMPPs, defined by their cell surface phenotype, in E2A deficient mice lack expression of lymphoid-related genes such as RAG1 and IL-7Rα [95]. However, PU.1 and Ikaros expression is normal in E2A deficient LMPPs. E2A is widely expressed, including in HSCs [110]. In fact, gene expression in HSCs is altered in the absence of E2A [95]. Therefore, E2A may be a prerequisite for global lymphoid-related gene expression, but it cannot be a determinant or the trigger of lymphoid lineage priming at the LMPP stage. The combination and ordered expression of E2A, Ikaros, PU.1, and other transcription factors might be the key to initiation of the lymphocyte developmental program prior to lymphoid lineage commitment. E2A, Ikaros and PU.1 have roles in T and/or B cell development. From gene targeting studies, various other transcription factors are known to be involved in T and B cell development. These transcription factors play a role in lymphoid lineage priming in collaboration with other transcription factors.

Many transcription factors form complexes with other nuclear factors to regulate chromosomal structures. In part, this explains how a transcription factor turns on (and off) in a vast number of genes. For example, Ikaros actually has dual roles as a transcriptional activator and repressor. The latter role is evident in that Ikaros co-localizes to foci of pericentromeric heterochromatin with some transcriptionally inactive genes [111]. This suppressive function of Ikaros might be dependent on its association with components of the nucleosome remodeling deacetylase (NuRD) complex [112]. In fact, targeted disruption of Mi-2β, a SNF2-like ATPase in the NuRD complex leads to up-regulation of some genes that are normally silenced in HSCs, including lymphoid lineage affiliated RAG1 [113]. Further investigations of the regulation of global gene expression in MPPs are necessary and will help us to better understand lymphoid lineage priming.

Stochastic and Instructive Roles of Cytokines in Hematopoietic Differentiation

Precise characterization of hematopoietic progenitors has been possible since the development of semi-solid cultures using agar or methylcellulose; these require the presence of cytokines, either derived from conditional medium from cell cultures or as recombinant

proteins [114]. B cell culture systems have been also developed by culturing bone marrow cells with either primary bone marrow stromal cells or established stromal cell lines [115,116]. Under optimal conditions, both lymphoid and myeloid potential in progenitors can be examined in liquid, semi-solid and stromal cell cultures.

The cDNAs of various cytokines have been cloned based on the unique supportive role of each cytokine, as revealed by the formation of a specific type of colony in methylcellulose. Historically, these cytokines were called colony-stimulating factors (CSFs). For example, erythropoietin (EPO) supports and is indispensable for erythroid colony formation by bone marrow cells *in vitro* [117]. More strikingly, EPO or EPO receptor deficient mice die at E13.5 due to severe anemia , and demonstrate a fundamental role of EPO during erythropoiesis. Similarly, GM-CSF was identified and its cDNA was cloned based on its ability to support GM colony formation from bone marrow cells [118].

Two different modes of action of cytokines in regulating lineage commitment have been proposed, namely stochastic and instructive [76]. Several studies with gene modified mice have indicated that the role of cytokines in lineage commitment (or specification) is stochastic (or supportive) rather than instructive [119-122]. Although lineage-specific cytokines exist, in many cases lineage specificity is due to the lineage specific expression of the cytokine receptors, rather than the transduction of unique differentiation signals. For example, while signaling through the thrombopoietin (TPO) receptor is indispensable for megakaryocyte differentiation, replacement of the cytoplasmic region of TPO receptor with that of the granulocyte colony stimulating factor (G-CSF) receptor supports normal megakaryopoiesis and platelet formation [123]. Similarly, swapping the cytoplasmic domains of G-CSF receptor for those of the EPO receptor does not preferentially drive erythoid differentiation at the expense of granulopoiesis in mice [121,124]. These results suggest that different cytokine receptors can deliver the same signals to support the maturation of multiple cell lineages.

A supportive rather than instructive role of cytokine receptor signaling is also illustrated by studies of the effects of CSFs on myeloid cell differentiation. IL-3 (multi-CSF), GM-CSF, M-CSF, and G-CSF can potently stimulate hematopoietic progenitors to undergo GM differentiation *in vitro*. However, animals deficient in GM-CSF do not have major perturbations in hematopoiesis [125]. M-CSF and G-CSF deficient mice have reduced, but not a complete absence of circulating monocytes and neutrophils, respectively [126]. Monocytes and neutrophils are still present in the absence of G-CSF, GM-CSF and M-CSF [127]. These results suggest that CSFs are not involved in providing lineage specific signals in HSCs or MPPs to promote GM differentiation, but rather are involved in the steady state survival and/or expansion of progenitors *in vivo*. These cytokines may be necessary for terminal maturation stages rather than the commitment stage. The role of cytokines in promoting the survival of developing hematopoietic progenitors was further demonstrated when the anti-apoptotic factor Bcl-xL was over-expressed in EPO deficient mice, which was sufficient to rescue erythroid differentiation in the absence of EPO [128]. Similarly, enforced expression of Bcl-2 can relieve the blockade of T cell maturation in mice deficient with IL-7Rα or γ_c, the components of the IL-7 receptor complex [129,130].

Though the role of cytokines in survival and proliferation of hematopoietic progenitors is well documented, there are also examples of the involvement of cytokines in lineage specification or differentiation events. Study of the role of IL-7 receptor signaling in B cell

development has revealed its function in the differentiation of this cell lineage. While Bcl-2 over-expression can reverse the T cell developmental blockade in IL-7Rα deficient mice as mentioned above, B cell development is not rescued [130]. IL-7 is also not involved in lymphoid specification or commitment, as IL-7 deficient mice do not have a decreased number of CLPs [131]. Rather, it has been shown that IL-7 receptor signaling can directly regulate immunoglobulin gene rearrangement via STAT5 at the pro-B cell stage after B lineage commitment [132]. More recently, it has also been shown that IL-7 stimulation is important in maintaining EBF expression, a transcription factor indispensable for B cell development, in pre-pro-B cells [131,133,134]. This is critical for maintaining B cell differentiation potential and progression of pre-pro-B cells to the pro-B cell stage [131].

There are also examples of instructive effects of cytokines in determining cell fate decisions in oligopotent hematopoietic progenitors. As mentioned previously, stimulation of IL-2 through ectopically expressed IL-2 receptor in CLPs can convert cell fate from lymphoid to myeloid [77]. Similarly, GM-CSF receptor signaling cannot substitute for signals delivered by IL-7 in lymphoid progenitors, but instead redirects CLPs to the myeloid cell lineage [135]. More recently, G-CSF has also been shown to up-regulate the level of expression of C/EBPα, a transcription factor critical for granulopoiesis, to specify granulocyte cell fate in GM bipotent progenitors [70]. It remains unclear at this moment, however, whether stimulation by extrinsic factors is involved in the determination of lymphoid *versus* myeloid cell fate by MPPs.

Influence of Bone Marrow Microenvironments in Lymphoid and Myeloid Lineage Differentiation

Microenvironments, also known as niches, are specialized spatial structures or cellular components where HSCs or progenitor cells are localized so that they can receive the critical stimuli that support their differentiation and function [136-139]. Bone marrow is present in the bone cavity and is, therefore, surrounded by hard cortical bone. Osteoblasts are present at the marginal (endosteal) area between bones and marrow and are present in the HSC niche [140,141]. These osteoblasts express adhesion molecules such as N-cadherin and VCAM-1, which function to retain HSCs in their niche [140,142]. Osteoblasts also provide important signals to keep HSCs in a quiescent and undifferentiated state through various factors such as angiopoietin [143], and Jagged-1 [141], respectively. It is postulated that HSCs migrate away from their niche into the core of bone marrow upon differentiation [144]. There is another type of HSC niche, namely a vascular niche that is formed by the endosteum [17]. The vascular niche contains reticular cells that secrete CXCL12, which contributes to promoting HSC maintenance [145]. The interaction of HSCs with niche forming cells is necessary for HSCs to maintain their self-renewal potential. Specialized niches have also been shown to support different developmental stages of B cell differentiation in bone marrow [146]. While CXCL12/SDF-1α expressing cells in the bone marrow form the niche for pre-pro-B cells, pro-B cells are preferentially localized with IL-7 producing stromal cells that are distinct from those secreting SDF-1α [146]. Thus, the bone marrow microenvironment is composed of heterogenous cell populations, which may be the key to regulation of homoeostasis of

lymphoid and myeloid cell numbers *in vivo*. Recently, we demonstrated that lymphoid-biased MPPs are localized to a different region of the bone marrow than the more primitive MPPs. Disruption of the specific localization of lymphoid-biased MPPs allows them to regain myeloid lineage differentiation potential. These results strongly suggest that the localization of MPPs has implications to lineage specification and commitment, and that distinct microenvironments that support exclusively lymphoid or myeloid lineage differentiation do exist. More investigations are necessary to carefully define and characterize these distinct bone marrow microenvironments.

In the steady state, the numbers of hematopoietic cells in the periphery are stable. On some occasions, however, the pattern of hematopoiesis changes drastically. At the early phase of a microbial infection, there is a massive production of granulocytes (mostly neutrophils), leading to neutrophilia [147]. This emergency granulopoiesis is important in defense against pathogens because patients with impairment of emergency granulopoiesis are extremely susceptible to bacterial infection [148]. Invasion of pathogens is recognized by cells that are involved in innate immunity. These cells produce pro-inflammatory cytokines such as GM-CSF, IL-3, etc that enhance granulopoiesis. At the same time, mainly by the action of TNFα, immature B cells leave the bone marrow such that more space is made presumably for further granulopoiesis [149,150]. Notably, the amount of available SDF-1 in the bone marrow is reduced after TNFα administration *in vivo* [150], suggesting that inflammatory cytokines affect the bone marrow microenvironment. Presumably when SDF-1 levels in the bone marrow decrease, there is both a reduction in immature B cell retention and mobilization of developing B cells. Various inflammatory cytokines are known to induce HSC mobilization [151]. Since SDF-1 plays a crucial role in HSC retention in the bone marrow [152], HSC mobilization might occur via a similar mechanism. It seems that myeloid progenitors and lymphoid progenitors compete within the limited space in bone cavity [149]. If this is the case, the numbers of lymphoid and myeloid progenitors would correlate with the numbers of putative lymphoid and myeloid niches, respectively, and such maintain homeostasis as to the number of mature hematopoietic cells *in vivo*.

While several extrinsic factors have been identified in regulating hematopoietic differentiation, it is equally important to understand the signaling pathways that are involved. One of the MAP kinase pathways, the MEK/ERK pathway seems to be necessary for proper myeloid differentiation, most likely at the onset of myeloid lineage commitment [153]. Various stimuli can activate the MEK/ERK pathway [153]. Since constitutive activation of the MEK/ERK pathway negatively affects the lymphoid lineage choice of MPPs [153], activation of the MEK/ERK pathway might be uniquely provided by bone marrow stromal cells that form the putative myeloid niche. It is important to uncover roles of bone marrow microenvironments in hemato/lymphopoiesis in order to fully understand the regulation of homeostasis in the hematopoietic system.

Conclusion

Subfractionation and characterization of bone marrow and fetal liver progenitors have been instrumental in advancing the understanding of hematopoiesis. These studies enabled the identification of many critical lineage- and stage-specific transcription factors involved in cell differentiation, and we are beginning to understand the role of extrinsic factors and microenvironments during this differentiation process. However, most in vivo studies completed thus far untilizes the bone marrow transplantation method, which may introduce variables that affect the physiological differentiation potentials of progenitors. To examine the biological potential of bone marrow and fetal liver progenitor cells *in vivo*, we injected the cells of interest into irradiated mice, which presumably have more space in the bone marrow compared to non-irradiated mice.

However, after total body irradiation, massive systemic inflammation is observed. Therefore, some data obtained using this experimental approach may not be readily integrated into the hematopoietic tree at the steady state even if the data were obtained in an *in vivo* setting. Effects of the bone marrow microenvironment on lymphoid and myeloid specification/commitment during the steady state and specialized conditions, such as inflammation, are not new concepts, but have yet to be extensively investigated by researchers in the field of hematology and immunology. We have tried to better understand the molecular regulation of lineage commitment at the cellular and molecular levels, mainly by focusing on hematopoietic progenitors.

To this end, it may be time to consider the influence of extracellular factors on the lineage choice by MPPs. In addition, we also need to clarify the key transcription or nuclear factors that play a role in specification and/or commitment to the lymphoid and myeloid lineages. Although we have made significant advances in understanding the mechanisms that are responsible for regulating lymphoid and myeloid lineage commitment, significant efforts are still required to answer these long-standing questions in hematology and developmental immunology. FACS has become an indispensable tool for analyzing hematopoietic cell populations. However; other technical breakthroughs may be necessary to provide us with new insights into this field. We believe these advancements are coming soon.

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References

[1] Kondo, M., Wagers, A.J., Manz, M.G., Prohaska, S.S., Scherer, D.C., Beilhack, G.F., Shizuru, J.A. and Weissman, I.L.Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu Rev Immunol* (2003) 21, 759-806

[2] Weissman, I.L.Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* (2000) 287 (5457), 1442-1446

[3] Spangrude, G.J., Heimfeld, S. and Weissman, I.L.Purification and characterization of mouse hematopoietic stem cells [published erratum appears in Science 1989 Jun 2;244(4908):1030]. *Science* (1988) 241 (4861), 58-62

[4] Jordan, C.T., McKearn, J.P. and Lemischka, I.R.Cellular and developmental properties of fetal hematopoietic stem cells. *Cell* (1990) 61 (6), 953-963

[5] Smith, L.G., Weissman, I.L. and Heimfeld, S.Clonal analysis of hematopoietic stem-cell differentiation in vivo. *Proceedings of the National Academy of Sciences of the United States of America* (1991) 88 (7), 2788-2792

[6] Osawa, M., Hanada, K., Hamada, H. and Nakauchi, H.Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* (1996) 273 (5272), 242-245

[7] Matsuzaki, Y., Kinjo, K., Mulligan, R.C. and Okano, H.Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. *Immunity* (2004) 20 (1), 87-93

[8] Uchida, N. and Weissman, I.L.Searching for hematopoietic stem cells: evidence that Thy-1.1lo Lin- Sca-1+ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. *Journal of Experimental Medicine* (1992) 175 (1), 175-184

[9] Morrison, S.J. and Weissman, I.L.The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* (1994) 1 (8), 661-673

[10] Purton, L.E. and Scadden, D.T.Limiting factors in murine hematopoietic stem cell assays. *Cell Stem Cell* (2007) 1 (3), 263-270

[11] Orkin, S.H. and Zon, L.I.Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* (2008) 132 (4), 631-644

[12] Kondo, M., Scherer, D.C., King, A.G., Manz, M.G. and Weissman, I.L.Lymphocyte development from hematopoietic stem cells. *Curr Opin Genet Dev* (2001) 11 (5), 520-526

[13] Iwasaki, H. and Akashi, K.Myeloid lineage commitment from the hematopoietic stem cell. *Immunity* (2007) 26 (6), 726-740

[14] Murre, C.Defining the pathways of early adult hematopoiesis. *Cell Stem Cell* (2007) 1 (4), 357-358

[15] Morrison, S.J., Wandycz, A.M., Hemmati, H.D., Wright, D.E. and Weissman, I.L.Identification of a lineage of multipotent hematopoietic progenitors. *Development* (1997) 124 (10), 1929-1939

[16] Christensen, J.L. and Weissman, I.L.Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. *Proc Natl Acad Sci U S A* (2001) 98 (25), 14541-14546

[17] Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C. and Morrison, S.J.SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* (2005) 121 (7), 1109-1121

[18] Yang, L., Bryder, D., Adolfsson, J., Nygren, J., Mansson, R., Sigvardsson, M. and Jacobsen, S.E.Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term

hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood* (2005) 105 (7), 2717-2723

[19] Lai, A.Y. and Kondo, M.T and B lymphocyte differentiation from hematopoietic stem cell. *Semin Immunol* (2008) 20 (4), 207-212

[20] Kondo, M., Weissman, I.L. and Akashi, K.Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* (1997) 91 (5), 661-672

[21] Akashi, K., Traver, D., Miyamoto, T. and Weissman, I.L.A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* (2000) 404 (6774), 193-197

[22] Peschon, J.J., Morrissey, P.J., Grabstein, K.H., Ramsdell, F.J., Maraskovsky, E., Gliniak, B.C., Park, L.S., Ziegler, S.F., Williams, D.E., Ware, C.B., Meyer, J.D. and Davison, B.L.Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* (1994) 180 (5), 1955-1960

[23] von Freeden-Jeffry, U., Vieira, P., Lucian, L.A., McNeil, T., Burdach, S.E. and Murray, R.Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med* (1995) 181 (4), 1519-1526

[24] Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C.T., Bryder, D., Yang, L., Borge, O.J., Thoren, L.A., Anderson, K., Sitnicka, E., Sasaki, Y., Sigvardsson, M. and Jacobsen, S.E.Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell* (2005) 121 (2), 295-306

[25] Traver, D., Miyamoto, T., Christensen, J., Iwasaki-Arai, J., Akashi, K. and Weissman, I.L.Fetal liver myelopoiesis occurs through distinct, prospectively isolatable progenitor subsets. *Blood* (2001) 98 (3), 627-635

[26] Mebius, R.E., Miyamoto, T., Christensen, J., Domen, J., Cupedo, T., Weissman, I.L. and Akashi, K.The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3- cells, as well as macrophages. *Journal of Immunology* (2001) 166 (11), 6593-6601

[27] Kawamoto, H., Ohmura, K. and Katsura, Y.Presence of progenitors restricted to T, B, or myeloid lineage, but absence of multipotent stem cells, in the murine fetal thymus. *Journal of Immunology* (1998) 161 (8), 3799-3802

[28] Lai, A.Y. and Kondo, M.Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *J Exp Med* (2006) 203 (8), 1867-1873

[29] Lai, A.Y., Lin, S.M. and Kondo, M.Heterogeneity of Flt3-expressing multipotent progenitors in mouse bone marrow. *J Immunol* (2005) 175 (8), 5016-5023

[30] Forsberg, E.C., Serwold, T., Kogan, S., Weissman, I.L. and Passegue, E.New evidence supporting megakaryocyte-erythrocyte potential of flk2/flt3+ multipotent hematopoietic progenitors. *Cell* (2006) 126 (2), 415-426

[31] Katsura, Y. and Kawamoto, H.Stepwise lineage restriction of progenitors in lympho-myelopoiesis. *International Reviews of Immunology* (2001) 20 (1), 1-20

[32] Perry, S.S., Welner, R.S., Kouro, T., Kincade, P.W. and Sun, X.H.Primitive lymphoid progenitors in bone marrow with T lineage reconstituting potential. *J Immunol* (2006) 177 (5), 2880-2887

[33] Petrie, H.T. and Kincade, P.W.Many roads, one destination for T cell progenitors. *J Exp Med* (2005) 202 (1), 11-13

[34] Bhandoola, A. and Sambandam, A.From stem cell to T cell: one route or many? *Nat Rev Immunol* (2006) 6 (2), 117-126

[35] Allman, D., Sambandam, A., Kim, S., Miller, J.P., Pagan, A., Well, D., Meraz, A. and Bhandoola, A.Thymopoiesis independent of common lymphoid progenitors. *Nat Immunol* (2003) 4 (2), 168-174

[36] Sambandam, A., Maillard, I., Zediak, V.P., Xu, L., Gerstein, R.M., Aster, J.C., Pear, W.S. and Bhandoola, A.Notch signaling controls the generation and differentiation of early T lineage progenitors. *Nat Immunol* (2005) 6 (7), 663-670

[37] Wada, H., Masuda, K., Satoh, R., Kakugawa, K., Ikawa, T., Katsura, Y. and Kawamoto, H.Adult T-cell progenitors retain myeloid potential. *Nature* (2008) 452 (7188), 768-772

[38] Bell, J.J. and Bhandoola, A.The earliest thymic progenitors for T cells possess myeloid lineage potential. *Nature* (2008) 452 (7188), 764-767

[39] Benz, C., Martins, V.C., Radtke, F. and Bleul, C.C.The stream of precursors that colonizes the thymus proceeds selectively through the early T lineage precursor stage of T cell development. *J Exp Med* (2008) 205 (5), 1187-1199

[40] Lai, A.Y. and Kondo, M.Identification of a bone marrow precursor of the earliest thymocytes in adult mouse. *Proc Natl Acad Sci U S A* (2007) 104 (15), 6311-6316

[41] Schmitt, T.M. and Zuniga-Pflucker, J.C.Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* (2002) 17 (6), 749-756

[42] Mercher, T., Cornejo, M.G., Sears, C., Kindler, T., Moore, S.A., Maillard, I., Pear, W.S., Aster, J.C. and Gilliland, D.G.Notch signaling specifies megakaryocyte development from hematopoietic stem cells. *Cell Stem Cell* (2008) 3 (3), 314-326

[43] Welner, R.S., Pelayo, R. and Kincade, P.W.Evolving views on the genealogy of B cells. *Nat Rev Immunol* (2008) 8 (2), 95-106

[44] Miyamoto, T., Iwasaki, H., Reizis, B., Ye, M., Graf, T., Weissman, I.L. and Akashi, K.Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment.PG - 137-47. *Dev Cell* (2002) 3 (1), 137-147.

[45] Hu, M., Krause, D., Greaves, M., Sharkis, S., Dexter, M., Heyworth, C. and Enver, T.Multilineage gene expression precedes commitment in the hemopoietic system. *Genes Dev* (1997) 11 (6), 774-785

[46] Zardo, G., Cimino, G. and Nervi, C.Epigenetic plasticity of chromatin in embryonic and hematopoietic stem/progenitor cells: therapeutic potential of cell reprogramming. *Leukemia* (2008) 22 (8), 1503-1518

[47] Ye, M., Iwasaki, H., Laiosa, C.V., Stadtfeld, M., Xie, H., Heck, S., Clausen, B., Akashi, K. and Graf, T.Hematopoietic stem cells expressing the myeloid lysozyme gene retain long-term, multilineage repopulation potential. *Immunity* (2003) 19 (5), 689-699.

[48] Akashi, K., He, X., Chen, J., Iwasaki, H., Niu, C., Steenhard, B., Zhang, J., Haug, J. and Li, L.Transcriptional accessibility for genes of multiple tissues and hematopoietic lineages is hierarchically controlled during early hematopoiesis. *Blood* (2003) 101 (2), 383-389

[49] Terskikh, A.V., Miyamoto, T., Chang, C., Diatchenko, L. and Weissman, I.L.Gene expression analysis of purified hematopoietic stem cells and committed progenitors. *Blood* (2003) 102 (1), 94-101. Epub 2003 Mar 2006.

[50] Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R.C. and Melton, D.A."Stemness": transcriptional profiling of embryonic and adult stem cells. *Science* (2002) 298 (5593), 597-600

[51] Ivanova, N.B., Dimos, J.T., Schaniel, C., Hackney, J.A., Moore, K.A. and Lemischka, I.R.A stem cell molecular signature. *Science* (2002) 298 (5593), 601-604

[52] Lai, A.Y., Watanabe, A., O'Brien, T. and Kondo, M.Pertussis toxin-sensitive G proteins regulate lymphoid lineage specification in multipotent hematopoietic progenitors. *Blood* (2009) 113 (23), 5757-5764

[53] Kuwata, N., Igarashi, H., Ohmura, T., Aizawa, S. and Sakaguchi, N.Cutting edge: absence of expression of RAG1 in peritoneal B-1 cells detected by knocking into RAG1 locus with green fluorescent protein gene. *J Immunol* (1999) 163 (12), 6355-6359

[54] Igarashi, H., Gregory, S.C., Yokota, T., Sakaguchi, N. and Kincade, P.W.Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity* (2002) 17 (2), 117-130

[55] Yokota, T., Kouro, T., Hirose, J., Igarashi, H., Garrett, K.P., Gregory, S.C., Sakaguchi, N., Owen, J.J. and Kincade, P.W.Unique properties of fetal lymphoid progenitors identified according to RAG1 gene expression. *Immunity* (2003) 19 (3), 365-375

[56] Muller, C. and Leutz, A.Chromatin remodeling in development and differentiation. *Curr Opin Genet Dev* (2001) 11 (2), 167-174

[57] Orkin, S.H.Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet* (2000) 1 (1), 57-64

[58] Zhang, P., Behre, G., Pan, J., Iwama, A., Wara-Aswapati, N., Radomska, H.S., Auron, P.E., Tenen, D.G. and Sun, Z.Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1. *Proc Natl Acad Sci U S A* (1999) 96 (15), 8705-8710

[59] Zhang, P., Zhang, X., Iwama, A., Yu, C., Smith, K.A., Mueller, B.U., Narravula, S., Torbett, B.E., Orkin, S.H. and Tenen, D.G.PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. *Blood* (2000) 96 (8), 2641-2648

[60] Rekhtman, N., Radparvar, F., Evans, T. and Skoultchi, A.I.Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells. *Genes Dev* (1999) 13 (11), 1398-1411

[61] Querfurth, E., Schuster, M., Kulessa, H., Crispino, J.D., Doderlein, G., Orkin, S.H., Graf, T. and Nerlov, C.Antagonism between C/EBPbeta and FOG in eosinophil lineage commitment of multipotent hematopoietic progenitors. *Genes & Development* (2000) 14 (19), 2515-2525

[62] Fuxa, M., Skok, J., Souabni, A., Salvagiotto, G., Roldan, E. and Busslinger, M.Pax5 induces V-to-DJ rearrangements and locus contraction of the immunoglobulin heavy-chain gene. *Genes & Development* (2004) 18 (4), 411-422

[63] Nutt, S.L., Morrison, A.M., Dorfler, P., Rolink, A. and Busslinger, M.Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. *EMBO Journal* (1998) 17 (8), 2319-2333

[64] Tagoh, H., Ingram, R., Wilson, N., Salvagiotto, G., Warren, A.J., Clarke, D., Busslinger, M. and Bonifer, C.The mechanism of repression of the myeloid-specific c-fms gene by Pax5 during B lineage restriction. *EMBO Journal* (2006) 25 (5), 1070-1080

[65] Hsu, C.L., King-Fleischman, A.G., Lai, A.Y., Matsumoto, Y., Weissman, I.L. and Kondo, M.Antagonistic effect of CCAAT enhancer-binding protein-alpha and Pax5 in myeloid or lymphoid lineage choice in common lymphoid progenitors. *Proc Natl Acad Sci U S A* (2006) 103 (3), 672-677

[66] Nutt, S.L., Heavey, B., Rolink, A.G. and Busslinger, M.Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature* (1999) 401 (6753), 556-562

[67] Rolink, A.G., Nutt, S.L., Melchers, F. and Busslinger, M.Long-term in vivo reconstitution of T-cell development by Pax5-deficient B-cell progenitors.[see comment]. *Nature* (1999) 401 (6753), 603-606

[68] Scott, E.W., Simon, M.C., Anastasi, J. and Singh, H.Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* (1994) 265 (5178), 1573-1577

[69] DeKoter, R.P. and Singh, H.Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science* (2000) 288 (5470), 1439-1441

[70] Dahl, R., Walsh, J.C., Lancki, D., Laslo, P., Iyer, S.R., Singh, H. and Simon, M.C.Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. *Nat Immunol* (2003) 4 (10), 1029-1036. Epub 2003 Sep 1027.

[71] Laslo, P., Spooner, C.J., Warmflash, A., Lancki, D.W., Lee, H.J., Sciammas, R., Gantner, B.N., Dinner, A.R. and Singh, H.Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell* (2006) 126 (4), 755-766

[72] Xie, H., Ye, M., Feng, R. and Graf, T.Stepwise reprogramming of B cells into macrophages. *Cell* (2004) 117 (5), 663-676.

[73] Iwasaki, H., Mizuno, S., Arinobu, Y., Ozawa, H., Mori, Y., Shigematsu, H., Takatsu, K., Tenen, D.G. and Akashi, K.The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. *Genes & Development* (2006) 20 (21), 3010-3021

[74] Medina, K.L. and Singh, H.Genetic networks that regulate B lymphopoiesis. *Curr Opin Hematol* (2005) 12 (3), 203-209

[75] Ye, M. and Graf, T.Early decisions in lymphoid development. *Current Opinion in Immunology* (2007) 19, 1-6

[76] Metcalf, D.Stem cells, pre-progenitor cells and lineage-committed cells: are our dogmas correct? *Annals of the New York Academy of Sciences* (1999) 872, 289-303; discussion 303-284

[77] Kondo, M., Scherer, D.C., Miyamoto, T., King, A.G., Akashi, K., Sugamura, K. and Weissman, I.L.Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. *Nature* (2000) 407 (6802), 383-386

[78] Laiosa, C.V., Stadtfeld, M., Xie, H., de Andres-Aguayo, L. and Graf, T.Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBP alpha and PU.1 transcription factors. *Immunity* (2006) 25 (5), 731-744

[79] Iwasaki, H., Mizuno, S., Wells, R.A., Cantor, A.B., Watanabe, S. and Akashi, K.GATA-1 converts lymphoid and myelomonocytic progenitors into the megakaryocyte/erythrocyte lineages. *Immunity* (2003) 19 (3), 451-462

[80] Kee, B.L. and Murre, C.Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix-loop-helix transcription factor E12. *J Exp Med* (1998) 188 (4), 699-713

[81] Pongubala, J.M., Northrup, D.L., Lancki, D.W., Medina, K.L., Treiber, T., Bertolino, E., Thomas, M., Grosschedl, R., Allman, D. and Singh, H.Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5. *Nat Immunol* (2008) 9 (2), 203-215

[82] Mikkola, I., Heavey, B., Horcher, M. and Busslinger, M.Reversion of B cell commitment upon loss of Pax5 expression. *Science* (2002) 297 (5578), 110-113

[83] Nutt, S.L. and Kee, B.L.The transcriptional regulation of B cell lineage commitment. *Immunity* (2007) 26 (6), 715-725

[84] Zhang, Z., Cotta, C.V., Stephan, R.P., deGuzman, C.G. and Klug, C.A.Enforced expression of EBF in hematopoietic stem cells restricts lymphopoiesis to the B cell lineage. *EMBO J* (2003) 22 (18), 4759-4769

[85] Ting, C.N., Olson, M.C., Barton, K.P. and Leiden, J.M.Transcription factor GATA-3 is required for development of the T-cell lineage. *Nature* (1996) 384 (6608), 474-478

[86] Chen, D. and Zhang, G.Enforced expression of the GATA-3 transcription factor affects cell fate decisions in hematopoiesis. *Exp Hematol* (2001) 29 (8), 971-980

[87] Tan, J.B., Visan, I., Yuan, J.S. and Guidos, C.J.Requirement for Notch1 signals at sequential early stages of intrathymic T cell development. *Nat Immunol* (2005) 6 (7), 671-679

[88] Tsuji, M., Shinkura, R., Kuroda, K., Yabe, D. and Honjo, T.Msx2-interacting nuclear target protein (Mint) deficiency reveals negative regulation of early thymocyte differentiation by Notch/RBP-J signaling. *Proc Natl Acad Sci U S A* (2007) 104 (5), 1610-1615

[89] Ciofani, M. and Zuniga-Pflucker, J.C.Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. *Nat Immunol* (2005) 6 (9), 881-888

[90] Hoflinger, S., Kesavan, K., Fuxa, M., Hutter, C., Heavey, B., Radtke, F. and Busslinger, M.Analysis of Notch1 function by in vitro T cell differentiation of Pax5 mutant lymphoid progenitors. *J Immunol* (2004) 173 (6), 3935-3944

[91] Feng, R., Desbordes, S.C., Xie, H., Tillo, E.S., Pixley, F., Stanley, E.R. and Graf, T.PU.1 and C/EBPalpha/beta convert fibroblasts into macrophage-like cells. *Proc Natl Acad Sci U S A* (2008) 105 (16), 6057-6062

[92] Takahashi, K. and Yamanaka, S.Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* (2006) 126 (4), 663-676

[93] Hanna, J., Markoulaki, S., Schorderet, P., Carey, B.W., Beard, C., Wernig, M., Creyghton, M.P., Steine, E.J., Cassady, J.P., Foreman, R., Lengner, C.J., Dausman, J.A. and Jaenisch, R.Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. *Cell* (2008) 133 (2), 250-264

[94] Cobaleda, C., Jochum, W. and Busslinger, M.Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors. *Nature* (2007) 449 (7161), 473-477

[95] Dias, S., Mansson, R., Gurbuxani, S., Sigvardsson, M. and Kee, B.L.E2A Proteins Promote Development of Lymphoid-Primed Multipotent Progenitors. *Immunity* (2008) 29 (2), 217-227

[96] Wang, J.H., Nichogiannopoulou, A., Wu, L., Sun, L., Sharpe, A.H., Bigby, M. and Georgopoulos, K.Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity* (1996) 5 (6), 537-549

[97] Georgopoulos, K., Bigby, M., Wang, J.H., Molnar, A., Wu, P., Winandy, S. and Sharpe, A.The Ikaros gene is required for the development of all lymphoid lineages. *Cell* (1994) 79 (1), 143-156

[98] Kaufmann, C., Yoshida, T., Perotti, E.A., Landhuis, E., Wu, P. and Georgopoulos, K.A complex network of regulatory elements in Ikaros and their activity during hemo-lymphopoiesis. *Embo J* (2003) 22 (9), 2211-2223

[99] Yoshida, T., Ng, S.Y., Zuniga-Pflucker, J.C. and Georgopoulos, K.Early hematopoietic lineage restrictions directed by Ikaros. *Nat Immunol* (2006) 7 (4), 382-391

[100] Klemsz, M.J., McKercher, S.R., Celada, A., Van Beveren, C. and Maki, R.A.The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. *Cell* (1990) 61 (1), 113-124
[101] Rosenbauer, F. and Tenen, D.G.Transcription factors in myeloid development: balancing differentiation with transformation. *Nat Rev Immunol* (2007) 7 (2), 105-117
[102] McKercher, S.R., Torbett, B.E., Anderson, K.L., Henkel, G.W., Vestal, D.J., Baribault, H., Klemsz, M., Feeney, A.J., Wu, G.E., Paige, C.J. and Maki, R.A.Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *Embo J* (1996) 15 (20), 5647-5658
[103] Scott, E.W., Fisher, R.C., Olson, M.C., Kehrli, E.W., Simon, M.C. and Singh, H.PU.1 functions in a cell-autonomous manner to control the differentiation of multipotential lymphoid-myeloid progenitors. *Immunity* (1997) 6 (4), 437-447
[104] Iwasaki, H., Somoza, C., Shigematsu, H., Duprez, E.A., Iwasaki-Arai, J., Mizuno, S., Arinobu, Y., Geary, K., Zhang, P., Dayaram, T., Fenyus, M.L., Elf, S., Chan, S., Kastner, P., Huettner, C.S., Murray, R., Tenen, D.G. and Akashi, K.Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood* (2005) 106 (5), 1590-1600
[105] Nutt, S.L., Metcalf, D., D'Amico, A., Polli, M. and Wu, L.Dynamic regulation of PU.1 expression in multipotent hematopoietic progenitors. *J Exp Med* (2005) 201 (2), 221-231
[106] Arinobu, Y., Mizuno, S., Chong, Y., Shigematsu, H., Iino, T., Iwasaki, H., Graf, T., Mayfield, R., Chan, S., Kastner, P. and Akashi, K.Reciprocal activation of GATA-1 and PU.1 marks initial specification of hematopoietic stem cells into myeloerythroid and myelolymphoid lineages. *Cell Stem Cell* (2007) 1 (4), 416-427
[107] DeKoter, R.P., Lee, H.J. and Singh, H.PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors. *Immunity* (2002) 16 (2), 297-309
[108] Nerlov, C., Querfurth, E., Kulessa, H. and Graf, T.GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription. *Blood* (2000) 95 (8), 2543-2551
[109] Murre, C.Helix-loop-helix proteins and lymphocyte development. *Nat Immunol* (2005) 6 (11), 1079-1086
[110] Zhuang, Y., Jackson, A., Pan, L., Shen, K. and Dai, M.Regulation of E2A gene expression in B-lymphocyte development. *Mol Immunol* (2004) 40 (16), 1165-1177
[111] Brown, K.E., Guest, S.S., Smale, S.T., Hahm, K., Merkenschlager, M. and Fisher, A.G.Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell* (1997) 91 (6), 845-854
[112] Kim, J., Sif, S., Jones, B., Jackson, A., Koipally, J., Heller, E., Winandy, S., Viel, A., Sawyer, A., Ikeda, T., Kingston, R. and Georgopoulos, K.Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes. *Immunity* (1999) 10 (3), 345-355
[113] Yoshida, T., Hazan, I., Zhang, J., Ng, S.Y., Naito, T., Snippert, H.J., Heller, E.J., Qi, X., Lawton, L.N., Williams, C.J. and Georgopoulos, K.The role of the chromatin remodeler Mi-2beta in hematopoietic stem cell self-renewal and multilineage differentiation. *Genes Dev* (2008) 22 (9), 1174-1189
[114] Metcalf, D.Hematopoietic cytokines. *Blood* (2008) 111 (2), 485-491
[115] Dorshkind, K.In vitro differentiation of B lymphocytes from primitive hematopoietic precursors present in long-term bone marrow cultures. *J Immunol.* (1986) 136 (2), 422-429

[116] Collins, L.S. and Dorshkind, K.A stromal cell line from myeloid long-term bone marrow cultures can support myelopoiesis and B lymphopoiesis. *J Immunol.* (1987) 138 (4), 1082-1087

[117] Krantz, S.B.Erythropoietin. *Blood* (1991) 77 (3), 419-434

[118] Gough, N.M., Gough, J., Metcalf, D., Kelso, A., Grail, D., Nicola, N.A., Burgess, A.W. and Dunn, A.R.Molecular cloning of cDNA encoding a murine haematopoietic growth regulator, granulocyte-macrophage colony stimulating factor. *Nature* (1984) 309 (5971), 763-767

[119] Fairbairn, L.J., Cowling, G.J., Reipert, B.M. and Dexter, T.M.Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors. *Cell* (1993) 74 (5), 823-832

[120] Socolovsky, M., Lodish, H.F. and Daley, G.Q.Control of hematopoietic differentiation: lack of specificity in signaling by cytokine receptors. *Proc Natl Acad Sci U S A* (1998) 95 (12), 6573-6575

[121] Semerad, C.L., Poursine-Laurent, J., Liu, F. and Link, D.C.A role for G-CSF receptor signaling in the regulation of hematopoietic cell function but not lineage commitment or differentiation. *Immunity* (1999) 11 (2), 153-161

[122] Jacob, J., Haug, J.S., Raptis, S. and Link, D.C.Specific signals generated by the cytoplasmic domain of the granulocyte colony-stimulating factor (G-CSF) receptor are not required for G-CSF-dependent granulocytic differentiation. *Blood* (1998) 92 (2), 353-361

[123] Goldsmith, M.A., Mikami, A., You, Y., Liu, K.D., Thomas, L., Pharr, P. and Longmore, G.D.Absence of cytokine receptor-dependent specificity in red blood cell differentiation in vivo. *Proc Natl Acad Sci U S A* (1998) 95 (12), 7006-7011

[124] Stoffel, R., Ziegler, S., Ghilardi, N., Ledermann, B., de Sauvage, F.J. and Skoda, R.C.Permissive role of thrombopoietin and granulocyte colony-stimulating factor receptors in hematopoietic cell fate decisions in vivo. *Proceedings of the National Academy of Sciences of the United States of America* (1999) 96 (2), 698-702

[125] Stanley, E., Lieschke, G.J., Grail, D., Metcalf, D., Hodgson, G., Gall, J.A., Maher, D.W., Cebon, J., Sinickas, V. and Dunn, A.R.Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc Natl Acad Sci U S A* (1994) 91 (12), 5592-5596

[126] Lieschke, G.J., Stanley, E., Grail, D., Hodgson, G., Sinickas, V., Gall, J.A., Sinclair, R.A. and Dunn, A.R.Mice lacking both macrophage- and granulocyte-macrophage colony-stimulating factor have macrophages and coexistent osteopetrosis and severe lung disease. *Blood* (1994) 84 (1), 27-35

[127] Hibbs, M.L., Quilici, C., Kountouri, N., Seymour, J.F., Armes, J.E., Burgess, A.W. and Dunn, A.R.Mice lacking three myeloid colony-stimulating factors (G-CSF, GM-CSF, and M-CSF) still produce macrophages and granulocytes and mount an inflammatory response in a sterile model of peritonitis. *J Immunol* (2007) 178 (10), 6435-6443

[128] Dolznig, H., Habermann, B., Stangl, K., Deiner, E.M., Moriggl, R., Beug, H. and Mullner, E.W.Apoptosis protection by the Epo target Bcl-X(L) allows factor-independent differentiation of primary erythroblasts. *Current Biology* (2002) 12 (13), 1076-1085

[129] Kondo, M., Akashi, K., Domen, J., Sugamura, K. and Weissman, I.L.Bcl-2 rescues T lymphopoiesis, but not B or NK cell development, in common gamma chain-deficient mice. *Immunity* (1997) 7 (1), 155-162

[130] Akashi, K., Kondo, M., von Freeden-Jeffry, U., Murray, R. and Weissman, I.L.Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. *Cell* (1997) 89 (7), 1033-1041
[131] Kikuchi, K., Lai, A.Y., Hsu, C.L. and Kondo, M.IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF. *J Exp Med* (2005) 201 (8), 1197-1203
[132] Bertolino, E., Reddy, K., Medina, K.L., Parganas, E., Ihle, J. and Singh, H.Regulation of interleukin 7-dependent immunoglobulin heavy-chain variable gene rearrangements by transcription factor STAT5. *Nature Immunology* (2005) 6 (8), 836-843
[133] Dias, S., Silva, H., Jr., Cumano, A. and Vieira, P.Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors. *J Exp Med* (2005) 201 (6), 971-979
[134] Kikuchi, K., Kasai, H., Watanabe, A., Lai, A.Y. and Kondo, M.IL-7 specifies B cell fate at the common lymphoid progenitor to pre-proB transition stage by maintaining early B cell factor expression. *J Immunol* (2008) 181 (1), 383-392
[135] Iwasaki-Arai, J., Iwasaki, H., Miyamoto, T., Watanabe, S. and Akashi, K.Enforced granulocyte/macrophage colony-stimulating factor signals do not support lymphopoiesis, but instruct lymphoid to myelomonocytic lineage conversion. *J Exp Med* (2003) 197 (10), 1311-1322.
[136] Heissig, B., Ohki, Y., Sato, Y., Rafii, S., Werb, Z. and Hattori, K.A role for niches in hematopoietic cell development. *Hematology* (2005) 10 (3), 247-253
[137] Arai, F. and Suda, T.Maintenance of quiescent hematopoietic stem cells in the osteoblastic niche. *Ann N Y Acad Sci* (2007) 1106, 41-53
[138] Nagasawa, T.Microenvironmental niches in the bone marrow required for B-cell development. *Nat Rev Immunol* (2006) 6 (2), 107-116
[139] Morrison, S.J. and Spradling, A.C.Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell* (2008) 132 (4), 598-611
[140] Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., Harris, S., Wiedemann, L.M., Mishina, Y. and Li, L.Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* (2003) 425 (6960), 836-841
[141] Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R., Milner, L.A., Kronenberg, H.M. and Scadden, D.T.Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* (2003) 425 (6960), 841-846
[142] Rafii, S., Mohle, R., Shapiro, F., Frey, B.M. and Moore, M.A.Regulation of hematopoiesis by microvascular endothelium. *Leukemia & Lymphoma* (1997) 27 (5-6), 375-386
[143] Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y. and Suda, T.Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche.[see comment]. *Cell* (2004) 118 (2), 149-161
[144] Suda, T., Arai, F. and Hirao, A.Hematopoietic stem cells and their niche. *Trends Immunol* (2005) 26 (8), 426-433
[145] Sugiyama, T., Kohara, H., Noda, M. and Nagasawa, T.Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in th bone marrow stromal cell niches. *Immunity* (2006) 25 (6), 977-988

[146] Tokoyoda, K., Egawa, T., Sugiyama, T., Choi, B.I. and Nagasawa, T.Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity* (2004) 20 (6), 707-718

[147] Burg, N.D. and Pillinger, M.H.The neutrophil: function and regulation in innate and humoral immunity. *Clin Immunol* (2001) 99 (1), 7-17

[148] Panopoulos, A.D. and Watowich, S.S.Granulocyte colony-stimulating factor: molecular mechanisms of action during steady state and 'emergency' hematopoiesis. *Cytokines* (2008) 42 (3), 277-288

[149] Ueda, Y., Kondo, M. and Kelsoe, G.Inflammation and the reciprocal production of granulocytes and lymphocytes in bone marrow. *J Exp Med* (2005) 201 (11), 1771-1780

[150] Ueda, Y., Yang, K., Foster, S.J., Kondo, M. and Kelsoe, G.Inflammation controls B lymphopoiesis by regulating chemokine CXCL12 expression. *J Exp Med* (2004) 199 (1), 47-58

[151] Lane, T.A., Law, P., Maruyama, M., Young, D., Burgess, J., Mullen, M., Mealiffe, M., Terstappen, L.W., Hardwick, A. and Moubayed, M.Harvesting and enrichment of hematopoietic progenitor cells mobilized into the peripheral blood of normal donors by granulocyte-macrophage colony-stimulating factor (GM-CSF) or G-CSF: potential role in allogeneic marrow transplantation. *Blood* (1995) 85, 275-282

[152] Ara, T., Tokoyoda, K., Sugiyama, T., Egawa, T., Kawabata, K. and Nagasawa, T.Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. *Immunity* (2003) 19 (2), 257-267

[153] Hsu, C.L., Kikuchi, K. and Kondo, M.Activation of mitogen-activated protein kinase kinase (MEK)/extracellular signal regulated kinase (ERK) signaling pathway is involved in myeloid lineage commitment. *Blood* (2007) 110 (5), 1420-1428

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Chapter 3

Intracellular Signaling for Granulocytic and Monocytic Differentiation

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Abstract

In this chapter we discuss the current understanding of the development of myeloid cells from hematopoietic stem cells (HSC), step-wise to their mature forms: granulocytes and monocytes. We delineate the characteristics of mature myeloid cells and show which transcription factors are necessary for their appearance. We also describe cytokines, signal transduction pathways and microRNAs (miRNAs) which modulate expression and activities of transcription factors that drive myeloid differentiation of HSC.

Introduction

The focus of this chapter is on myeloid cells, that is, granulocytes, monocytes and their immature forms. Despite there being a plethora of information regarding cellular signaling, and although the key regulators of the process of differentiation to the mature forms have been identified, as summarized in Fig 1, we are far from fully understanding how myelopoiesis works.

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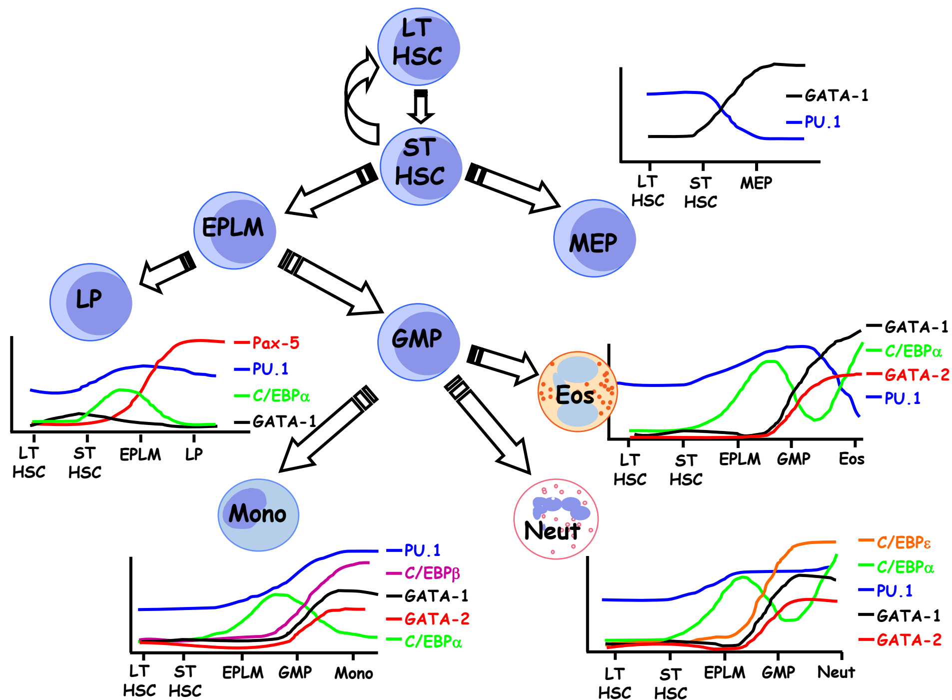

Figure 1. The principal factors in granulocytic and monocytic differentiation.

Cell fate during hematopoiesis is governed by spatiotemporal fluctuations in transcription factor concentrations, which either cooperate or compete in regard to driving target gene expression. The sequence of fluctuations of the transcription factors currently considered the most important for the differentiation of megakaryocyte and erythrocyte progenitors (MEPs), lymphocyte progenitors, and cells of the myeloid lineage is presented as a series of graphs. Arbitrary units are assigned to the abundance levels of these transcription factors; they do not reflect real protein concentrations, but indicate changes that drive the differentiation process. Further, the choice of one differentiation pathway option does not totally preclude other options, as lymphocyte progenitors still retain myeloid differentiation potential [1,2]. LT-HSCs, long-term reconstituting HSCs; ST-HSCs, short-term reconstituting HSCs; EPLM, early progenitor with lymphoid and myeloid potentials; LP, lymphoid progenitor; GMP, granulocyte and monocyte progenitor, Mono, monocyte; Eos, eosinophil; Neut, neutrophil. (This diagram is based on and modified from references [3] and [4]).

As shown in the Figure 1, a current emphasis is on the role of transcription factors, and although many of these participate, there is evidence that some have critical roles in lineage selection. In a wider context, discussed in detail in chapter 10 [5,6], a currently accepted model of hematopoiesis posits that differentiation of particular blood cell lineages consists of sequential losses and the appearance of specific lineage potentials, which are driven by asymmetric cell divisions and spatiotemporal fluctuations in transcription factor concentrations. It is now well established in the murine model that mature neutrophils and macrophages develop in a step-wise manner from lymphoid-myeloid progenitors (LMPs), which have already lost the potential to become platelets and erythrocytes, but are still able to

become lymphocytes [5,7]. Initially, through asymmetric divisions the long-term reconstituting hematopoietic stem cells (LT-HSCs) self-renew and give rise to short-term reconstituting HSCs (ST-HSCs), which in turn lose self-renewal capacity, but still possess the ability to initiate differentiation into all hematopoietic lineages [8]. LT-HSCs, which retain their capacity for self-renewal, can be defined by the absence of CD34 and low expression of FMS-like tyrosine kinase-3 receptor (Flt-3; CD135). ST-HSCs have high expression of CD34 and low expression of CD135, and at this stage they either differentiate to megakaryocyte-erythrocyte progenitors (MEPs) with low expression of CD135, or LMPs, in which CD135 expression is high [9,10]. At this stage of lineage commitment the PU.1 transcription factor seems to be the key player. PU.1 is present at moderate levels in HSCs and in LMPs, while its expression decreases in MEPs [11]. PU.1 levels rise in cells undergoing the myeloid pathway of differentiation, and a high expression level favors monocyte development, while a medium level determines granulopoiesis [12]. In contrast, entrance into the megakaryocyte and erythrocyte pathway requires the GATA-1 transcription factor, which competes with PU.1 for DNA binding [13]. In cells that enter the myeloid pathway PU.1 expression is accompanied by transient expression of CCAAT/enhancer binding protein α (C/EBPα), but the expression of GATA-1 and GATA-2 transcription factors cannot be detected [14]. These two GATA factors re-emerge at later stages of granulopoiesis, but not in lymphoid development. C/EBPα also reappears, and its presence favors granulocytic over lymphocytic development [3], by competition with Pax5, which seems crucial for expansion of lymphocytes, particularly B lymphocytes. The cells that still express PU.1 together with a second wave of C/EBPα will become granulocytes, while a subsequent decrease in C/EBPα [3,14] and increased levels of C/EBPβ together with a continuously high level of PU.1 will lead to monocyte and macrophage development [15,16]. Additionally, at the late stages of granulopoiesis another member of C/EBP family, C/EBPε, is up-regulated and leads to neutrophil differentiation [17]. However, when the wave of C/EBPα is followed by expression of GATA-2 granulocyte precursors become redirected to the eosinophilic lineage [14].

The current knowledge of precisely what signals cause changes in the expression and activity of transcription factors, and further details of the transcriptional control of myeloid lineage selection and maintenance, will be discussed below. It is important to note, however, that since normal human material is difficult to obtain and study, the preponderance of data has been obtained in murine systems and from analysis of alterations in human leukemic cells. While attempts are being made to unify the picture, not all current models may apply to normal human hematopoiesis.

The Mature Myeloid Phenotype

The process of differentiation leads to the acquisition of functional properties by the terminally differentiated cells. In order to comprehend the individual steps, it is useful to be acquainted with the characteristics of these mature end products.

Granulocytes and **monocytes** are white blood cells that are very important for innate immunity. Their early development occurs initially in the yolk-sac, then in the dorsal aorta, fetal liver and spleen and finally in a bone marrow of adult organisms [18]. As mentioned

above, development is driven by a sequential emergence of transcription factors [3], but requires also parallel signals provided by cytokines, growth factors and their receptors [19].

Granulocytes constitute the majority of leukocytes and there are approximately 2.5-7.5x10^9 of them in each liter of human blood. The term “granulocytes” refers to three lineages of cells, neutrophils being most numerous (2.5-7 x 10^9), eosinophils less numerous (0.4-4x10^8/L), and basophils the rarest (0.1-1x10^8/L). The nomenclature is based on differences in cytoplasmic granules and in nuclear staining patterns**,** but they also differ in their physiological roles. Neutrophils constitute the major phagocytic force of the immune system; eosinophils fight parasitic infections, and basophils are important regulators of the inflammatory response. The turnover of neutrophils in the organism is very rapid, since these cells are short-lived: their half-life in circulating blood is 6-10 hours. In appropriate circumstances they enter inflamed tissues, where they can persist for some more days before they die by apoptosis [20].

The number of monocytes in circulating blood is much lower than that of granulocytes, usually less than 1 x 10^9/L. They phagocytose pathogens and other solid particles, and later may present ingested antigens to lymphocytes, thus linking the innate and acquired immune systems. Monocytes persist longer than granulocytes, several days in circulating blood and even months in tissues, where they further differentiate into macrophages [20].

This comparison shows that *in vivo* the production of granulocytes must greatly outnumber that of monocytes. In perfectly functioning organisms this production is precisely regulated and occurs in the right proportions.

The Most Characteristic Proteins Expressed in Granulocytes and Monocytes

Neutrophils store their most characteristic proteins in cytoplasmic secretory vesicles and in granules [21]. Secretory vesicles contain a range of adhesion molecules, receptors for many different cytokines and chemotactic agents, while the granules contain various antibacterial proteins and enzymes. The ability to pre-form all of the proteins that are necessary to the inflammation process, to store them intracellularly and to empty the stores in response to appropriate signals allows the neutrophils to respond quickly, and to minimize potential tissue damage during inflammation [21]. In order to achieve this effect, neutrophils must be able to activate transcription factors that drive the production of stored proteins at different stages of their differentiation. There are dozens of proteins contained in secretory vesicles and granules, including adhesion molecules such as L-selectin, CD11b, CD18; receptors for tumor necrosis factor (TNF), interferon α (IFNα), transforming growth factor beta (TGFβ) and many chemokine receptors. The most characteristic antibacterial proteins are defensins, lysozyme and lactoferrin, while proteases are represented by elastase, gelatinase, collagenase and cathepsin G [21].

Eosinophils are believed to be the major anti-parasitic forces of the organism. Their granules contain four of the most characteristic proteins: eosinophil peroxidase, major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN)

[22]. As ECP and EDN are ribonucleases, members of the RNase A family with proven activity against respiratory syncytial virus, an antiviral role has been attributed to eosinophils as well [22].

Basophils posses high-affinity receptors for immunoglobulin E (FcεR-I), which allow them to release mediators of inflammation in response to immunoglobulin of this class [23]. Their granules contain pre-formed mediators of inflammation that are released immediately, such as histamine, heparin or TNFα [20]. These cells are also able to produce some later mediators of inflammation, such as interleukins 4 and 13 (IL-4 and IL-13), which induce T helper cell differentiation to the type 2 (Th2) phenotype [24]. In this regard, basophils also constitute a link between innate and acquired immunity.

Monocytes are not abundant, but are very important cells capable of presenting antigens via their MHC class II molecules. Their population in peripheral blood is heterogenous and consists of two main subsets of cells. The larger one (80-95%) is defined by the presence of CD14, and absence of CD16, while the remaining monocytes have low expression of CD14 and high CD16 [25]. MHC-II molecules are present on cells of both populations, but the level of expression is higher on the second subset, making them more efficient in antigen presentation. Moreover monocytes that belong to this first and more abundant subset carry two receptors on their surface: CD32 (FcγR-II) and CD64 (FcγR-I) [26], and are potent phagocytes which is important to the innate immune response. All these cells contain monocyte-specific esterase (MSE) [27], localized in the endoplasmic reticulum [28], and myeloperoxidase (MPO) [26] enclosed in granules [29]. Monocytes migrate to tissues, where they differentiate to macrophages, and some to dendritic cells [25].

Transcription Factors in Developmental Steps from Hematopoietic Stem Cell to Granulocytes and Monocytes

As illustrated in Figure 1 the preponderance of current data suggests that **lineage selection** within the myeloid cell series depends on the appropriate levels of key transcription factors. C/EBPα and PU.1 work in concert with a variety of other factors which include other members of the C/EBP family, as well as components of the transcription factor AP-1, VDR, RXR and RAR, EGR-1/2, and perhaps AML-1. The latter factors modulate the effects of the key regulators such as C/EBPs, and may have a greater role in **lineage maintenance**, rather than in their initiation. Also of importance are the CCAAT displacement protein (CDP) and members of the homeobox (HOX) family of transcription factors, the levels of which must be reduced for differentiation to proceed.

Box 1. PU.1 Transcription Factor

PU.1 belongs to the Ets family of transcription factors. Proteins that belong to this family share a unique DNA binding domain with a winged helix-turn-helix structure [30] and recognize a consensus sequence of GGAA/T in the target DNA. PU.1 is expressed in hematopoietic cells, predominantly in B lymphocytic and myeloid lineages [31,32], but is down-regulated in cells undergoing erythroid differentiation [32]. The *PU.1* gene is an oncogene, because in mice viral activation of its 5' flanking region, which results in continued expression of PU.1, causes the onset of erythroleukemia [31,32]. The PU.1 protein has a transactivation domain at its N-terminus, followed by a protease-sensitive domain, and by a DNA binding domain located at the C-terminus. PU.1 is an interacting partner for many other transcription factors and signaling proteins. The N-terminal part of PU.1 interacts with the C-terminal portion of the basal transcription factor TFIID or with the pocket of retinoblastoma (Rb) protein, while the C-terminal part can interact with C/EBPδ, heat shock protein (Hsp) 90, CREB binding protein and some other proteins [32]. PU.1 directly regulates transcription of numerous genes which encode proteins characteristic of B lymphocytes, monocytes and granulocytes. In B lymphocytes it regulates the expression of heavy and light chains of immunoglobulin, CD20, MHC class II and Bruton's tyrosine kinase [32]. In cells undergoing myeloid differentiation, PU.1 up-regulates transcription of characteristic proteins such as: receptors for the granulocyte, macrophage and granulocyte-macrophage colony-stimulating factors (G-CSF, M-CSF and GM-CSF), scavenger receptors, CD11b, CD11c and CD18, lysozyme, MPO, EDN, FcγR and many others [32]. It is noteworthy that PU.1 also regulates its own expression. Disruption of the *PU.1* gene in mice is lethal. PU.1-deficient embryos have normal numbers of megakaryocytes and proerythroblasts, but their myelopoiesis is blocked at a very early stage. Fetal livers of such animals do not contain myeloid progenitors: granulocyte, macrophage, granulocyte-macrophage and granulocyte-erythroid-megakaryocyte-monocyte colony-forming units (CFU-G, CFU-M, CFU-GM and CFU-GEMM) [31].

CCAAT Enhancer-Binding Proteins are Master Regulators of Myelopoiesis

C/EBPα is one of the master regulators of myeloid lineage selection. Its expression predominates in immature cells and granulocytes, but declines in developing monocytes/macrophages (Fig 1). Initially, it may stabilize myeloid lineage commitment by cross inhibition of Pax5 expression, a transcription factor necessary for the B lymphoid lineage [33]. C/EBPα inhibits the G1 to S phase cell cycle progression in a variety of cell types, and suggested mechanisms include direct binding to cyclin-dependent kinases 2/4 and induction of their inhibitor p21^{cip1} [34], as well as a direct binding of E2F1 and repression of the c-myc gene [35]. Importantly, C/EBPα binds and activates the PU.1 promoter, of particular importance for myelopoiesis, as discussed above (Box 1). There are multiple target genes for C/EBP transcription factors, but in myeloid cells the most important ones seem to

be those encoding G-CSF, M-CSF and GM-CSF receptors, CD14, lysozyme, MPO, lactoferrin, elastase and collagenase [36].

The role of C/EBPβ in myelopoiesis is less well established than that of C/EBPα, though it has a clear role in macrophage function [37]. In some murine systems C/EBPβ can compensate for the loss of C/EBPα [38], and may promote myeloid cell proliferation of all lineages [39]. In human cells, however, evidence has been provided that C/EBPβ is required for 1α,25-dihydroxyvitamin D_3-induced monocytic differentiation of leukemic cells [15,40]. It has also been suggested that an increased level of C/EBPβ relative to that of C/EBPα can redirect malignant cells from an aborted granulocytic pathway to normal monocytic differentiation [41]. Further studies are needed to determine if the role of C/EBPβ is specific to a particular cell context. It has been also shown in mouse models that enforced expression of either C/EBPα or C/EBPβ in differentiated B or T lymphocytes can reprogram these cells to macrophages, thus indicating the importance of these transcription factors to expression of genes typical of myeloid cells, and their capacity to cross-inhibit the activity of Pax5 [42,43]. Interestingly, the transcription factor PU.1 converted committed T cell progenitors into myeloid dendritic cells in this system [43]. It should be stressed that myeloid potential remains active for a long time in lymphocyte lineage, since in the mouse thymus even the T-cell progenitors that have already lost B-cell potential still retain the ability to become macrophages [1,2].

Box 2. C/EBP Family of Transcription Factors

C/EBPs are a family of basic leucine zipper transcription factors with similar, modular structures. C/EBPs contain an activation domain, a DNA-binding basic domain and a leucine-rich dimerization domain [44]. There is substantial sequence homology among all C/EBP isoforms in the C-terminal portion containing DNA-binding region, which is followed by a dimerization motif. The dimerization domain is a heptad of leucine repeats that intercalate with repeats of the dimer partner, forming a coiled coil of alpha-helices in parallel orientation. The C/EBPs form dimers prior to the binding to the consensus sequence 5'-TT/GNNGNAAT/G-3' in DNA. The most variable portion of C/EBP isoforms is the N-terminal part, which contains activation domains that interact with components of the basal transcription apparatus to stimulate transcription [45]. There are six genes encoding C/EBP family members, and, due to a leaky ribosomal scanning mechanism, two different products of C/EBPα, three products of C/EBPβ and four products of C/EBPε genes are translated [45]. Because the activation domains are at the N-termini of these proteins, the different protein products have different transcriptional activities, and some isoforms that lack the entire activation domain exert inhibitory functions [45], presumably by a dominant negative mechanism. Initially, it was believed that the only physiological role of C/EBP proteins is to regulate gene expression by binding to the common sequence in regulatory regions of many gene promoters. However, later studies showed another level of gene expression regulation through protein:protein interactions of C/EBPs with other proteins that are important to cell cycle and proliferation, such as cdk2, cdk4, the Rb protein and the E2F transcription factors [46-48]. E2F transcription factors drive transcription of the genes that are necessary for the

transition from the G1 to the S phase of the cell cycle. When C/EBP transcription factors bind to E2Fs, the latter are sequestered from the promoters of their target genes, which arrests cell cycle progression. At this stage tumor suppressor protein Rb comes into play. When this protein binds to a C/EBP protein its transcriptional activity is enhanced, which usually results in the transcription of differentiation-related genes. Still one more level of regulation can be achieved by means of post-translational modifications of C/EBP family members, such as phosphorylation [49,50].

Many experiments have shown that C/EBPε is crucial and indispensable for the terminal stages of granulopoiesis. The most striking results come from murine knockout models. C/EBPε-deficient mice display defects in granulocyte development; they have an increased number of granulocyte progenitors, but their neutrophils are defective in chemotaxis, superoxide production, release of granule contents and bactericidal activity [51]. These mice die at the age of 3-5 months because of opportunistic infections. The neutrophils of these mice do not contain characteristic proteins, such as lactoferrin, gelatinase and collagenase and are not able to up-regulate granulocyte colony-stimulating factor (G-CSF) receptors, but contain elastase, lysozyme and myeloperoxidase [17,52]. The eosinophils are also deficient in the most characteristic proteins: EPO and MBP [52]. However, C/EBPε itself is not sufficient to induce expression of genes encoding secondary granule contents and requires cooperation of C/EBPα, GATA-1 and PU.1, which are expressed during granulopoiesis [52]. It is noteworthy that patients suffering from the rare disorder, neutrophil-specific granule deficiency, carry a mutation in the C/EBPε gene resulting in a loss of the full-length protein [53]. Another level of regulation of granulopoiesis by C/EBPε is achieved by its interaction with E2F1 and Rb proteins. By binding E2F1, C/EBPε sequesters it from the promoters of genes that are necessary for cell cycle progression. On the other hand, when C/EBPε interacts with Rb in cells during granulopoiesis, this interaction enhances the transcriptional activity of C/EBPε [54], in contrast to C/EBPβ-Rb association in monocytic differentiation, which inhibits C/EBPβ activity [40].

Other transcription factors cooperate with the above in the expression of proteins that are characteristic of white blood cells. For example, the integrin chains CD11d and CD18 require Sp1 for their expression [55,56]. Attenuation of the activities of C/EBP transcription factors is mediated by the CCAAT displacement protein (CDP). It has been documented that this protein, when over-expressed, inhibits C/EBPε-mediated granulocytic differentiation [57]. As an example, one of the myeloid-specific genes that is negatively regulated by CDP is lactoferrin [58].

Another group of genes that may interfere with myeloid differentiation is the *Homeobox* (*HOX*) gene family. These genes are in principle master regulators of embryonic development, but they continue to be expressed in some tissues in post-natal life [59]. Human HSCs express *HOXA* and *HOXB* genes which are normally down-regulated at later stages of differentiation [60], but their over-expression leads to excessive proliferation of progenitor cells, as has been shown for HOXA10 [61]. Interesting mechanistic studies showed that HOXA10 represses the gp91-phox and p67-phox promoters in immature myeloid cells and SHP2 tyrosine phosphatases act directly on HOXA10 to permit DNA binding [62,63]. Conversely, tyrosine phosphorylation of HOXA9 increases binding and activation of phox

genes in maturing myeloid cells [64]. Other studies showed that the inability to switch off the *HOXA9* gene, caused by chromosomal translocation which produces a fusion-gene between nucleoporin and HOXA9, leads to the onset of human acute myeloid leukemia [65], while over-expression of *HOXB8* inhibits myeloid differentiation of murine cells [66] and produces myeloid leukemia in mice [67]. Also of interest is the report that HOXB7 is induced during 1α,25-dihydroxyvitamin D_3-driven monocytic differentiation of HL60 cells, and its over-expression inhibits neutrophilic differentiation [68]. HOX target genes in mammals are multiple, and apart from the *phox* genes it is not clear which of them are involved in hematopoiesis, but the most likely are those encoding cell adhesion molecules and proteins that regulate the cell cycle [69].

Also important to the hematopoietic developmental pathways are members of the Kruppel-like family (KLF) of transcription factors. In particular, KLF4/GKLF, one of the small group of genes which can re-program mature cells back to pluripotent stem cells, termed induced pluripotent stem cells (iPS) [70], has recently been reported to promote monocytic differentiation in a lineage-specific manner [71]. Interestingly, KLF4 is a target of PU.1 and can activate a monocyte-specific promoter which appears to contribute to the development of the morphologic and functional characteristics of the mature monocyte.

Ligand-Responsive Nuclear Receptors Enhance Myeloid Differentiation

It is not clear how retinoic acid receptors (RARs) and the vitamin D receptor (VDR) are involved in the terminal stages of granulocyte and monocyte development. RARα is expressed during myelopoiesis, and a reciprocal t(15;17) translocation, that fuses the PML and RARα genes impairs function of the RARα protein, causes arrest of granulocytic differentiation and initiates acute promyelocytic leukemia [72], which can, to a degree, be corrected by high concentrations of retinoic acid. However, RARα-deficient mice have normal numbers of neutrophils in their blood and in hematopoietic organs [73]. Some experimental data suggest that the unoccupied RARα antagonizes granulocytic differentiation, whereas the ligand-occupied receptor enhances it [73]. Thus, RARα-null mice lack this negative regulator of differentiation, yet their phenotype does not resemble vitamin A-deficiency or receptor mutation.

Similarly, VDR is expressed in human cells undergoing monocytic differentiation and in experimental models 1α,25-dihydroxyvitamin D_3, the natural ligand of VDR, induces differentiation of GMPs towards monocytes [74]. Moreover, in patients with vitamin D resistant rickets (type II), who have defective VDRs resulting from various mutations, myeloid progenitors are resistant to 1α,25-dihydroxyvitamin D_3-induced differentiation [75]. However since VDR-null mice show no defects in hematopoiesis [76], it is possible that VDR is not essential for the development of monocytes and macrophages, if this finding can be applied to humans.

Box 3. RAR and VDR Nucelar Receptors

RARα and VDR belong to the super-family of nuclear receptors, which consists of the receptors for thyroid and steroid hormones, retinoids, 1α,25-dihydroxyvitamin D_3 and some receptors with unknown ligands. RARα is a nuclear receptor for all-*trans* retinoic acid (RA) and 9-*cis* retinoic acid, and there are two other related receptors (RARβ and RARγ). All nuclear receptors show structural and functional similarities which reflect their evolutional relationships [77]. They are composed of five domains designated A to E. The N-terminal, highly variable A/B domain has ligand-independent transactivation function. The DNA binding domain C is the most conserved domain within the family of nuclear receptors. It contains two zinc-fingers and confers the ability to recognize specific sequences in target gene promoters [78]. Region D is variable and serves as a flexible joint, which allows rotation of the neighboring domains. Domain E, the ligand binding domain, is conserved within receptors that have the same ligand, but varies between receptors with various ligands. In addition to ligand binding, the E domain is responsible for the receptor dimerization and the ligand-dependent transactivation, and is composed of 12 α-helices which create a ligand-binding cavity formed by hydrophobic amino acids. Subsequent to binding of the ligand, helix 12 shifts its position and becomes accessible to the co-activators [77]. Nuclear receptors regulate transcription by binding to specific hormone response elements (HREs) in promoters of target genes. HREs usually are bipartite, composed of two hexameric core sequences, configured as palindromes, inverted palindromes or direct repeats [78]. The response elements for RAR receptors are usually composed of two directly repeated AGGTCA half-sites, separated by five nucleotides (so called DR5) [79]. In the case of VDR the most common responsive elements (VDREs), designated DR3, consist of two six-base elements, with the consensus sequence AGGTCA, that are separated by a spacer of three nucleotides. Another type of VDRE, named IP9, is composed of two inverted palindromic consensus sequences separated by a nine-base spacer [80].

RARα complexes with RXR and the complex has two main functions. In the absence of a ligand, the heterodimer binds co-repressors that interact with histone deacetylases, leading to silencing of target genes, while after ligand binding, the RAR/RXR heterodimer interacts with a large protein complex containing the transcriptional co-activators with histone acetyltransferase activity, which unfolds and exposes the DNA helix for transcription [73]. VDR, after ligation with 1α,25-dihydroxyvitamin D_3 and dimerization with RXR, binds to a VDRE in the proximal, or sometimes distal, promoter regions of target genes [81]. In the case of DR3 type VDRE, when RXR binds to 5’ half-site and the VDR to 3’, the regulation of gene transcription is positive, while a changed polarity of the VDR/RXR-VDRE complex results in negative regulation [80]. In general, for up-regulation of gene transcription VDR recruits coactivators with histone acetyltransferase activity, while for the transcriptional repression VDR recruits corepressors with histone deacetylase activity, which prevents chromatin decondensation [80].

Lineage–Specific Myeloid Growth Factors Ensure Survival and Proliferation

Although lineage commitment is principally driven by an intrinsically regulated emergence of transcription factors, a variety of growth factors are needed to ensure the survival and proliferation of myeloid cells at all stages of their development. The presence of these growth factors can also influence the relative abundance of cells of different lineages by supporting the differentiation of progenitor cells to a specific cell type [19]. The principal myeloid-specific growth factors are the granulocyte-macrophage colony stimulating factor (GM-CSF), which supports the survival and proliferation of granulocyte and monocyte progenitor (GMP) cells and their progeny, the macrophage colony stimulating factor (M-CSF) which targets monocytes and macrophages, and among the granulocytes, granulocyte colony stimulating factor (G-CSF), interleukin-5 (IL-5), and the stem-cell factor (SCF), which support neutrophils, eosinophils, and basophils or mast cells, respectively [82]. G-CSF is of considerable importance in the therapy of human diseases, so there is a wealth of knowledge regarding its properties. For instance, this cytokine facilitates the release of neutrophils from the bone marrow, and enhances their functional capacities such as phagocytosis and generation of superoxide anions. G-CSF production, increased in inflammation, is stimulated by cytokines such as TNFα, IL-1 and IL-6, all of which can be secreted by activated monocytes [83].

In order to react to CSFs, cells have to recognize these growth factors by their cognate surface receptors, which belong to a large cytokine-receptor super-family. These are transmembrane proteins that share some common features. Most CSF receptors are composed of homo- or heterodimers, while some are composed of three chains [84]. The heterodimeric receptors can be grouped into four subfamilies which share a common β-chain, γ-chain, gp130 chain or gp140 chain [84]. The receptors within a particular group have a similar signaling unit, but specific extracellular ligand-binding domains [19]. Ligand binding results in a dimerization of the specific receptor and induces a conformational change which usually activates Janus kinases (JAKs), which are constitutively associated with the receptors. Activated JAKs autophoshorylate themselves and phosphorylate cytokine receptors at tyrosine residues, which leads to the activation of secondary signaling molecules [84]. Activated JAKs directly interact with transcription factors named signal transducers and activators of transcription (STATs) and induce their phosphorylation, dimerization and translocation to the cell nucleus [85,86]. Some new data suggest that JAKs are not the only activators of STATs, and in some instances, such as signaling from the IL-3 receptor, Src kinases participate in signal transduction [84]. Seven genes encoding STAT proteins are known, but more isoforms are generated by alternative splicing and proteolytic cleavage [86]. There are multiple target genes, that are mostly STAT isoform-specific and many of them are involved in regulation of cell proliferation and survival [84]. Cytokine receptors can also activate the Ras/MAPK [87] and phosphatidylinositol 3-kinase (PI3K) [88] pathways. G-CSF is representative of cytokines that activate these two pathways through their receptors. The signals transmitted through mitogen activated protein kinases/extracellular-signal regulated kinases (MAPK/Erk) and MAPK/p38 signal transduction modules are necessary for cell proliferation in response to G-CSF [89], while activation of PI3K is necessary for

neutrophil migration [90]. These signals are then transmitted to an overlapping group of differentiation-related transcription factors, which modulate the expression of the cell cycle and cell survival regulators, as well as proteins essential to the function of the mature myeloid cells. For instance, the promoters of all three myeloid CSF receptors have binding sites for C/EBP and PU.1 transcription factors, an arrangement that is likely to be of developmental significance [91].

Intracellular Signaling Pathways Modulate the Activity of Master Regulators

It was recently reported that C/EBPα, one of the key regulators of myeloid differentiation discussed above, can form a heterodimer with a member of the AP-1 transcription factor complex, which may then contribute to monocytic lineage development [92]. This can offer a rationale for the earlier observations in model systems that the MAPK pathways are involved in monocytic differentiation. First, several laboratories demonstrated that activated Erk1/2 MAP kinases contribute to 1α,25-dihydroxyvitamin D_3-induced monocytic differentiation of human leukemia HL60 cells [93-97]. Then the JNK pathway branch of MAPK signaling was shown to enhance this form of differentiation [98]. Both of these pathways activate the AP-1 transcription factor by up-regulating the expression of its components such as c-fos and c-jun. The importance of AP-1 for monocytic differentiation was then further established by experiments based on a dominant negative strategy using AP-1 oligonucleotide decoys, which showed the requirement for the AP-1 complex in this system [99]. These experiments were complemented by a similar approach, using C/EBP decoys, which demonstrated that members of the C/EBP family of transcription factors are also required for optimal differentiation [40], and this was also linked to phosphorylation of C/EBPβ by the MEK/Erk pathway [15]. Thus, Friedman's suggestion [3] of a transcription factor binding DNA site consisting of AP-1 and C/EBP half sites could explain a number of disparate findings discussed above, and provide a basis for the influence of MAPK signaling on transcription factors critical for monocytic differentiation.

Another, perhaps complementary, way in which c-jun can influence differentiation lineage choice is its role as a co-activator for the transcriptional activity of PU.1 [100]. It has been found that an interaction between c-jun and PU-1 favors myeloid lineage, whereas GATA-1 binding to PU.1 displaces c-jun from PU.1 leading to erythroid differentiation (Fig 2). Although an active JNK was not found to be required for c-jun participation in these events [100], other studies found that the JNK pathway can stabilize c-jun and thus increase its cellular abundance [98,101]. In this way the JNK pathway can also enhance monocytic differentiation. Conversely, displacement of c-jun from PU.1 by the fusion protein AML-ETO, the result of the chromosomal translocation t(8:21), interferes with normal differentiation, resulting in the development of a form of AML, exemplified by the Kasumi–1 human leukemia cell line [102].

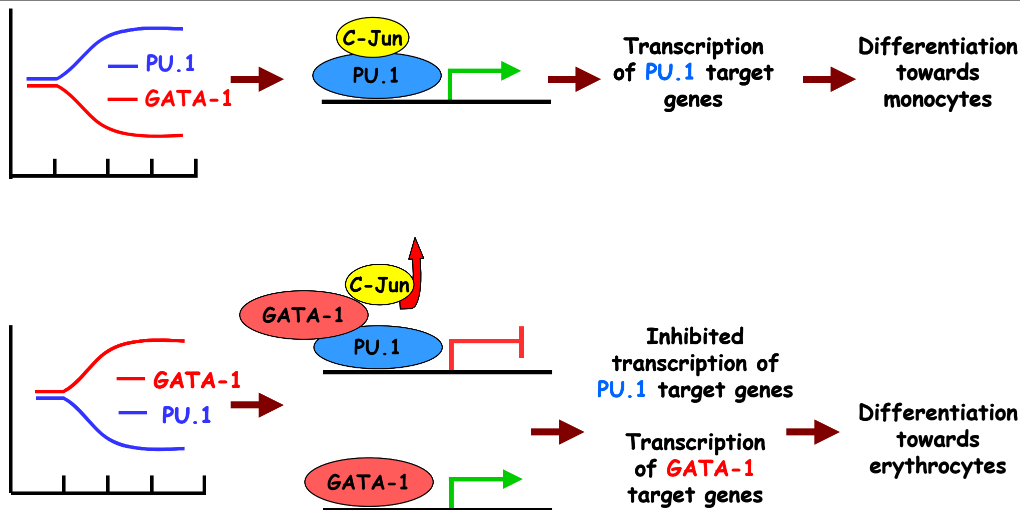

Figure 2. PU.1 and GATA-1 compete in cell fate determination.

High levels of PU.1 favor transcription of monocyte-related genes. Transcriptional activity of PU.1 is enhanced by its interaction with c-jun through the winged region of the DNA-binding domain. However, an excess of GATA-1 causes disruption of PU.1/c-jun complexes and represses transcription of PU.1-dependent genes. GATA-1 then activates GATA-1-dependent genes, which are important for erythroid differentiation. This diagram is based on and modified from reference [4].

There is evidence that the PI3K/AKT pathway also plays a role in monocytic differentiation perhaps by ensuring the survival of differentiating cells. First, it has been reported that PI3K activation is necessary for 1α,25-dihydroxyvitamin D_3-induced monocytic differentiation of HL60 [103] and THP-1 cells [104]. The proposed mechanism linked PI3K signal transduction pathway with the action of VDR, indicating a physical interaction of these proteins [104]. It has been shown that the inhibition of PI3K pathway impairs the function of monocytes, through disruption of the mechanisms responsible for cell adherence and the response to lipopolysaccharide (LPS) [105], FcγR- and mannose receptor-mediated phagocytosis, and for the oxidative burst [106]. Activation of PI3K/AKT pathway has an anti-apoptotic action in human leukemia HL60 cells induced to differentiate with 1α,25-dihydroxyvitamin D_3 [107,108] which may contribute to the relatively high survival potential of monocytes.

More recently, hKSR2, a novel regulator of Ras signaling [109,110], has been reported to participate in the control of monocytic cell survival [111]. The survival-enhancing action of hKSR2 is due, at least in part, to its ability to increase the expression of the anti–apoptotic protein Bcl-2. An interesting question is whether this effect can be found in cells which do not have an activated Ras oncogene, as the studies to date have been performed only in HL60 cells, which harbor the activated N-Ras [112]. Another question is whether hKSR2 signals cell survival via a pathway that includes AKT, described above. A summary of the signal transduction pathways that are involved in myeloid differentiation of HL60 cells is shown in Figure 3.

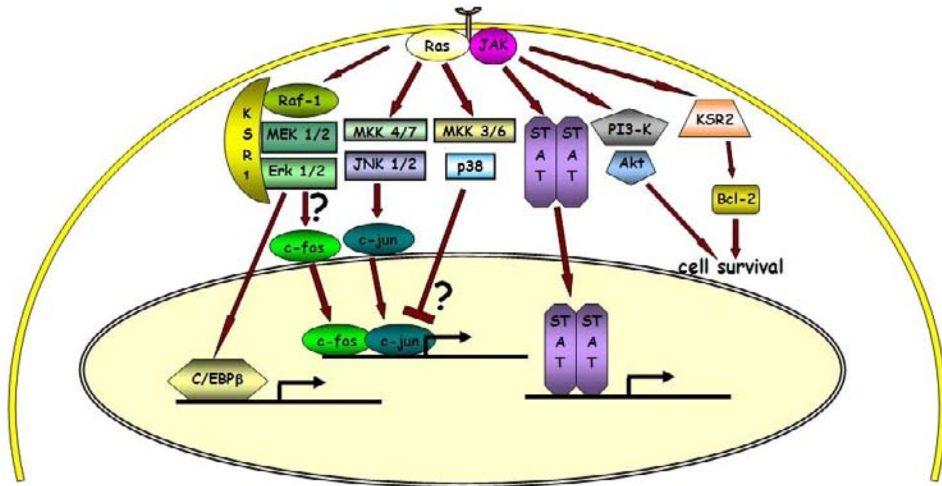

Figure 3. Signal transduction pathways contributing to myeloid differentiation.

This scheme highlights **some of** the pathways which may contribute to the signaling of myeloid differentiation and to the differentiation-associated increase in cell survival potential. The AP-1 transcription factor family is activated by at least two branches of MAPK cascades, directly (e.g. c-jun) or indirectly (e.g. c-fos), while the C/EBPβ transcription factor can be activated by Erk1/2 [49,50]. The role of p38 MAPK is unclear at this time. The kinase suppressor of Ras 1 (KSR1) can facilitate Raf-1 signaling [113,114], while KSR2 and AKT participate in mechanisms that increase cell survival under adverse conditions [108]. JAK kinases are constitutively associated with the CSF receptors. Upon activation and oligomerization of these receptors JAKs become activated by autophosphorylation. Activated JAKs directly interact with STATs, induce their phosphorylation, dimerization and translocation to the cell nucleus, where STAT-target genes are regulated [85,86].

Control of Monomyelopoiesis by MicroRNAs

MicroRNAs repress protein expression at the post-transcriptional level, and are coming into prominence as regulators of most biological functions, including differentiation and hematopoiesis [115-118]. One of the earliest reports in this field was the demonstration that several miRNAs, miR–181, miR-223 and miR-142s, are differentially expressed in hematopoietic lineages *in vivo*, and are able to alter the choice of lineage differentiation [119]. In these studies miR–181 was found to be preferentially expressed in mouse bone marrow B-lineage cells, and its ectopic expression led to an increased fraction of B lymphocytes in both tissue culture differentiation assays and in adult mice.

The relevance of miRNAs to myelopoiesis was then quickly demonstrated by the discovery that miR-223 is specifically activated in RA-induced granulocytic differentiation of human cells in culture, and that miR-223 is required for optimal differentiation-response to

RA [120]. Evidence was also obtained for the mechanism of this effect, which centers on the C/EBPα and nuclear factor I/A (NFI-A) transcription factors as targets for miR-223. It was proposed that NFI-A, a CCAAT-related binding protein that functions in growth control [121], competes with C/EBPα for binding to the region that controls miR-223 upregulation by RA. When NFI-A prevents C/EBPα binding, miR-223 is not expressed and the cells do not differentiate. It was also found that there is a negative regulatory loop whereby NFI-A is negatively controlled by miR-223, which explains how expression of miR-223, once initiated by RA or other signals, can be maintained during granulocytic differentiation.

While this seems to be a convincingly coherent story, a more recent paper provided evidence that although miR223 is myeloid-specific, it actually negatively regulates progenitor cell proliferation, granulocytic differentiation and activation [122]. The authors speculated that the discrepancy from the results of Fazi *et al.* who, as indicated above, concluded that miR223 is a positive regulator of granulocytic differentiation [120], could be attributed to disparate experimental approaches used in their studies. For instance, Fazi *et al.* utilized over-expression strategies, while Johnnidis *et al.* extrapolated from deletion studies. Furthermore, manipulating cells in different stages of myeloid development may provide different responses since miRNAs are likely to change expression dynamically during development. Importantly, these disparate results may illustrate the differences in the roles of miRNAs between human [120] and murine [122] myeloid lineage development, as already noted in the report by Ramkisson *et al.* [123].

In another study that used human cells it was found that in the acute promyelocytic cell line NB4 retinoic acid up-regulated a number of miRNAs, including miR-107 which targets NFI-A, and along with miR-223, discussed above, negatively regulates NFI-A [124]. It was also reported that the RA-induced down-regulation of the Ras gene correlated with activation of let-7a, and that down-regulation Bcl-2 correlated with the activation of miR-15/miR-16-1, the known regulators of these genes. Curiously, although a seeming plethora of miRNA genes up-regulated in response to RA was found in this study, only miR-181b was down-regulated. Since miRNAs attenuate the expression of coding genes, miR-181b may have a special role in granulocytic differentiation by removing a barrier to this lineage choice, perhaps by allowing the appropriate level of expression of C/EBPα.

More recently, involvement of miRNAs in monocytopoiesis was also described, and here the down-regulation involved three miRNAs, 17-5p, 20a and 106a [125]. In uni-lineage cultures generated from cord blood $CD34^+$ cells incubated with saturating amounts of M-CSF 95% of the cells are monocytes, which become terminally differentiated macrophages during a 3-4 week period. While these miRNAs are down-regulated, the Runt-related transcription factor, Runx-1, also known as AML-1, becomes up-regulated at protein but not at mRNA level, presumably because these miRNAs bind to the 3'UTR of Runx-1 mRNA, and the decline of 17-5p, 20a and 106a miRNAs unblocks translation of Runx-1 These events also lead to M-CSFR down-regulation. The authors suggested that the monocytic lineage is controlled by miRNAs, 17-5p, 20a and 106a, which function to repress Runx-1/AML-1 and M-CSFR. These culture studies of human cells contrast with *in vivo* studies of mouse monocytopoiesis, where knock outs of the miR 17-92 cluster, which includes miRNAs studied by Fontana et al, do not show defects in granulocytes, monocytes, or T-lymphocytes, though there is a marked deficiency of pre-B lymphocytes [126]. This raises the possibility

that there are important differences between human and mouse monocytopoiesis, though the physiological significance of the cord blood $CD34^+$ system may require further validation. In any case, it seems that miRNAs are not the sole regulators of monocytic differentiation, and it is likely that miRNAs act as differentiation lineage modulators, rather than direct switches.

Conclusion

Taking all these data together, it is clear that transcription factors such as C/EBPα, β, ε and PU.1, with contributions from AP-1, EGR1/2, HOX family, and nuclear receptors-transcription factors RAR and VDR have predominant roles in granulocytic and monocytic differentiation. Rapid progress in understanding signaling networks and lineage selection have been hampered by the inherent complexity of the mechanisms involved. However, it can be assumed that lineage selection depends on the relative amounts of transcription factors which either cooperate with one another, or perhaps more frequently compete for DNA binding sites. Protein:protein interactions are also very important for lineage selection. For instance, GATA-1, which drives erythroid differentiation, can bind to PU.1, which drives myeloid lineages, and this binding inhibits each other's transcriptional activity. Thus, the simple view is that whichever of these transcription factors is present in greater concentration, this lineage will be favored, whereas the other pathway is inhibited, i.e., if GATA-1 is relatively deficient, PU.1 will drive the myelopoiesis at the expense of the erythropoiesis, and *vice versa*. Of course, differentiation is further modulated by epigenetic modifications (Chapter 11) of chromatin and associated proteins by processes such as phosphorylation, methylation, acetylation, ubiquitinylation, and SUMOylation (eg [127-131]).

Transcription factor interactions are also dependent on a changing cellular environment, which is communicated to the transcription machinery by pathways such as the MAPK and PI3K/AKT cascades, which can elicit gene expression changes at both transcriptional and post-transcriptional levels. These transcription factors and other regulatory proteins may be under the control of miRs, but there is still a lack of full information and a flux of ideas how this control is exerted. Finally, it should be noted that one fundamental difference between granulocytic and monocytic differentiation is that the former is a form of slow cell death, whereas the latter entails acquisition of considerable survival potential. In both cases, however, the cell cycle is gradually arrested. Therefore, genes controlling apoptosis and cell cycle progression may also need to be fitted into the picture of lineage selection, providing rich opportunities for further exploration of this exciting facet of cell biology.

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References

[1] Bell JJ and Bhandoola A. (2008) The earliest thymic progenitors for T cells possess myeloid lineage potential. *Nature*, 452, 764-767.

[2] Wada H, Masuda K, Satoh R, Kakugawa K, Ikawa T, Katsura Y and Kawamoto H. (2008) Adult T-cell progenitors retain myeloid potential. *Nature*, 452, 768-772.

[3] Friedman AD. (2007) Transcriptional control of granulocyte and monocyte development. *Oncogene*, 26, 6816-6828.

[4] Gangenahalli GU, Gupta P, Saluja D, Verma YK, Kishore V, Chandra R, Sharma RK and Ravindranath T. (2005) Stem cell fate specification: role of master regulatory switch transcription factor PU.1 in differential hematopoiesis. *Stem Cells Dev*, 14, 140-152.

[5] Brown G, Hughes PJ, Michell RH, Rolink AG and Ceredig R. (2007) The sequential determination model of hematopoiesis. *Trends Immunol*, 28, 442-448.

[6] Brown G, Hughes PJ, Michell RH, Rolink A and Ceredig R. (2008) *Ordered commitment of hematopoietic stem cells to lineage options*, Nova Science Publishers Inc.

[7] Adolfsson J, Månsson R, Buza-Vidas N, Hultquist A, Liuba K, Jensen CT, Bryder D, Yang L, Borge OJ, Thoren LA, Anderson K, Sitnicka E, Sasaki Y, Sigvardsson M and Jacobsen SE. (2005) Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell*, 121, 295-306.

[8] Lai AY and Kondo M. (2006) Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *J Exp Med* (203), 1867-1873.

[9] Rlink AG, Massa S, Balciunaite G and Ceredig R. (2006) Early lymphocyte development in bone marrow and thymus. *Swiss Med Wkly*, 136, 679-683.

[10] Rappold I, Ziegler BL, Köhler I, Marchetto S, Rosnet O, Birnbaum D, Simmons PJ, Zannettino AC, Hill B, Neu S, Knapp W, Alitalo R, Alitalo K, Ullrich A, Kanz L and Bühring HJ. (1997) Functional and phenotypic characterization of cord blood and bone marrow subsets expressing FLT3 (CD135) receptor tyrosine kinase. *Blood*, 90, 111-125.

[11] Iwasaki H, Somoza C, Shigematsu H, Duprez EA, Iwasaki-Arai J, Mizuno S, Arinobu Y, Geary K, Zhang P, Dayaram T, Fenyus ML, Elf S, Chan S, Kastner P, Huettner CS, Murray R, Tenen DG and Akashi K. (2005) Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood*, 106, 1590-1600.

[12] Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, Sciammas R, Gantner BN, Dinner AR and Singh H. (2006) Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell*, 126, 755-766.

[13] Zhang P, Zhang X, Iwama A, Yu C, Smith KA, Mueller BU, Narravula S, Torbett BE, Orkin SH and Tenen DG. (2000) PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. *Blood*, 96, 2641-2648.

[14] Iwasaki H, Mizuno S, Arinobu Y, Ozawa H, Mori Y, Shigematsu H, Takatsu K, Tenen DG and Akashi K. (2006) The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. *Genes Dev*, 20, 3010-3021.

[15] Marcinkowska E, Garay E, Gocek E, Chrobak A, Wang X and Studzinski GP. (2006) Regulation of C/EBPbeta isoforms by MAPK pathways in HL60 cells induced to differentiate by 1,25-dihydroxyvitamin D_3. *Exp Cell Res*, 312, 2054-2065.

[16] Pham TH, Langmann S, Schwarzfischer L, El Chartouni C, Lichtinger M, Klug M, Krause SW and Rehli M. (2007) CCAAT enhancer-binding protein beta regulates constitutive gene expression during late stages of monocyte to macrophage differentiation. *J Biol Chem*, 282, 21924-21933.

[17] Lekstrom-Himes JA. (2001) The role of C/EBPepsilon in the terminal stages of granulocyte differentiation. *Stem Cells*, 19, 125-133.

[18] Martinez-Agosto JA, Mikkola HK, Hartenstein V and Banerjee U. (2007) The hematopoietic stem cell and its niche: a comparative view. *Genes Dev*, 21, 3044-3060.

[19] Robb L. (2007) Cytokine receptors and hematopoietic differentiation. *Oncogene*, 26, 6715-6723.

[20] Hoffbrand AV, Moss PAH and Pettit JE. (2006) *Essential Haematology*, Blackwell Publishing.

[21] Borregaard N, Sørensen OE and Theilgaard-Mönch K. (2007) Neutrophil granules: a library of innate immunity proteins. *Trends Immunol*, 28, 340-345.

[22] Rosenberg HF and Domachowske JB. (2001) Eosinophils, eosinophil ribonucleases, and their role in host defense against respiratory virus pathogens. *J Leukoc Biol*, 70, 691-698.

[23] Arock M, Schneider E, Boissan M, Tricottet V and Dy M. (2002) Differentiation of human basophils: an overview of recent advances and pending questions. *J Leukoc Biol*, 71, 557-564.

[24] Falcone FH, Zillikens D and Gibbs BF. (2006) The 21st century renaissance of the basophil? Current insights into its role in allergic responses and innate immunity. *Exp Dermatol*, 15, 855-864.

[25] Tacke F and Randolph GJ. (2006) Migratory fate and differentiation of blood monocyte subsets. *Immunobiology*, 211, 609-618.

[26] Grage-Griebenow E, Flad HD and Ernst M. (2001) Heterogeneity of human peripheral blood monocyte subsets. *J Leukoc Biol*, 69, 11-20.

[27] Uphoff CC, Hu ZB, Gignac SM, Ma W, Rainey FA, Kreutz M, Ludwig WD and Drexler HG. (1994) Characterization of the monocyte-specific esterase (MSE) gene. *Leukemia*, 8, 1510-1526.

[28] Patel D, Hooper NM and Scott CS. (1993) Subcellular fractionation studies indicate an intracellular localization for human monocyte specific esterase (MSE). *Br J Haematol*, 84, 608-614.

[29] Deimann W. (1984) Endogenous peroxidase activity in mononuclear phagocytes. *Prog Histochem Cytochem*, 15, 1-58.

[30] Dittmer J. (2003) The biology of the Ets1 proto-oncogene. *Mol Cancer*, 2, 1-21.

[31] Fisher RC and Scott EW. (1998) Role of PU.1 in hematopoiesis. *Stem Cells*, 16, 25-37.

[32] Oikawa T, Yamada T, Kihara-Negishi F, Yamamoto H, Kondoh N, Hitomi Y and Hashimoto Y. (1999) The role of Ets family transcription factor PU.1 in hematopoietic cell differentiation, proliferation and apoptosis. *Cell Death Differ*, 6, 599-608.

[33] Hsu CL, King-Fleischman AG, Lai AY, Matsumoto Y, Weissman IL and Kondo M. (2006) Antagonistic effect of CCAAT enhancer-binding protein-alpha and Pax5 in myeloid or lymphoid lineage choice in common lymphoid progenitors. *Proc Natl Acad Sci U S A*, 103, 672-677.

[34] Johnson PF. (2005) Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors. *J Cell Sci*, 118, 2545-2555.

[35] Johansen LM, Iwama A, Lodie TA, Sasaki K, Felsher DW, Golub TR and Tenen DG. (2001) c-Myc is a critical target for C/EBPalpha in granulopoiesis. *Mol Cell Biol*, 3789-3806.

[36] Rosmarin AG, Yang Z and Resendes KK. (2005) Transcriptional regulation in myelopoiesis: Hematopoietic fate choice, myeloid differentiation, and leukemogenesis. *Exp Hematol*, 33, 131-143.

[37] Poli V. (1998) The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *J Biol Chem*, 273, 29279-29282.

[38] Hirai H, Zhang P, Dayaram T, Hetherington CJ, Mizuno S, Imanishi J, Akashi K and Tenen DG. (2006) C/EBPbeta is required for "emergency" granulopoiesis. *Nat Immunol* (732-9)

[39] Sebastian T and Johnson PF. (2006) Stop and go: anti-proliferative and mitogenic functions of the transcription factor C/EBPbeta. *Cell Cycle*, 5, 953-957.

[40] Ji Y and Studzinski GP. (2004) Retinoblastoma protein and CCAAT/Enhancer-binding protein β are required for 1,25-dihydroxyvitamin D_3-induced differentiation of HL60 cells. *Cancer Res*, 64, 370-377.

[41] Studzinski GP, Wang X, Ji Y, Wang Q, Zhang Y, Kutner A and Harrison JS. (2005) The rationale for deltanoids in therapy for myeloid leukemia: role of KSR-MAPK-C/EBP pathway. *J Steroid Biochem Mol Biol*, 97, 47-55.

[42] Xie H, Ye M, Feng R and Graf T. (2004) Stepwise reprogramming of B cells into macrophages. *Cell*, 117, 663-676.

[43] Laiosa CV, Stadtfeld M, Xie H, de Andres-Aguayo L and Graf T. (2006) Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBP alpha and PU.1 transcription factors. *Immunity*, 25, 731-744.

[44] Landschulz WH, Johnson PF and McKnight SL. (1989) The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science*, 243, 1681-1688.

[45] Ramji DP and Foka P. (2002) CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem J*, 365, 561-575.

[46] Charles A, Tang X, Crouch E, Brody JS and Xiao ZX. (2001) Retinoblastoma protein complexes with C/EBP proteins and activates C/EBP-mediated transcription. *J Cell Biochem*, 83, 414-425.

[47] Timchenko NA, Wilde M and Darlington GJ. (1999) C/EBPalpha regulates formation of S-phase-specific E2F-p107 complexes in livers of newborn mice. *Mol Cell Biol*, 19, 2936-2945.

[48] Timchenko NA, Wilde M, Iakova P, Albrecht JH and Darlington GJ. (1999) E2F/p107 and E2F/p130 complexes are regulated by C/EBPalpha in 3T3-L1 adipocytes. *Nucleic Acids Res*, 27, 3621-3630.
[49] Buck M, Poli V, Hunter T and Chojkier M. (2001) C/EBP phosphorylation by RSK creates a functional XEXD caspase inhibitory box critical for cell survival. *Molecular Cell*, 8, 807-816.
[50] Nakajima T, Kinoshita S, Sasagawa T, Sasaki K, Naruto M, Kishimoto T and Akira S. (1993) Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc Natl Acad Sci U S A*, 90, 2207-2211.
[51] Yamanaka R, Barlow C, Lekstrom-Himes J, Castilla LH, Liu PP, Eckhaus M, Decker T, Wynshaw-Boris A and Xanthopoulos KG. (1997) Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. *Proc Natl Acad Sci USA*, 94, 13187-13192.
[52] Gombart AF, Kwok SH, Anderson KL, Yamaguchi Y, Torbett BE and Koeffler HP. (2003) Regulation of neutrophil and eosinophil secondary granule gene expression by transcription factors C/EBPepsilon and PU.1. *Blood*, 101, 3265-3273.
[53] Lekstrom-Himes JA, Dorman SE, Kopar P, Holland SM and Gallin JI. (1999) Neutrophil-specific granule deficiency results from a novel mutation with loss of function of the transcription factor CCAAT/enhancer binding protein epsilon. *J Exp Med*, 189, 1847-1852.
[54] Gery S, Gombart AF, Fung YK and Koeffler HP. (2004) C/EBPepsilon interacts with retinoblastoma and E2F1 during granulopoiesis. *Blood*, 103, 828-835.
[55] Noti JD, Johnson AK and Dillon JD. (2004) The zinc finger transcription factor transforming growth factor beta-inducible early gene-1 confers myeloid-specific activation of the leukocyte integrin CD11d promoter. *J Biol Chem*, 279, 26948-26958.
[56] Rosmarin AG, Luo M, Caprio DG, Shang J and Simkevich CP. (1998) Sp1 cooperates with the ets transcription factor, GABP, to activate the CD18 (beta2 leukocyte integrin) promoter. *J Biol Chem*, 273, 13097-13103.
[57] Khanna-Gupta A, Zibello T, Sun H, Lekstrom-Himes J and Berliner N. (2001) C/EBP epsilon mediates myeloid differentiation and is regulated by the CCAAT displacement protein (CDP/cut). *Proc Natl Acad Sci U S A*, 98, 8000-8005.
[58] Khanna-Gupta A, Zibello T, Sun H, Gaines P and Berliner N. (2003) Chromatin immunoprecipitation (ChIP) studies indicate a role for CCAAT enhancer binding proteins alpha and epsilon (C/EBPalpha and C/EBPepsilon) and CDP/cut in myeloid maturation-induced lactoferrin gene expression. *Blood*, 101, 3460-3468.
[59] Lappin TR, Grier DG, Thompson A and Halliday HL. (2006) HOX genes: seductive science, mysterious mechanisms. *Ulster Med J*, 75, 23-31.
[60] Lawrence HJ, Sauvageau G, Humphries RK and Largman C. (1996) The role of HOX homeobox genes in normal and leukemic hematopoiesis. *Stem Cells*, 14, 281-291.
[61] Thorsteinsdottir U, Sauvageau G, Hough MR, Dragowska W, Lansdorp PM, Lawrence HJ, Largman C and Humphries RK. (1997) Overexpression of HOXA10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia. *Mol Cell Biol*, 17, 495-505.

[62] Eklund EA, Jalava A and Kakar R. (2000) Tyrosine phosphorylation of HoxA10 decreases DNA binding and transcriptional repression during interferon gamma - induced differentiation of myeloid leukemia cell lines. *J Biol Chem*, 275, 20117-20126.

[63] Lindsey S, Huang W, Wang H, Horvath E, Zhu C and Eklund EA. (2007) Activation of SHP2 protein-tyrosine phosphatase increases HoxA10-induced repression of the genes encoding gp91(PHOX) and p67(PHOX). *J Biol Chem*, 282, 2237-2249.

[64] Bei L, Lu Y and Eklund EA. (2005) HOXA9 activates transcription of the gene encoding gp91Phox during myeloid differentiation. *J Biol Chem*, 280

[65] Nakamura T, Largaespada DA, Lee MP, Johnson LA, Ohyashiki K, Toyama K, Chen SJ, Willman CL, Chen IM, Feinberg AP, Jenkins NA, Copeland NG and Shaughnessy JD Jr. (1996) Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia. *Nat Genet*, 12, 154-158.

[66] Blatt C, Lotem J and Sachs L. (1992) Inhibition of specific pathways of myeloid cell differentiation by an activated Hox-2.4 homeobox gene. *Cell Growth Differ*, 3, 671-676.

[67] Perkins A, Kongsuwan K, Visvader J, Adams JM and Cory S. (1990) Homeobox gene expression plus autocrine growth factor production elicits myeloid leukemia. *Proc Natl Acad Sci U S A*, 87, 8398-8402.

[68] Lill MC, Fuller JF, Herzig R, Crooks GM and Gasson JC. (1995) The role of the homeobox gene, HOX B7, in human myelomonocytic differentiation. *Blood*, 85, 692-697.

[69] Svingen T and Tonissen KF. (2006) Hox transcription factors and their elusive mammalian gene targets. *Heredity*, 97, 88-96.

[70] Takahashi K and Yamanaka S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126, 663-676.

[71] Feinberg MW, Wara AK, Cao Z, Lebedeva MA, Rosenbauer F, Iwasaki H, Hirai H, Katz JP, Haspel RL, Gray S, Akashi K, Segre J, Kaestner KH, Tenen DG and Jain MK. (2007) The Kruppel-like factor KLF4 is a critical regulator of monocyte differentiation. *EMBO J*, 26, 4138-4148.

[72] Grignani F, Fagioli M, Ferrucci PF, Alcalay M and Pelicci PG. (1993) The molecular genetics of acute promyelocytic leukemia. *Blood Rev*, 7, 87-93.

[73] Kastner P and Chan S. (2001) Function of RARalpha during the maturation of neutrophils. *Oncogene*, 20, 7178-7185.

[74] Koeffler HP, Amatruda T, Ikekawa N, Kobayashi Y and DeLuca HF. (1984) Induction of macrophage differentiation of human normal and leukemic myeloid stem cells by 1,25-dihydroxyvitamin D_3 and its fluorinated analogues. *Cancer Res*, 44, 5624-5628.

[75] Nagler A, Merchav S, Fabian I, Tatarsky I, Weisman Y and Hochberg Z. (1987) Myeloid progenitors from the bone marrow of patients with vitamin D resistant rickets (type II) fail to respond to $1,25(OH)_2D_3$. *Br J Haematol*, 67, 267-271.

[76] Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T and Kato S. (1997) Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat Genet*, 16, 391-396.

[77] Kishimoto M, Fujiki R, Takezawa S, Sasaki Y, Nakamura T, Yamaoka K, Kitagawa H and Kato S. (2006) Nuclear receptor mediated gene regulation through chromatin remodeling and histone modifications. *Endocr J*, 53, 157-172.

[78] Aranda A and Pascual A. (2001) Nuclear hormone receptors and gene expression. *Physiol Rev*, 81, 1269-1304.

[79] Pemrick SM, Lucas DA and Grippo JF. (1994) The retinoid receptors. *Leukemia*, Suppl 3, S1-10.

[80] Dusso AS, Brown AJ and Slatopolsky E. (2005) Vitamin D. *Am J Physiol Renal Physiol*, 289, F8-28.

[81] Wang X, Wang TT, White JH and Studzinski GP. (2007) Expression of human kinase suppressor of Ras 2 (hKSR-2) gene in HL60 leukemia cells is directly upregulated by 1,25-dihydroxyvitamin D_3 and is required for optimal cell differentiation. *Exp Cell Res*, 313, 3034-3045.

[82] Kaushansky K. (2006) Lineage-specific hematopoietic growth factors. *N Engl J Med*, 354, 2034-2045.

[83] Hareng L and Hartung T. (2002) Induction and regulation of endogenous granulocyte colony-stimulating factor formation. *Biol Chem*, 383, 1501-1517.

[84] Baker SJ, Rane SG and Reddy EP. (2007) Hematopoietic cytokine receptor signaling. *Oncogene*, 26, 6724-6737.

[85] Ihle JN and Kerr IM. (1995) Jaks and Stats in signaling by the cytokine receptor superfamily. *Trends Genet* (11), 69-74.

[86] Lim CP and Cao X. (2006) Structure, function, and regulation of STAT proteins. *Mol Biosyst*, 2, 536-550.

[87] Miura Y, Miura O, Ihle JN and Aoki N. (1994) Activation of the mitogen-activated protein kinase pathway by the erythropoietin receptor. *J Biol Chem*, 269, 29962-29969.

[88] Miyakawa Y, Rojnuckarin P, Habib T and Kaushansky K. (2001) Thrombopoietin induces phosphoinositol 3-kinase activation through SHP2, Gab, and insulin receptor substrate proteins in BAF3 cells and primary murine megakaryocytes. *J Biol Chem*, 276, 2494-2502.

[89] Rausch O and Marshall CJ. (1999) Cooperation of p38 and Extracellular Signal-regulated Kinase Mitogen-activated Protein Kinase Pathways during Granulocyte Colony-stimulating Factor-induced Hemopoietic Cell Proliferation. *J Biol Chem*, 274, 4096-4105.

[90] Nakamae-Akahori M, Kato T, Masuda S, Sakamoto E, Kutsuna H, Hato F, Nishizawa Y, Hino M and Kitagawa S. (2006) Enhanced neutrophil motility by granulocyte colony-stimulating factor: the role of extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *Immunology*, 119, 393-403.

[91] Wang QF and Friedman AD. (2002) CCAAT/enhancer-binding proteins are required for granulopoiesis independent of their induction of the granulocyte colony-stimulating factor receptor. *Blood*, 99, 2776-2785.

[92] Friedman AD. (2007) C/EBPalpha induces PU.1 and interacts with AP-1 and NF-kappaB to regulate myeloid development. *Blood Cells Mol Dis*, 39, 340-343.

[93] Marcinkowska E. (2001) Evidence that activation of MEK1,2/erk1,2 signal transduction pathway is necessary for calcitriol-induced differentiation of HL-60 cells. *Anticancer Res*, 21(1A), 499-504.

[94] Wang X and Studzinski GP. (2001) Activation of extracellular signal-regulated kinases (ERKs) defines the first phase of 1,25-dihydroxyvitamin D_3-induced differentiation of HL60 cells. *J Cell Biochem*, 80, 471-482.

[95] Dwivedi PP, Hii CS, Ferrante A, Tan J, Der CJ, Omdahl JL, Morris HA and May BK. (2002) Role of MAP kinases in the 1,25-dihydroxyvitamin D_3-induced transactivation of the rat cytochrome P450C24 (CYP24) promoter. Specific functions for ERK1/ERK2 and ERK5. *J Biol Chem*, 277, 29643-29653.

[96] Studzinski GP, Garay E, Patel R, Zhang J and Wang X. (2006) Vitamin D receptor signaling of monocytic differentiation in human leukemia cells: role of MAPK pathways in transcription factor activation. *Curr Top Med Chem*, 6, 1267-1271.

[97] Hughes PJ and Brown G. (2006) 1Alpha,25-dihydroxyvitamin D_3-mediated stimulation of steroid sulphatase activity in myeloid leukaemic cell lines requires VDRnuc-mediated activation of the RAS/RAF/ERK-MAP kinase signalling pathway. *J Cell Biochem*, 98, 590-617.

[98] Wang Q, Wang X and Studzinski GP. (2003) The jun N-terminal kinase pathway enhances signaling of monocytic differentiation of human leukemia cells induced by 1,25-dihydroxyvitamin D_3. *J Cellular Biochem*, 89, 1087-1101.

[99] Wang X and Studzinski GP. (2006) The requirement for and changing composition of the activating protein-1 transcription factor during differentiation of human leukemia HL60 cells induced by 1,25-dihydroxyvitamin D_3. *Cancer Res*, 66, 4402-4409.

[100] Tenen DG. (2003) Disruption of differentiation in human cancer: AML shows the way. *Nat Rev Cancer*, 3, 89-101.

[101] Musti AM, Treier M and Bohmann D. (1997) Reduced ubiquitin-dependent degradation of c-Jun after phosphorylation by MAP kinases. *Science*, 275, 400-402.

[102] Asou H, Tashiro S, Hamamoto K, Otsuji A, Kita K and Kamada N. (1991) Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8;21 chromosome translocation. *Blood*, 77, 2031-2036.

[103] Marcinkowska E, Wiedlocha A and Radzikowski C. (1998) Evidence that phosphatidylinositol 3-kinase and p70S6K protein are involved in differentiation of HL-60 cells induced by calcitriol. *Anticancer Res*, 18, 3507-3514.

[104] Hmama Z, Nandan D, Sly L, Knutson KL, Herrera-Velit P and Reiner NE. (1999) 1alpha,25-dihydroxyvitamin D_3-induced myeloid cell differentiation is regulated by a vitamin D receptor-phosphatidylinositol 3-kinase signaling complex. *J Exp Med*, 190, 1583-1594.

[105] Lee JS, Hmama Z, Mui A and Reiner NE. (2004) Stable gene silencing in human monocytic cell lines using lentiviral-delivered small interference RNA. Silencing of the p110alpha isoform of phosphoinositide 3-kinase reveals differential regulation of adherence induced by 1alpha,25-dihydroxycholecalciferol and bacterial lipopolysaccharide. *J Biol Chem*, 279, 9379-9388.

[106] Lee JS, Nauseef WM, Moeenrezakhanlou A, Sly LM, Noubir S, Leidal KG, Schlomann JM, Krystal G and Reiner NE. (2007) Monocyte p110alpha phosphatidylinositol 3-

kinase regulates phagocytosis, the phagocyte oxidase, and cytokine production. *J Leukoc Biol*, 81, 1548-1561.

[107] Marcinkowska E, Chrobak A and Wiedłocha A. (2001) Evading apoptosis by calcitriol-differentiated human leukemic HL-60 cells is not mediated by changes in CD95 receptor system but by increased sensitivity of these cells to insulin. *Exp Cell Res*, 270, 119-127.

[108] Zhang Y, Zhang J and Studzinski GP. (2006) AKT Pathway is activated by 1,25-dihydroxyvitamin D_3 and participates in its anti-apoptotic effect and cell cycle control in differentiating HL60 cells. *Cell Cycle*, 5, 447-451.

[109] Ohmachi M, Rocheleau CE, Church D, Lambie E, Schedl T and Sundaram MV. (2002) C. elegans ksr-1 and ksr-2 have both unique and redundant functions and are required for MPK-1 ERK phosphorylation. *Curr Biol*, 12, 427-433.

[110] Channavajhala PL, Wu L, Cuozzo JW, Hall JP, Liu W, Lin LL and Zhang Y. (2003) Identification of a novel human kinase supporter of Ras (hKSR-2) that functions as a negative regulator of Cot (Tpl2) signaling. *J Biol Chem*, 278, 47089-47097.

[111] Wang X, Patel R and Studzinski GP. (2008) hKSR-2, a vitamin D-regulated gene, inhibits apoptosis in AraC-treated HL60 leukemia cells. *Mol Cancer Ther*, 7, 2798-2806.

[112] Janssen JW, Steenvoorden AC, Collard JG and Nusse R. (1985) Oncogene activation in human myeloid leukemia. *Cancer Res*, 45, 3262-3267.

[113] Wang X and Studzinski GP. (2002) Kinase suppressor of RAS (KSR) amplifies the differentiation signal provided by low concentrations vitamin D receptor (nVDR) & non nVDR-mediated pathways. *J Cell Physiol*, 191, 198-207.

[114] Wang X, Wang T-T, White JH and Studzinski GP. (2006) Induction of kinase suppressor of RAS-1(KSR-1) gene by 1,25-dihydroxyvitamin D_3 in human leukemia HL60 cells through a vitamin D response element in the 50-flanking region. *Oncogene*, 25, 7078-7085.

[115] Bartel DP. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116, 281-297.

[116] Ambros V. (2004) The functions of animal microRNAs. *Nature*, 431, 350-355.

[117] He L and Hannon GJ. (2004) MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*, 5, 522-531.

[118] Mendell JT. (2008) miRiad roles for the miR-17-92 cluster in development and disease. *Cell*, 133, 217-222.

[119] Chen CZ, Li L, Lodish HF and Bartel DP. (2004) MicroRNAs modulate hematopoietic lineage differentiation. *Science*, 303, 83-86.

[120] Fazi F, Rosa A, Fatica A, Gelmetti V, De Marchis ML, Nervi C and Bozzoni I. (2005) A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell*, 123, 819-831.

[121] Gronostajski RM. (2000) Roles of the NFI/CTF gene family in transcription and development. *Genes Dev*, 249, 31-45.

[122] Johnnidis JB, Harris MH, Wheeler RT, Stehling-Sun S, Lam MH, Kirak O, Brummelkamp TR, Fleming MD and Camargo FD. (2008) Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature*, 451, 1125-1129.

[123] Ramkissoon SH, Mainwaring LA, Ogasawara Y, Keyvanfar K, McCoy JP Jr, Sloand EM, Kajigaya S and Young NS. (2006) Hematopoietic-specific microRNA expression in human cells. *Leuk Res*, 30, 643-647.

[124] Garzon R, Pichiorri F, Palumbo T, Visentini M, Aqeilan R, Cimmino A, Wang H, Sun H, Volinia S, Alder H, Calin GA, Liu CG, Andreeff M and Croce CM. (2007) MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. *Oncogene*, 26, 4148-4157.

[125] Fontana L, Pelosi E, Greco P, Racanicchi S, Testa U, Liuzzi F, Croce CM, Brunetti E, Grignani F and Peschle C. (2007) MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. *Nat Cell Biol*, 9, 775-787.

[126] Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkeland SJ, Newman J, Bronson RT, Crowley D, Stone JR, Jaenisch R, Sharp PA and Jacks T. (2008) Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell*, 132, 875-886.

[127] Deng H, Bao X, Cai W, Blacketer MJ, Belmont AS, Girton J, Johansen J and Johansen KM. (2008) Ectopic histone H3S10 phosphorylation causes chromatin structure remodeling in Drosophila. *Development*, 135, 699-705.

[128] Hodge DR, Cho E, Copeland TD, Guszczynski T, Yang E, Seth AK and Farrar WL. (2007) IL-6 enhances the nuclear translocation of DNA cytosine-5-methyltransferase 1 (DNMT1) via phosphorylation of the nuclear localization sequence by the AKT kinase. *Cancer Genomics Proteomics*, 4, 387-398.

[129] Hackanson B, Bennett KL, Brena RM, Jiang J, Claus R, Chen SS, Blagitko-Dorfs N, Maharry K, Whitman SP, Schmittgen TD, Lübbert M, Marcucci G, Bloomfield CD and Plass C. (2008) Epigenetic modification of CCAAT/enhancer binding protein alpha expression in acute myeloid leukemia. *Cancer Res*, 68, 3142-3151.

[130] Khan AN, Gregorie CJ and Tomasi TB. (2008) Histone deacetylase inhibitors induce TAP, LMP, Tapasin genes and MHC class I antigen presentation by melanoma cells. *Cancer Immunol Immunother*, 57, 647-654.

[131] Nathan D, Ingvarsdottir K, Sterner DE, Bylebyl GR, Dokmanovic M, Dorsey JA, Whelan KA, Krsmanovic M, Lane WS, Meluh PB, Johnson ES and Berger SL. (2006) Histone sumoylation is a negative regulator in *Saccharomyces cerevisiae* and shows dynamic interplay with positive-acting histone modifications. *Genes Dev*, 20, 966-976.

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Chapter 4

Dendritic Cell Development, Lineage Issues and Haematopoiesis at the Single Cell Level*

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Abstract

Dendritic cells perform some of the most important functions of the immune system. They were originally named based on their dendritic extensions, and their ability to activate naïve T lymphocytes. However, the diversity of functionally and developmentally distinct DC subtypes precludeprecludes them from all being categorized as the one cell type – in the same way immunologists would never lump all lymphocytes together anymore. Considering their rarity, the pathways of DC development were previously poorly characterized. However, rRecent studies utilizing progenitor transfer studies, culture models, or the perturbation of transcription factors, cytokines or signaling molecules, have unraveled some of their complexity. This review outlines the progress made in understanding the diversity of the DC family and regulators of their differentiation. It will also make a case for the importance of single cell studies in light of evidence for a new model termed 'graded' commitment, which is a departure from the classic binary models of haematopoiesis.

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Introduction

This review will attempt to set the known players in the dendritic cell (DC) development scene with respect to the location of precursors, their steps in development, the molecular regulators of their development and their ultimate progeny in various locations in the body. It is an overlap, but also an extension, of a previously published review [1]. There are some extended sections on DC classification, phenotypic comparisons, developmental pathways and technical issues in field of DC development. There are also some thoughts on aspects of lineage commitment (a model termed 'graded' commitment is presented), a tentative idea that DCs are a '3rd lineage' in addition to the myeloid and lymphoid lineages, and descriptions of new lineage tracing tools for single cells. But first, this review will address some definitions and questions for creating a context for understanding DC subtype and leukocyte development, in general.

The first issue that should be addressed is; what defines a DC as being a DC? The short answer is there is not yet a clear answer, and the term DC is partly artificially constructed by us DC researchers. CD11c expression, MHC class II expression, the presence of dendrites, the ability to process and present antigen, to activate naïve T lymphocytes etc. have all been proposed as key features of DC. But clearly, none of these characteristics in isolation is unique amongst DC within the entire repertoire of leukocytes – NK cells express CD11c, B lymphocytes express MHCII, many leukocytes can have dendrites, all cells can process and present antigen (via MHC class I), and not all DCs can activate naïve T lymphocytes. To pool all of these attributes within the one cell type comes closer, but this is still not perfect. This review will not attempt to create such a definition, but merely highlight here that the term 'DC' is not always the best term for all members that are currently in this family. Nevertheless, there are some properties emerging that do allow us to group cells by origin, genetics, evolution and function for a working model of this constructed family of cells.

The second issue is; what makes a *subtype* a real *subtype*? Some argue that further and further micro dissection of leukocytes by phenotype will lead to an infinite diversity of cells [2]. That's probably true, technically speaking. One only needs to consider that most cell surface molecules are expressed not at a fixed level, but in a lognormal distribution – for example the 'bell-shaped curve' expression of most cell surface markers by flow cytometry. Thus, it has been argued that grouping cells by a common phenotype is artifactual. That could be true for some cases considering the potentially infinite phenotypic variations one could give to a cell. However, if a researcher-defined group of cells exhibit a unique set of functional features – for example, a unique toll-like receptor signature to recognize a pathogen, distinct location in terms of accessibility to that pathogen, and a unique T lymphocyte activation outcome – should this cell type be ignored as merely a colour in the rainbow of immune cells? Moreover, if this cell type has a unique gene expression pattern compared to other cell subtypes and has a conserved counterpart amongst different species, I would argue we're getting closer to a biologically distinct subset of cells that has evolved for a reason and should not be ignored merely as 'stamp collecting'.

Presuming there is such thing as a DC subtype, the third issue is; must all cells of one subtype have a common origin? This question raises the follow-on question of; does one mean a common *cellular* origin or a common *molecular* origin? While not mutually

exclusive, this distinction is important to make. This issue will be discussed later in the review, as while the classic binary branching models of hematopoiesis have served us well so far, there has been a flurry of data to suggest they are simply not accurate.

DC Classifiers

As previously described, DCs can be categorized by some general properties of their life history[3]. Our group has preferred to classify the DC family by the following criteria (which has been elaborated upon here, and in a previous review [1]) (Figure 1); first, according to whether the DCs migrate from the peripheral tissues via the lymphatics to the draining lymphoid organs ('migratory'), develop and reside within the lymphoid organs ('resident') or develop in the BM and are circulating in the bloodstream ('circulating'); second, whether they are present in the uninfected steady-state or whether they arise with infection or inflammation ('inflammatory'); third, whether the DC itself is 'unactivated' or 'activated' (the latter could result in T lymphocyte activation, tolerisation or other outcomes); fourth, into the different subtypes; and fifth, either as precursors of DC (Pre-DC) or the DC products themselves (Figure 1). Each of these classifiers could, in turn, be given more levels of complexity (e.g. which organ they are resident in, different types of activation, etc), but for the purpose of this review it will be left there. If one categorises precursors of DCs, or the DCs themselves within these criteria, placement in the context of the immune system becomes easier. No doubt there will be brushstrokes to this picture in the future.

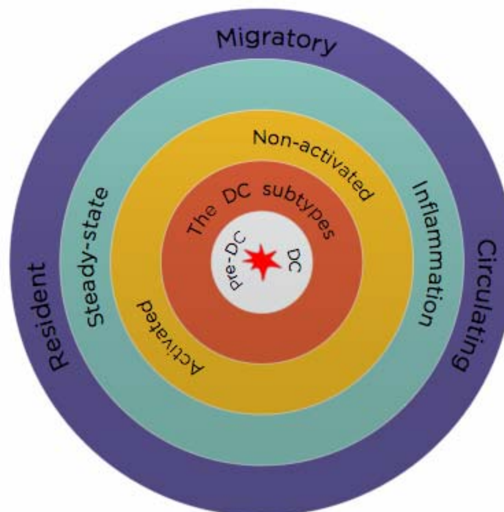

Figure 1. The DC classification wheel.

Pre-DCs versus DCs

Cells just prior to differentiation into MHC class II^{+} DCs are classified as pre-DCs. This may happen by default in the steady-state, or may require a stimulus as provided by infection or inflammation [3]. This is an important distinction to make as circulating monocytes and plasmacytoid cells, which are classed as pre-DCs, also have unique functions in the steady-state, irrespective of their additional capacity for DC differentiation. Nevertheless, their ability to generate DCs assigns them a feature that distinguishes them from non-DC antigen-presenting cells, such as B lymphocytes and macrophages. Other pre-DCs, such as pre-conventional DCs (pre-cDCs) will differentiate by default in the steady-state (also discussed later). 'Developed' DCs, by contrast, are 'dendritic' when freshly isolated, even though their functional properties can vary widely depending on their environmental exposure.

Migratory

Migratory DCs develop from precursors in the periphery then migrate via the lymphatics to their draining lymphoid organ. This process is usually accompanied by a type of 'maturation', which occurs at a basal rate in the steady-state [4-6] such that once reaching the lymphoid organ, they have a 'mature' DC phenotype [7]. Surprisingly, this process occurs even in germ-free or toll-like receptor (TLR)-signaling-independent scenarios [8, 9] but can be enhanced upon infection or inflammation [10-14]. Thus, one cannot simply use major histocompatibility (MHC) class II levels as the only activation marker to discriminate 'mature' steady-state DCs from 'mature' pathogen-activated DCs. Such factors that differentially influence T lymphocyte fate may encompass co-stimulatory molecules and cytokine production, and must always be taken into account [15]. Langerhans cells and dermal DCs belong to the 'migratory' DC category.

Resident

Lymphoid-tissue resident, or simply 'resident' DCs, are located in all lymphoid organs of the mouse, including the spleen, and are found in an MHC II^{int} 'immature' state [7], which allows their clear distinction from the mature MHC II^{hi} migratory DC [5]. Resident DCs include the $CD11c^{hi}$ $CD45RA^{lo}$ MHC II^{int} conventional DC (cDC), which can be further broken into two broad subsets; the $CD8^{+}$ cDC and the $CD8^{-}$ cDC (also known as $CD11b^{+}$ cDCs) [16, 17]. Most $CD8^{-}$ cDCs also express CD4 but are largely overlapping with those that don't [18, 19]. Unlike migratory DC, resident DC appears to be 'born' within the lymphoid organ itself and perform their functions and turn over *in situ* [1, 3].

Circulating

While it is clear that some pre-DCs, including plasmacytoid cells (pDCs) and Ly6C^{hi} monocytes, can circulate in blood and extravasate tissues depending on the circumstances, one should take care not to refer to these as circulating DCs *per se*. Some evidence suggests DCs can migrate via the blood into the lymphoid organs [20, 21]. However, CD11c^{hi} cDC are actually not found in significant numbers in the blood [22, 23], and only some in bone marrow (BM) [24]. The majority of CD11c^{+} cells in the blood are B220^{+} pDC, DX5^{+} NK cells, MHCII^{-} pre-DC [25], or Ly6C^{lo} monocytes (which might also overlap with blood pre-DC1) [23, 26, 27]. It appears that no freshly isolated cell from mouse blood is a good naïve T lymphocyte activator without prior haematopoietic development [23]. Why this conflicts with the observation of DCs in human blood [28] is unknown. One potential hypothesis could be the influence of the specific pathogen-free environment of mice, versus our daily exposure to microbes.

The Players

Monocytes

Monocytes are a form of pre-DC, but more importantly are part of the mononuclear phagocyte system (MPS) [29]. They are found in many organs including blood, BM and spleen. In the steady-state, some monocytes function by patrolling tissues for damage/inflammation, while others appear to be a reserve to function only upon infection or inflammation ([30] and references therein). They probably derive from macrophage-DC precursors (MDP) [31, 32], which, in turn, probably derive from common myeloid progenitors (CMP) [33]. The Leenen and Geissmann groups [26, 27] recently separated Ly6C^{hi} and Ly6C^{lo} monocytes and found they were the mouse equivalents of human CD14^{+} and CD16^{+}CD14^{+} monocytes, respectively. In both mice and humans, monocytes have the capacity to differentiate into macrophages in response to M-CSF [34] and into CD11c^{+} MHCII^{+} DCs in the presence of GM-CSF, with or without IL-4 [3, 35-42]. This DC transition can occur for both monocyte subsets *in vitro*, and under some conditions *in vivo* [26, 27, 43-45]. However, the precise role of monocytes in DC generation appears to be more complex than this, and is discussed later.

Plasmacytoids

Plasmacytoid cells, or pDCs [46], are another type of pre-DC in the steady-state and are found in many tissues of the mouse including blood, thymus, BM, liver, and lymphoid organs [23, 47-53]. Their best described role is in pathogen surveillance where, upon exposure to viruses [54], bacteria [49, 55] and certain toll-like receptor (TLR) agonists [56-59], they produce a host of inflammatory chemokines [60] and cytokines, including a characteristic type I interferon (IFN) burst. pDCs also have an overlapping role as antigen-presenting cells,

and express low levels of MHC I, MHC II and costimulatory molecules in the steady state, all of which are upregulated upon activation [52, 61]. At such time, pDCs acquire dendritic processes and are thus converted, in some cases [62, 63] but not all, with the ability to activate T lymphocytes [64, 65]. Collectively, these studies enlist pDCs as crucial mediators linking the innate and adaptive arms of the immune system.

Langerhans Cells

Langerhans cells (LCs) are stellate DCs that make up 2-4% of total cellularity in the epidermis[66]. Despite some initial clues about their immune activating role [67-74], and the popular dogma that LCs are the T lymphocyte-priming DCs for cutaneous infection, clear *in vivo* evidence is still lacking. There are several recent insights into LC function including virus trapping, antigen transport, and in tolerance [4, 75-83].

The origin of LCs has received some attention recently including knowledge that they can proliferate themselves or develop from skin-resident precursors [84-87]. Upon exposure to UV light or skin damage, LCs are depleted from skin and replaced by circulating precursors in a CCR2-dependent process [86] that recruits $CCR2^{+}$ $Ly6C^{hi}$ monocytes [43]. However, in the absence of inflammation, LCs homeostatically divide or derive from local precursors.

A monocyte origin of LCs in these circumstances fits with prior evidence, that LCs are members of the MPS [88-90], that *op/op* mice (deficient for M-CSF and, thus, monocytes) have reduced LCs [91], and that LCs can be derived from monocytes with GM-CSF + TGF-β [10, 92, 93].

Interstitial DCs

Interstitial DCs are found throughout the body and comprise those found in all peripheral tissues, excluding the LCs of the epidermis. They include the well-characterised dermal DCs of the skin [94] and DCs of the mucosae [95, 96]. These DCs share some characteristics with LCs but also have many differences [97-99]. While there are indications that interstitial DC are an immune regulating APC based on response to injected antigen or hypersensitivity responses[100, 101], a role for interstitial DCs during an actual infection has only been demonstrated after infection with intravaginal HSV-2[76] and Leishmania [102, 103]. Dermal DCs also appear to be derived locally from as yet unknown precursors [104].

A combination of observations over the last years has highlighted at least 2 interstitial types of DCs in many organs and their draining lymphoid tissue. Although not phenotypically correlated across all studies, it appears that there are $CD11b^{+}CD103^{-}$ $Langerin^{-}$ DCs and $CD11b^{-}CD103^{+}Langerin^{+}$ DCs. Organs of residence include the lung, gut, kidney, liver and skin, and their draining lymphoid organs [87, 105-111]. In particular, several lines of evidence identify the $CD103^{+}$ DCs as a migratory cell that shares many phenotypic similarities to resident $CD8^{+}$ DCs of the spleen including that they are both $CD11b^{lo}CD103^{+}CD24^{hi}$, as well as being able to cross-present, amongst others. While there

is some indirect evidence that these subsets derive from the monocyte subtypes [112], definitive proof is lacking. In fact, a recent study suggested they do indeed relate developmentally to splenic CD8^{+} DCs as dermal CD103^{+} DCs did not develop in mice with a knockout for the transcription factor Batf3 [113]. The development and function of these cells appear to be part of ongoing studies across many laboratories, and one expects some exciting findings to emerge about the role of these cells in the immune system (see 'notes added in proof: 1').

Resident CD8^{+} DC

CD8^{+} cDCs have been the focus of many exciting studies over the years. Unlike other resident cDCs, they are found characteristically in the T cell areas of the spleen and lymph nodes in the steady-state [114], and probably correspond to the interdigitating cell (IDC) that had been described previously [115-117]. There are many roles ascribed to this subtype in tolerance, anti-tumour, anti-viral, and anti-bacterial immunity, as discussed elsewhere [1, 118]. The recently described *batf3*$^{-/-}$ mouse was examined as a surrogate "CD8^{+} DC knockout" and demonstrated their crucial role in virus and tumour clearance, probably via cross-presentation [113]. Furthermore, the elusive human counterpart to this subtype has been insinuated to be the rare BDCA3^{+} DC human DCs ([119-123] and discussed in [1]). Clinical trials that target activity of this subtype in patients could be worth pursuing.

Resident CD8^{-} cDC

CD8^{-} cDCs are the most numerous of the resident cDCs in spleen, comprising roughly 80% of the total, but are a minor population in lymph nodes. In contrast to CD8^{+} cDCs in the T cell areas, CD8^{-} cDCs tend to be found in the marginal zones [114, 116, 124], which appear to be dependent on the CCR6/MIP-3α axis [125, 126]. However, upon stimulation by lipopolysaccharide (LPS) or other TLR agonists, CD8^{-} cDCs relocate to the T cell areas of lymphoid organs [114, 127]. Despite a recent report of their potential importance in yeast infection [128], other infections where they play an important role is yet to be determined. Considering their proximity to B lymphocytes, their high numbers in spleen (which is the lymphoid organ for pathogens circulating in blood), and their preference for CD4^{+} T cell activation [129-132] one expects future studies might highlight their importance in humoral immunity to blood-borne pathogens.

Molecular Requirements of DC Development

Cytokines

There are several cytokines that are important for DC development, and some of these are specific for particular subtypes. Certain cytokines appear to be obligatory for DC

development, some enhance DC generation and/or survival, while others are produced during certain infections to boost numbers.

Fms-like tyrosine kinase 3 (Flt3) ligand (Flt3L) is a hematopoietic cytokine that was first described to have a role for multipotent stem cell and lymphoid differentiation [133, 134]. Genetic knockouts of Flt3L and its associated signaling pathways have the most striking defects in DC development. Flt3L$^{-/-}$ mice have drastically reduced numbers of pDC, cDC and NK cells [135, 136] and Flt3$^{-/-}$ mice have a reduced DC compartment, as well as other lineages, especially early in age [33]. Despite all DC BM progenitors expressing Flt3, it has recently been proposed that flt3 ligand affects pre-DC differentiation into DCs rather than numbers of the earlier precursors [33]. The necessity of FL for DC generation *in vivo* correlates with the observation that blocking its signaling leads to reduced DC numbers [137], and that injection of recombinant FL can boost numbers of pDCs and cDCs both *in vivo* [138, 139] and in *in vitro* models of DC development (see below) [126, 136, 140, 141].

GM-CSF was originally identified as a cytokine able to promote the generation of granulocyte and macrophage colonies in soft agar cultures [142]. An important boon for DC research was the realization that large numbers of MHC II^{+} DC could be generated *in vitro* when monocytes or BM were cultured with GM-CSF, with or without IL-4 [35, 39]. However, it did not seem to be required for steady-state pDC or cDC development as mice deficient for GM-CSF or its receptor have normal numbers and function of pDC and cDC ([143, 144] and my unpublished observations) (see ‘notes added in proof: 2’). Interestingly, injection of pegylated GM-CSF has no reported effects on pDC or CD8^{+} cDC numbers but did generate a CD11b^{+} DC population. GM-CSF is also found to enhance pDC and cDC survival *in vitro* [49]. The most striking role for GM-CSF *in vivo* is probably during inflammatory DC generation (discussed later).

Downstream of cytokine signaling are the molecular regulators that ‘instruct’ or ‘permit’ the development of DCs to their respective lineage. A few studies have shed light on the interplay between members of the JAK/STAT pathway for such an influence. Previously, it was established that STAT3 was essential for FL-mediated signaling within DC precursors as *stat3*$^{-/-}$ mice had a severe reduction in DC numbers in the steady-state and in FL cultures of *stat3*$^{-/-}$ BM [145]. In contrast, there was no aberrant GM-CSF derived DC development but macrophage numbers were increased. More recently, Watowich and colleagues describe the dominance of GM-CSF over Flt3L in generation of particular DC subtypes – GM-CSF generated myeloid DCs, Flt3L generated cDCs and pDCs, and GM-CSF + Flt3L only generated myeloid DC. This regulation appeared to relate to GM-CSF-dependent STAT5 signaling versus Flt3L-dependent STAT3 signaling[146]. This was a unique study in that it definitively linked a pathway from cytokine, to signaling molecule to transcription factor. Similar conclusions were found in the human system, although a thorough characterization of subsets was not undertaken in that study [147]

IL-6 is well established as an important haematopoietic regulator [148], although the role for this cytokine in DC development has only recently been questioned. Initial indications came from the first descriptions of FL BM cultures. The addition of blocking IL-6 antibody or the use of IL-6$^{-/-}$ BM abrogated significant CD11c^{+} DC generation [140]. IL-6 has also been implicated in monocyte to DC conversion [149]. In contrast, another study stated that numbers of splenic pDC and cDC in IL-6$^{-/-}$ mice were unchanged, although data was not

shown [150]. Rather, the role for IL-6 was suggested to be suppressive in that study as CD11c$^+$ MHC II^{int} cells in the lymph nodes of IL-6$^{-/-}$ mice, corresponding to immature cDC and pDC, had spontaneously developed *in vivo* into activated MHC II^{hi} cells. Both IL-6 and FL act through STAT3 so that fits with the STAT3$^{-/-}$ data [145]. There may be a more complex relationship between these factors in DC development that has still to be identified.

G-CSF, a myeloid cytokine that can immobilize haematopoietic progenitors, is a potent inducer of numbers of pDC, but not cDC. This effect has been observed either when administered alone or in combination with FL or thrombopoietin [139, 151-153]. Whether this cytokine exerts its affect by boosting numbers of the early precursors and/or specific pDC-restricted precursors is not clear.

M-CSF is another myeloid cytokine whose role in DC development is still under debate. M-CSF null mice (*op/op*) and M-CSF receptor$^{-/-}$ have severely perturbed monocyte development in early life and an absence of some macrophage subsets [43, 154-156]. While total DC numbers are down in these mice [157], it is in accordance with total spleen size and so probably does not reflect a specific defect in the DC compartment ([158] and David Vremec, personal communication). Of the DCs that are present, the splenic subtypes are also in their expected ratio and density and exhibit normal functions (in the assays tested), suggesting M-CSF is not obligatory for steady-state DC precursors, nor are the precursors monocytes. However, M-CSF might play a role in the maintenance of the pool of DCs [28] and in augmenting DC development[159]. M-CSF has also recently been described as a novel DC poietin. In that study, the non-adherent cells ordinarily discarded in M-CSF BM cultures (which are employed to generate macrophages) contained DCs [160].

Transcription Factors

In recent years, the generation of mice genetically manipulated for specific transcription factors has highlighted their role in the development of particular cell types, including DCs [3, 161]. Below is a description of some of these regulators.

The interferon regulatory factor (IRF) family of proteins (IRF-1 to -9) waswere originally identified as transcription factors involved in gene regulation in response to type I and II interferons [162]. IRF-8, also known as interferon consensus sequence binding protein (ICSBP), is involved in several developmental and functional roles of the immune system. *Irf8*$^{-/-}$ have a chronic myelogenous leukaemia-like phenotype and are severely immuno-compromised [163, 164]. Interestingly, these mice have reduced splenic CD8$^+$ cDC, pDC and LC numbers [165-168]. *Irf4*$^{-/-}$ mice have a converse phenotype, with an absence of most CD4$^+$ cDC, some pDC, but no effect on CD8$^+$ cDC [169]. *Irf4*$^{-/-}$*Irf-8*$^{-/-}$ double knockout mice have further reductions in these subsets [170]. *Irf2*$^{-/-}$ mice also have reduced CD8$^-$ cDC numbers, but normal numbers of pDC and CD8$^+$ cDC [171]. Collectively, these studies place IRFs as crucial drivers of the DC compartments. There is the formal possibility the observed susceptibility to infections in IRF null mice may not simply be due to defective interferon responsive gene induction after infection, but also a result of DC and other immune cell deficiencies in the first place, prior to infection.

The nuclear factor of κB (NF-κB) family has several subunits and, like the IRFs, is crucial in both steady-state and induced immune responses. NF-κB is downstream of a host of signaling pathways [172, 173]. Recently, defects in steady-state pDC and cDC development have been observed in *Nfκb1*$^{-/-}$ and/or c-*Rel*$^{-/-}$ [174]. To add complexity, it appears that different members of this family can contribute independently to processes that govern DC development, cytokine secretion and morphological activation.

Id2 and Id3 are members of the helix-loop-helix (HLH) transcription factors and exert inhibitory effects on the transcriptional activities of other HLH transcription factors. A transcriptional profiling study revealed that Id2 was upregulated during DC development and that mice deficient for Id2 lacked Langerhans cells, and had markedly reduced numbers of splenic CD8^{+} cDC, but no change in pDC numbers [175]. However, ectopic expression of Id2 and Id3 can strongly inhibit the development of pDC without effect on the development of cDC, at least when DCs are derived from human CD34^{+} progenitors [176]. These findings suggest that Id2 and Id3 are obligatory for cDC development, and inhibitory for pDC.

Spi-B is a member of the *ets* familiy of TFs. In human DC cultures and humanized mouse models, knockdown of Spi-B led to the abrogation of pDC development, suggesting a crucial role for this factor in pDC lineage commitment [177]. Conversely, over-expression led to the suppression of NK, B and T cell development [178]. Considering Spi-B binds to member of the IRFs, like its homologue PU.1, it makes sense that it could be a factor involved in skewing DC lineage decision-making.

E2-2 is a member of the E family of transcription factors, which are involved in many biological processes including lymphoid and neuronal development. Recently, E2-2 was found highly expressed in mouse and human pDCs, and a binder of many pDC-related gene promoters including IRF-8 and SpiB. Knockdown and knockout of this TF in both mouse and human models reveals a non-redundant role of this factor in pDC development [179, 180]. E2-2 appears to affect pDC specification at the final pre-pDC to pDC transition. There may even be feed-forward loop involving E2-2 and Spi-B in combination.

There has been solid progress in understanding the role of many individual as well as families of transcription factors in DC development. Identification of yet more players as well as the biochemical and bioinformatics study of their interactions and relationships to each other should illuminate the developmental programming of the DC subtypes. Better insight into lineage fate may also be achieved by visualizing simultaneous co-expression of these regulators under different fluorochromes, for example, as a means to separate lineage decisions – this could be achieved either by flow cytometry or by real-time tracing of lineage commitment and may be more informative than current surface phenotype-based separations.

Table 1. Molecular regulators of dendritic cell subtype development

	pDC	$CD8^+$	$CD8^-$	LC	Inflammatory Mo-DC	References
Flt3/L	+	+	+	–	–	[33, 126, 135, 138, 140, 181-185]
GM-CSF/R	–	–	–	?	+	[143, 181, 186-188]
M-CSF/R	+/–	+/–	+/–	+	+	[43, 154, 158-160]
TGF-β	–	–	–	+	?	[189, 190]
IRF-8	+	+	–	+	?	[165-168, 170, 191]
IRF-4	–	–	+	–	?	[169, 170]
IRF-2	?	–	+	+	?	[192]
RelB	–	–	+	–	+	[193-195]
Id2	–	+	–	+	?	[175, 176, 196]
Gfi1	+	+	+	+	+	[197]
Runx3	?	+	+	+	+	[198]
E2-2	+	–	–	?	?	[179, 180]
Ikaros	+	+	+	–	?	[199]
Spi-B	+	–	–	+	?	[177-180]
Batf3	–	+	–	–	–	[113]
Stat3	+	+	+	?	–	[145, 146]
Stat5	+	?	?	?	+	[146]

+ Has an effect
– Does not have an effect
+/– Has a partial effect
? Effect has not been explored

GM-CSF-Derived DCs and Inflammatory Tip DCs: One and the Same?

It is now clear that monocytes are generally not the major precursors for steady-state DCs, with some exceptions (see above). Rather, they readily convert to DCs in inflammatory situations [26, 45, 200-203]. Related to this is that the DCs generated when monocytes or earlier progenitors are cultured with GM-CSF represent these inflammatory scenarios [204]. Considering this culture method is the most widely used protocol for DC generation when assessing genetic, synthetic, biological or pathogenic factors in DC biology, the interpretation of numerous GM-CSF-derived DC studies that claim to represent the steady-state situation may need to be revisited.

As monocytes generate a relatively homogeneous population of $CD11c^{+}CD11b^{+}$ $CD8^{-}$ MHC II^{+} 'myeloid' DCs in GM-CSF cultures, they have often been referred to as the equivalent of $CD8^{-}$ cDCs of the spleen and lymphoid organs, which share this pattern of marker expression. However, formal proof of such a correlation is lacking. Indeed, several lines of evidence suggest monocyte-derived DCs are not related to the development of steady-state DCs, apart from LCs and interstitial DCs in some situations.

These include the observations that *(i)* GM-CSF levels are low in the steady-state[205-210], *(ii)* that knockouts for GM-CSF and its receptor have normal steady-state DC representation and function [143, 144] (see 'notes added in proof: 2'), *(iii)* that null mice for M-CSF (*op/op*) and its receptor have monocyte/macrophage defects [43, 154-156] but a normal density and ratio of DCs ([158] and David Vremec, personal communication), and *(iv)* that the major cytokine driving steady-state DC generation is Flt3 ligand (FL) [135, 145], which does not affect monocyte numbers[211] or GM-CSF derived DC development [145].

Moreover, it is important to note that *in vivo* $CD8^{-}$ DCs and GM-CSF-derived DCs are fundamentally different in terms of their derivation from precursors [200, 212], in their function [204], and in their genetic make-up [122]. Thus, despite popular assumption, they may represent distinct DC types.

GM-CSF is more relevant for DC generation upon infections, such as with *Listeria monocytogenes* [208] and other situations [213]. The first clear hint of the *in vivo* equivalent of GM-CSF-driven monocyte-derived DCs came when a novel DC subtype was identified in mice infected with *Listeria monocytogenes* [214]. This TNF-α (tumour necrosis factor α) and iNOS (inducible nitric oxide synthase) producing DC (Tip DC) was distinct from steady-state DC. While it was assumed that Tip DCs derived from $Ly6C^{hi}$ monocytes in a CCR2-dependent process [26, 214, 215], formal evidence came from our *in vivo* studies using adoptive transfer in conditions of inflammation and *Listeria* infection [200, 204]. Since the description of Tip DCs, and even before, other $CD11c^{int}CD11b^{hi}$ DCs have been described that arise during many bacterial, viral and parasitic infections, and autoimmune sequelae (summarized in Table 2 in [1]). Whether these are involved in immunity, infection control or regulation will be very important to dissect in the future.

Considering their similarites, Xu and colleagues thoroughly investigated whether GM-CSF-derived DC cultures represent Tip DCs rather than those of the steady-state [204]. By all parameters tested, this appeared to be the case. Specifically, GM-CSF-derived DCs, but not FL-derived DCs (see below), were large and granular, had the phenotype $CD11c^{+}MHCII^{+}Mac\text{-}3^{+}$, could produce TNF–α and iNOS in response to TLR agonists, and could develop from $Ly6C^{hi}$ monocytes – all parameters which aligned them with Tip DCs, and not those of the steady-state or from FL cultures.

Flt3 Ligand-Derived DCs Represent Steady-State $CD8^{-}$, $CD8^{+}$ and pDCs

If GM-CSF cultures do not best represent the steady-state DC pathway, then can any other culture methods substitute? Immunex scientists provided the answer in a key publication [140], with a more thorough examination of the question from our laboratory

[126]. In view of the observations that FL treatment increased DC numbers [138, 216], and that $FL^{-/-}$ mice had abrogated DC numbers [135], the Immunex group devised a novel culture system to generate DCs[140]. BM cell cultures supplemented with FL for 9 days generated DCs to roughly the same number as total BM cells initially seeded. Interestingly, and in an often-overlooked paper, these DCs were morphologically and phenotypically distinct from GM-CSF-derived DCs [217], with further characterization of these differences by Xu *et al.* [204] (see above).

Despite the lack of CD8 or CD4 expression, the investigators [136, 140, 141] classified three DC subtypes in these cultures; $CD11c^{+}B220^{+}$ pDCs, $CD11c^{hi}CD11b^{lo}$ DCs, and $CD11c^{lo}CD11b^{hi}$ DCs. Considering $CD11c^{hi}CD11b^{lo}$ DCs (which would have also contained pDCs) up-regulated surface CD8 after lipopolysacharide (LPS) activation and secreted IL-12 p70 upon activation, they were proposed to be similar to $CD8^{+}$ cDC of the spleen.

Using new markers, we better separated two cDC subtypes into $CD24^{hi}Sirp{-}\alpha^{-}$ and $CD24^{lo}Sirp{-}\alpha^{+}$ DCs, and demonstrated they were indeed *bona fide* equivalents of $CD8^{+}$ and $CD8^{-}$ cDCs – not only phenotypically, but also functionally (Figure 1 in [1]). For example, only the $CD8^{+}$ FL-DC equivalents expressed cystatin C and TLR3, produced IL-12 p70 in response to TLR agonists, could cross-present cell-associated antigen to $CD8^{+}$ T cells, and were dependent on the IRF-8 transcription factor for its development, among other unique features[126].

If the research purpose is to investigate the development or function of the steady-state pDC, $CD8^{+}$ or $CD8^{-}$ cDCs, then FL cultures are currently the simplest and most representative *in vitro* protocol. Most importantly, one can get up to 20 × more total DCs from BM FL cultures per mouse compared to spleen, and up to 80 × more $CD8^{+}$ DC equivalents. Access to a mouse FL-expressing Chinese Hamster Ovary cell line is available upon request.

The Steps in DC Development

Determined so Far...

So what are the steps in steady-state DC development? Monocytes had been the presumed precursor of DCs for many years. As mentioned earlier, our first set of experiments to match the observation that $Ly6C^{hi}$ monocytes and $CD8^{-}$ cDC could be precursors of $CD8^{+}$ cDC [44, 218] did not find the same [200, 219]. Transfer of the ‘resident’ $Ly6C^{lo}$ subtype could produce some DCs in spleen, lung and lamina propria [32, 203, 220]. However, in some of these cases, inflammation or the experimental clearance of DCs was required for efficient engraftment. Whether these latter situations represent an ‘emergency’ process, or simply amplify the normal process, is not clear. Thus, our results fit with previous and subsequent data [26, 27, 32, 43], which floated the idea that monocytes are not the only precursors of DCs, and that $CD8^{+}$ and $CD8^{-}$ DC were not simply precursor-product related.

Considering that few cells with the phenotype of $CD11c^{hi}MHCII^{hi}$ DCs are found in blood, but that DCs are found in large numbers in the spleen [23] we reasoned that precursors of DCs, rather than the DCs themselves, may be migrating from BM. There was also the

possibility that spleen contained a pool of long-term DC precursors. In any case, we logically sought for the immediate precursor stage before splenic DC in the spleen.

Through a process of exclusion, we sought the identity of cell types that could not generate DCs *in vivo*, in order to find the cell type/s that could. On the basis of density and cell surface markers, we whittled away the majority of leukocytes (by testing their *in vivo* DC generating potential) to reveal a population that represented only 0.05% of splenocytes. We termed this type of pre-DC a pre-conventional DC (pre-cDC), as they only generated $CD8^+$ and $CD8^-$ cDC *in vivo* in non-irradiated mice, but not pDC or any other cell lineage [200]. They were $CD11c^{int}MHCII^-CD43^+SIRP\text{-}\alpha^{int}B220^{lo}$, and presumably $flt3^+$ as they were responsive to ligand for $flt3^+$ (FL) in culture to differentiate into DCs.

The peak of DC generation by pre-cDC was 5 days after transfer, involved 0-3 divisions, and donor DC progeny disappeared by day 10. A time of clearance of 5 days fit with the reported turnover of splenic DCs [221, 222]. Thus, it appeared pre-cDCs were a transient precursor of DCs. They also reflected (despite SIRP-α or a similar marker to separate from $Gr\text{-}1^{lo}$ monocytes) those identified by the Cattral group in many other organs [25, 223], and may have been a component of heterogeneous populations identified by others [224-226]. These are the first studies cumulatively to identify pre-DCs.

Another observation by our group was that there were precursors already dedicated to making only $CD8^+$ cDC, or $CD8^-$ cDCs [200]. This was by far the most convincing evidence that $CD8^+$ DCs can be a distinct developmental lineage from $CD8^-$ DC, on the back of other indirect evidence from TFs and turnover data. These so called 'pre-$CD8^+$ DC' were characteristically $CD24^{hi}$, a phenotype similar to $CD8^+$ DC themselves. 'Pre-$CD8^-$ DCs' were correspondingly $CD24^{lo}$. However, the major $CD24^{int}$ population could make both subtypes as a population. Due to their small clonal burst size we were never able to tell if, as single cells, they were separate pre-$CD8^+$ and pre-$CD8^-$ DC precursors that overlapped in phenotype, or a common pre-cDC precursor. It still remains to be determined whether these precursors are a unique population that migrates from the BM and at what stage in haematopoiesis they branch.

While it was known that DCs could be generated from HSC, MPP, CMP and CLP [185, 227-230], the question remained whether there were intermediate precursors *en route* to pre-DC. Fogg *et al.* have identified a precursor within the CMP fraction that could produce cDCs (both $CD8^+$ and $CD8^-$) and macrophages, but not granulocytes, pDCs or any other lineage [31]. In contrast, our group [212] and that of Manz [159] identified a precursor in BM that could only generate pDCs, $CD8^+$ and $CD8^-$ DCs but less than 5% able to produce macrophages *in vivo* or *in vitro* [212]. We termed this cell a 'pro-DC'. As an aside, we have a preference for the term 'pro-DC' over CDP (common DC progenitor) as both studies found that not all single cells made all DC subtypes, thus were not truly 'common' by definition. In any case, this cell was able to generate DCs in unperturbed non-irradiated mice, and occurred without the addition of Flt3L or, in our case, sorting based on expression of the receptor for macrophage colony-stimulating factor (M-CSFR) – factors that others claim skews the *in vivo* pro-DC away from macrophage development and towards pDC development [33]. We found that this cell expanded significantly, and gave rise to a distinct pre-DC intermediate *en route* to the generation of the DC subtypes. Thus, the picture downstream of pro-DC was relatively complete – pro-DCs divide and differentiate into pre-DCs, which then differentiate into the

DC subtypes. One should note that the authors of MDPs have recently conceded that their precursors do actually make pDCs [231]. However, we have only observed a minority (<5%) of single pro-DCs able to generate macrophages. This may be due to the fact that pro-DCs and MDPs are distinct populations (discussed below) or that macrophages are only detectable by spleen sections and not by flow cytometry.

MDPs and pro-DCs are very similar in phenotype (both are $Lin^{-}Sca\text{-}1^{-}IL7R^{-}CD117^{int}$). However, at least in our hands, most but not all pro-DCs are $CD34^{-}CD16/32^{-}$ [212], whereas MDPs are largely $CD34^{+}CD16/32^{+}$ [31]. We also find that just over half of *in vivo* pro-DCs are M-$CSFR^{+}$, which is a distinction from the other groups [212]. Geissman *et al.* have noted unpublished observations that all MDPs are $Flt3^{+}$, and all pro-DCs (as sorted by the Manz criteria) fall within the MDP gate [30] (see 'notes added in proof': 4). CD16/32 and CD34 expression was not mentioned there. Another study found that $MCSFR^{+}$ cells alone could sort MDPs [33]. That latter study also claimed no pDC production, although this claim seems to have been reversed as well. A second important difference is that the majority of pro-DCs do not generate colonies in response to GM-CSF, whereas MDPs do. Considering GM-CSF-derived DCs better represent inflammatory DCs (above), and clonal studies of MDP used this cytokine, it is possible that MDPs are precursors of monocyte-derived DCs, but not monocyte-independent steady-state DCs. Arguing against this was that MDPs could generate large numbers of $CD8^{+}$ and $CD8^{-}$ cDCs *in vivo* [31]. However, the assays in that part of the study were not clonal, so there is a possibility that macrophage and DC precursors were distinct populations within cells classed as MDPs. Another possibility is that there are multiple routes to DC production and both pro-DCs and MDPs make a contribution. There is precedence for this considering both CLPs and CMPs can give rise to all splenic DC subtypes. Certainly further comparison between the two progenitors will be required.

That spleen contains cDC-only precursors, whereas BM contains pDC and cDC precursors, the question arises about the branchpoint of pDC development. As pDCs are found in most organs of the body, the location of pDC precursors has been difficult to pinpoint [232]. We have previously postulated that pDC develop in the BM and/or blood then traffic to the lymphoid organs via the blood as developed pDCs, similar to B lymphocytes [232]. Evidence for this comes from the observations that developed pDCs are found in significant numbers in the blood and BM [50, 54, 233], that pDC circulation is CD62L (L-selectin) dependent[51, 234, 235] akin to T and B lymphocytes, and that their immediate precursors ($CD11c^{int}B220^{+}Ly49Q^{-}MHCII^{-}$) are not detected in the peripheral lymphoid organs, whereas developed pDCs ($CD11c^{int}B220^{+}Ly49Q^{+}MHCII^{+}$) are [223, 236-238]. They can be derived from either *fms*-like tyrosine kinase 3 (Flt3)-expressing CMP or common lymphoid progenitors (CLP) and, independent of their derivation, can also have features that overlap with lymphocytes [233, 239-242]. See 'notes added in proof: 3'.

Blood-Derived or Organ-Resident Precursors

Since DCs are present in human blood, it was presumed that DCs are derived in the BM and circulate as DCs to the organs, and there is evidence for such a phenomenon[21, 243, 244]. However, as many have now identified or implied organ-resident DC precursors [25,

43, 104, 200, 212, 223], it suggests that the final step of differentiation may take place within the organ itself for some DC subtypes. We speculated in one study that the existence of pre-cDCs could indicate derivation from a long-term organ-resident reservoir of DC precursors, and/or that they represented 'in-transit' cells from the BM *en route* to DC development [200]. Liu *et al.* addressed this issue using mouse pairs that differ in CD45 allotype and are physically joined (parabiotic mice) [245]. Within days, each mouse had 17-35% of their DCs of donor origin (although not 50%), suggesting precursors migrate via the blood and seed the spleen to generate most, if not all DCs. Their most convincing data was from the tracking of DC origin after parabiont separation, where most DCs were host origin within 10-14 days. They also claim this necessitates a re-evaluation of DC lifespan. However, as they show precursors migrate in the bloodstream, and we have shown that the pre-cDC to DC transition involved cell division; the apparent longer life-span may be due to the lag between seeding of the pre-cDC, rather than their proliferation to generate DCs. An alternative explanation is that pre-cDCs do not proliferate but DCs do, and there is evidence for this. Formal proof would require an observation of pre-DC division without MHC class II up-regulation. Which precursor is migrating from BM is also not clear but could include one or more of the MDP, pro-DC or pre-DC stages. Pre-DCs appear to be the best candidate in light of the DC turnover data and their presence in BM, blood and spleen [25]. See 'notes added in proof: 3'.

All of the above apparently contradictory studies may tie together with the following scenario, which it is stressed is only speculation at this stage and may be an oversimplification; MDPs could peel away from the myeloid lineage with both macrophage and DC potential, these then split to true macrophage/monocyte precursors versus DC-only pro-DCs, which then generate pre-DCs. Somewhere during this process pDCs branch off, while other pre-DCs migrate to lymphoid organs, seed there, and develop into DCs *in situ*. Better lineage tracing tools would be required to definitely answer such questions.

Lineage Issues

Do DCs Represent a '3rd Lineage'?

Separating the leukocyte subtypes has historically relied on functional/phenotypic correlations. For example, lymphoid cells were first ultra-structurally separated from myeloid cells, then dissected into T and B lymphocytes, then T lymphocytes into CD4 and CD8 T lymphocytes, then CD4 T lymphocytes into conventional CD4 T lymphocytes and Treg, etc. While this approach has worked for most cell types, there are cases where there have been false relationships assigned [122, 246].

Grouping gene expression profiles is a more rigorous test of relationships, and which appears to give a better approximation of molecular and functional lineage relationships [246]. In this form of analysis a clear presence or absence of genes between two cell types, at least at the population level, denotes distinct cell types. This does not preclude further separations in the future if new sub-sub-types are found and does not formally demonstrate that every single cell within that population does or does not express that gene. However, some clear approximations can be made.

After the initial phenotypic separation of mouse DC subtypes [115, 143], two studies were the first to highlight genetic diversity between the DC subtypes [18, 19]. Here, both groups recognized that $CD8^+$ DCs were unique compared to the $CD4^+$ and $CD4^-$ subtypes of $CD8^-$ DCs, the latter two of which were very similar. As an aside, it is for that reason that grouping these last two subtypes as bulk $CD11b^+$ splenic DC is valid.

More recently, Robbins *et al.* compared these subtypes plus pDCs and other leukocytes within, and between, mouse and humans. They highlighted some striking correlations and hierarchy of relationships. While not unexpected, they nicely demonstrated correlations between mouse vs human pDCs, and mouse vs human GM-CSF-derived DCs [122]. They also demonstrated a hierarchical relationship within the species' DC subtypes; e.g. murine $CD8^+$ and $CD8^-$ DC subtypes were most related, followed by their relationship to pDC. The relationship of these DC subtypes, however, was relatively distant from the interrelated myeloid lineages including monocytes, monocyte-derived DCs (with GM-CSF), macrophages and granulocytes. They even postulated an interesting idea that pDCs, $CD8^+$ DCs and $CD8^-$ DCs represent a 3rd self-standing lineage of 'DCs' in addition to the classic myeloid and lymphoid distinction.

This genetic analysis fits perfectly with our findings of *(i)* late-branching between the cDC subtypes (there is a pre-cDC precursor in spleen that makes only $CD8^+$ and $CD8^-$ DC), *(ii)* their proximity with the pDC pathway (some of the earlier single pro-DCs from the BM can make all DC subtypes but most are biased to cDC-only generation), and *(iii)* their distinction from the myeloid pathway (pro- and pre-DCs do not make macrophages, and monocytes do not make splenic DCs) (see above and [3, 126, 200, 204, 212, 247]). It also emphasizes that, despite popular belief, $CD8^-$ splenic DC and GM-CSF driven monocyte-derived 'myeloid' DC are not the same cell, as discussed above and elsewhere [1].

Molecular versus Cellular Common Origins

I would argue that two cells can have a common cellular ancestor (of the same *cellular* lineage), or have different ancestors that give the same cell type due to molecular regulators (of the same *molecular* lineage). Why is this an important distinction to make when talking about lineage? Because, there is growing evidence that there are more and more exceptions to the simple binary decisions in oft-drawn haematopoietic diagrams.

For example, there is precedence that apparently divergent progenitors can give the same cell type. The clearest example is that both $Flt3^+$ CMP and CLP, opposite ends of the haematopoietic spectrum, can generate all DC subtypes both in the mouse, and in humans [185, 230, 248]. Therefore, does one ascribe a myeloid pDC to be different to a lymphoid pDC? Akashi and co-workers found this not to be the case as the DC subtypes were virtually genetically indistinguishable whether they derived from myeloid or lymphoid progenitors, in both mouse and humanized mouse models [241, 249]. This observation could also fit with the '3rd DC lineage' concept.

Graded Commitment Model

In classic models of haematopoiesis, progenitors progressively lose multipotency and become increasingly lineage-restricted (Figure 2, left panel). To identify such progenitors, the traditional approach has been to isolate a candidate progenitor by a combination of cell surface markers or a reporter, and then test it *in vivo* for it's lineage potential (usually in myeloablated recipients), followed by assessment of its clonal potential in *in vitro* assays that drive a progenitor in different directions. In that way, a multipotent progenitor (MPP) is said to be $Lin^{-}Sca1^{+}ckit^{+}flt3^{+}$ and the majority of single cells sorted from that population can make B lymphocytes in OP9 cultures, T lymphocytes in OP9-DL1, macrophages in M-CSF, granulocytes in G-CSF etc. But while these *in vitro* assays may demonstrate the *possibility* that a single cell can be driven down a particular direction, it' is not a true test of its *in vivo* fate, especially considering an *in vivo* environment will pull the cell in a multitude of directions.

Based on data that arose from our single cell DC progenitor experiments *in vitro*, a different model, which I have termed 'graded commitment', could better explain some of the phenomenon observed [1]. In this model, commitment to a restricted lineage/s can occur at any stage of the hematopoietic pathway with differing probability. In this way a phenotypically defined multipotent progenitor can already have committed to pDCs only, for example, but still divide and differentiate so that all downstream progeny go through a phenotypic CMP/CLP, then a pro-, then a pre-DC stage, but all the while having already committed to pDC-only development (Figure 2, right panels).

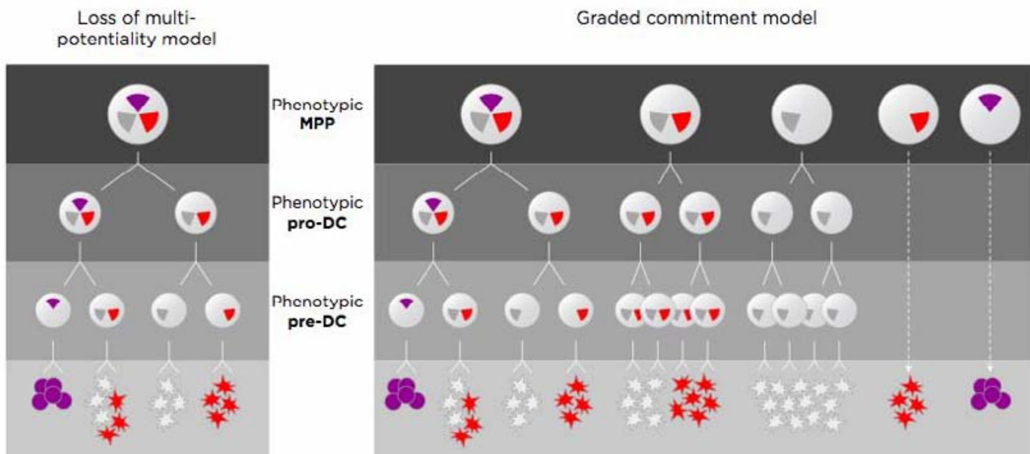

Figure 2. 'Loss of multipotentiality' versus the 'graded commitment' models.

The current paradigm states that every single cell in what is defined as a multipotent progenitor (MPP) is able to make every cell lineage. As MPPs divide and develop, they lose multipotentiality and progressively restrict to fewer lineages in their output. In this diagram each large circle represents a single cell, and their cell fates are inset. In the case of DC development in the loss of multipotentiality model, a multipotent MPP would go through phenotypic intermediates of pro- to pre- to DCs. But every MPP would be capable of generating every DC subtype (left panels). However, in the 'graded commitment model', levels of particular transcription factors or other such factors may already specify cell fate. In this way, while a single phenotypic MPP may divide and go through all phenotypic stages in DC development, its fate may be specified already at the MPP stage, or further downstream,. There may be single, double, triple etc. commitment already specified.

What is the evidence for such a model? As mentioned above, we established that FL cultures are the best culture model to reflect development of the $CD8^+$, $CD8^−$ and pDC lineages [126]. It allowed us for the first time to track the developmental outcome of a single pro-DC [212]. There, we seeded one GFP^+ pro-DC amongst 500 non-GFP 'filler' pro-DCs (a single pro-DC could not propagate on its own) in conditioned medium and waited 5 days. Of the clones that developed, to our surprise we found that only 16% of developed clones made all three DC subtypes, despite the seeded pro-DCs being homogeneous for over 45 tested phenotypic markers. The remainder of single cells made, in order of frequency, DC clones of only $CD8^+$/ & $CD8^−$ (47%), $CD8^+$ alone (18%), $CD8^−$ alone (9%), and the remainder, pDC alone or in combination with only one other subtype (9%).

To establish a 'control' where 100/100 cells would generate clones containing all subtypes we sorted $Lin^−Sca^+kit^+$ progenitors, which should contain $Flt3^+$ MPPs and ST-HSCs, expecting that all such cells were truly multipotent. However, of the clones that developed only 50% generated all DC subtypes. The rest gave, again, a mixed picture of commitment similar to that for pro-DC. Why might this be so? The *in vitro* conditions were not limiting as every well contained the internal control of 500 filler pro-DC that were perfectly capable of generating all DC subtypes on a population level. Since some of the single cell clones expanded to over 1000 cells in size, the precursors *en route* were exposed to many different cells, so a subtype-specific niche in the well can also not be the reason. Somehow, the cell's fate was predetermined specified down a certain DC lineage at some early stage. This could have been due to intrinsic and/or extrinsic cues before or after isolation, and is the subject of continued investigation.

The molecular mechanisms that would guide such fates may include the numerous transcription factors that have been described that differentially guide DC subtype development (see above and [3]). Evidence for TF expression being better predictors of commitment has come from reporter mice explored by Akashi and colleagues [250-252]. Their regulation may be stochastic within the cell, or depend on external cytokine or cell-associated signals. In the context of DC development, the Flt3/FL axis may be an 'instructive' mediator in such an 'all DC' program with evidence that enforced expression of committed $Flt3^−$ Megakaryocyte/Erythroid progenitors (MEPs) could divert DC development [253, 254]. This can also fit with the '3rd DC lineage' concept (above) such that a fixed DC program can be initiated in single cells, even as far down 'opposite ends' of haematopoiesis as the lymphoid and myeloid pathways [249]. Kincade and colleagues also provide important

evidence and arguments against the classical binary models of haematopoiesis[255], which apply here. While not discussed further, existing data for many cell types can fit such a phenomenon. An assertion of the relevance of this model *in vivo* would ultimately require good cell tracing technologies.

If this phenomenon occurs *in vivo* – of an early stage haematopoietic progenitor being able to generate a clone of only one DC cell subtype – then the classic models of haematopoiesis may not be accurate. Thus while individual cells of an early progenitor population all share a common surface phenotype, on the single cell level they may 'make their mind up' at any stage – conceivably as early as a stem cell, or as downstream as the immediate progenitor. It is emphasized that this only theoretical and based on *in vitro* data and *in vivo* proof is ongoing.

New Technologies for Single Cell Tracing

Haematopoietic studies have undergone several phases over the last decades punctuated by changes in assay. The original work of Till and McCulloch investigated the ability of transferred progenitors to generate, from a single cell, colony-forming units in the spleens of irradiated recipients [256], thus establishing the concept of a stem cell. Depending on the time colonies were harvested from recipients and the cells re-transferred into new recipients allowed researchers to determine that there were short, intermediate and long-term progenitors in the bone marrow. The advent of *in vitro* soft agar colony-forming assays, developed by Don Metcalf, recapitulated some of the *in vivo* findings and led to the discovery of different progenitors and colony-stimulating factors (CSFs) able to generate differentiated cells of the many lineages. Somewhat later, the ability to stain for specific cell surface markers and sort cells by flow cytometry revolutionized the dissection of progenitor stages with greater restriction to lineages, not to mention the identification of host of new lineages. Subsequently, the molecular mechanisms governing these processes were identified using knockdowns, knockouts and reporter mice. Combined, these technological progressions have given rise to our current models of haematopoiesis.

However, the missing link in all of these models is the ability to track single cell fate *in vivo* in large numbers, and to track single-cell decision-making processes real-time. Why are single cell studies important? As mentioned above, the fate of a single cell is ultimately required to establish true lineage fate. Simple *in vitro* assays are useful but they 'push' cells one way or another and certainly do not reflect the complexity of lineage fates. Moreover, the current maps are based on population-based outcomes, and there may be levels of commitment complexity not seen by these approaches.

Actually, there have been some valiant attempts to track cells at the single cell level *in vivo*. The most well noted are studies of reconstitution of a whole haematopoietic system from a single cell [257], and of tagging cells using retrovirus to track progenies of single cells [258]. While these were landmark studies assessing a handful of clones, high throughput studies of single cell outcomes were lacking. Some labs have made great strides by up scaling these types of experiments [259, 260]. However, the labour of analyzing one mouse per one

cell can be restrictive. A new technology termed cellular barcoding developed by Schumacher and co-workers may be one technology that helps to change that.

This system employes the use of a panel of 5000 artificially constructed barcodes. Barcodes are simply semi-random and non-coding stretches of 100-nucleotides. They are packaged into lenti- or retrovirus that is then used to infect progenitor cells of choice so that roughly one barcode is integrated into one cell. Barcode-labeled cells are then allowed to develop *in vivo* into the various lineages. As the barcode is genome-integrated, each subsequent daughter cell also inherits the barcode. In this way, different progeny cell types at a later time point can be isolated and their genomic DNA assessed for their barcode inheritance signature using a custom-made microarray. By comparing the shared and distinct barcodes between progeny cell types, one can assess lineage relationships at the single cell level in a high throughput fashion. Using this technology is akin to doing 5000 single cell assays simultaneously in the one mouse. The first demonstration of this technology was recently published to investigate the phenomenon of location-specific T cell imprinting [261]. The use of this system in haematopoietic studies is part of ongoing work.

The second leap in understanding development will be tracking the fate of single cells real-time. While clonal assays are informative in their capacity to generate multiple cell types, only the end of the experiment is generally assessed. However, the road from one cell to, for example, 100 cells is whole journey in itself. In fact, while there are a slew of maps in the literature from one progenitor to another, including by this author, ‘true’ maps can only be accomplished when every cell and it’s progeny, from start to finish, is traced without loss of identity – and the only way to achieve this is real-time imaging. This requires intermittent time of only minutes in order not to lose cell fate in a standard cell culture plates and tracing haematopoiesis over a period of days, not hours. Tracking such a process *in vivo* is an entirely other challenge but is already making strides [262]. Clearly, such investigations provide many technical and logistical burdens. The latest developments in this field are reviewed extensively elsewhere [263].

Conclusion

By combining knowledge of the location, phenotype and properties of precursors, their stages of development, their molecular regulators and, in the future, their outcome on the single-cell level, we will get closer to the true picture of the complex processes of haematopoietic development.

Notes added in proof

1. Additional studies have now been published on the development and functions of $CD103^+$ DCs, and their counterparts in humans. [264-268]
2. Recent data indicates that GM-CSF does play a role in steady-state development of many DC subtypes, and is highlighted in particular in mice lacking both *gm-csf* and *flt3l* [269].

3. A more recent study [270] has confirmed ours and others' previous data ([25, 31, 159, 200, 212, 223] and reviewed in [1]) and done some extension on the following linear transition pathway; MDP branch to give myeloid cells or pro-DC (CDP) in the BM. pDC development branches off from the pro-DC, and pre-cDC then migrate via the blood to seed lymphoid organs for cDC development. One should stress that this pathway has not been confirmed on the clonal level, so whether MDP are true 'common' precursors or two precursors overlapping in phenotype remains to be seen.
4. Some evidence now suggests an overlap between MDPs and pro-DCs (CDPs) [271], as all CX_3CR1^+ MDP overlapped with $CD115^+Flt3^+$ CDP, and vice versa. However, as mentioned, differences between groups' precursors include CD115 (M-CSFR), CD16/32 (FcR) and CD34, that still need to be resolved.

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References

[1] Naik, S. H., Demystifying the development of dendritic cell subtypes, a little. *Immunol Cell Biol* 2008. 86: 439-452.

[2] Hume, D. A., Macrophages as APC and the Dendritic Cell Myth. *J Immunol* 2008. 181: 5829-5835.

[3] Shortman, K. and Naik, S. H., Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* 2007. 7: 19-30.

[4] Hemmi, H., Yoshino, M., Yamazaki, H., Naito, M., Iyoda, T., Omatsu, Y., Shimoyama, S., Letterio, J. J., Nakabayashi, T., Tagaya, H., Yamane, T., Ogawa, M., Nishikawa, S., Ryoke, K., Inaba, K., Hayashi, S. and Kunisada, T., Skin antigens in the steady state are trafficked to regional lymph nodes by transforming growth factor-beta1-dependent cells. *Int Immunol* 2001. 13: 695-704.

[5] Hnri, S., Vremec, D., Kamath, A., Waithman, J., Williams, S., Benoist, C., Burnham, K., Saeland, S., Handman, E. and Shortman, K., The dendritic cell populations of mouse lymph nodes. *J Immunol* 2001. 167: 741-748.

[6] Jkob, T., Ring, J. and Udey, M. C., Multistep navigation of Langerhans/dendritic cells in and out of the skin. *J Allergy Clin Immunol* 2001. 108: 688-696.

[7] Wilson, N. S., El-Sukkari, D., Belz, G. T., Smith, C. M., Steptoe, R. J., Heath, W. R., Shortman, K. and Villadangos, J. A., Most lymphoid organ dendritic cell types are phenotypically and functionally immature. *Blood* 2003. 102: 2187-2194.

[8] Hang, F. P. and MacPherson, G. G., Continuing education of the immune system--dendritic cells, immune regulation and tolerance. *Curr Mol Med* 2001. 1: 457-468.

[9] Wilson, N. S., Young, L. J., Kupresanin, F., Naik, S. H., Vremec, D., Heath, W. R., Akira, S., Shortman, K., Boyle, J., Maraskovsky, E., Belz, G. T. and Villadangos, J. A., Normal proportion and expression of maturation markers in migratory dendritic cells in the absence of germs or Toll-like receptor signaling. *Immunol Cell Biol* 2007.

[10] Geissmann, F., Revy, P., Regnault, A., Lepelletier, Y., Dy, M., Brousse, N., Amigorena, S., Hermine, O. and Durandy, A., TGF-beta 1 prevents the noncognate maturation of human dendritic Langerhans cells. *J Immunol* 1999. 162: 4567-4575.

[11] Roake, J. A., Rao, A. S., Morris, P. J., Larsen, C. P., Hankins, D. F. and Austyn, J. M., Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J Exp Med* 1995. 181: 2237-2247.

[12] Forster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Muller, I., Wolf, E. and Lipp, M., CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 1999. 99: 23-33.

[13] Cumberbatch, M., Dearman, R. J. and Kimber, I., Langerhans cells require signals from both tumour necrosis factor alpha and interleukin 1 beta for migration. *Adv Exp Med Biol* 1997. 417: 125-128.

[14] Scheinecker, C., McHugh, R., Shevach, E. M. and Germain, R. N., Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. *J Exp Med* 2002. 196: 1079-1090.

[15] Reis e Sousa, C., Dendritic cells in a mature age. *Nat Rev Immunol* 2006. 6: 476-483.

[16] Vremec, D., Pooley, J., Hochrein, H., Wu, L. and Shortman, K., CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol* 2000. 164: 2978-2986.

[17] Vremec, D., Zorbas, M., Scollay, R., Saunders, D. J., Ardavin, C. F., Wu, L. and Shortman, K., The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J Exp Med* 1992. 176: 47-58.

[18] Edwards, A. D., Chaussabel, D., Tomlinson, S., Schulz, O., Sher, A. and Reis e Sousa, C., Relationships among murine CD11c(high) dendritic cell subsets as revealed by baseline gene expression patterns. *J Immunol* 2003. 171: 47-60.

[19] Lahoud, M. H., Proietto, A. I., Gartlan, K. H., Kitsoulis, S., Curtis, J., Wettenhall, J., Sofi, M., Daunt, C., O'Keeffe, M., Caminschi, I., Satterley, K., Rizzitelli, A., Schnorrer, P., Hinohara, A., Yamaguchi, Y., Wu, L., Smyth, G., Handman, E., Shortman, K. and Wright, M. D., Signal regulatory protein molecules are differentially expressed by CD8- dendritic cells. *J Immunol* 2006. 177: 372-382.

[20] Cavanagh, L. L. and Von Andrian, U. H., Travellers in many guises: the origins and destinations of dendritic cells. *Immunol Cell Biol* 2002. 80: 448-462.

[21] Randolph, G. J., Ochando, J. and Partida, S. N. S., Migration of Dendritic Cell Subsets and their Precursors. *Annu Rev Immunol* 2007.

[22] Steinman, R. M. and Cohn, Z. A., Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 1973. 137: 1142-1162.

[23] O'Keeffe, M., Hochrein, H., Vremec, D., Scott, B., Hertzog, P., Tatarczuch, L. and Shortman, K., Dendritic cell precursor populations of mouse blood: identification of the murine homologues of human blood plasmacytoid pre-DC2 and CD11c+ DC1 precursors. *Blood* 2003. 101: 1453-1459.

[24] Feuerer, M., Beckhove, P., Garbi, N., Mahnke, Y., Limmer, A., Hommel, M., Hammerling, G. J., Kyewski, B., Hamann, A., Umansky, V. and Schirrmacher, V., Bone marrow as a priming site for T-cell responses to blood-borne antigen. *Nat Med* 2003. 9: 1151-1157.

[25] Diao, J., Winter, E., Cantin, C., Chen, W., Xu, L., Kelvin, D., Phillips, J. and Cattral, M. S., In Situ Replication of Immediate Dendritic Cell (DC) Precursors Contributes to Conventional DC Homeostasis in Lymphoid Tissue. *J Immunol* 2006. 176: 7196-7206.

[26] Geissmann, F., Jung, S. and Littman, D. R., Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 2003. 19: 71-82.

[27] Snderkotter, C., Nikolic, T., Dillon, M. J., Van Rooijen, N., Stehling, M., Drevets, D. A. and Leenen, P. J., Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol* 2004. 172: 4410-4417.

[28] MacDonald, K. P., Munster, D. J., Clark, G. J., Dzionek, A., Schmitz, J. and Hart, D. N., Characterization of human blood dendritic cell subsets. *Blood* 2002. 100: 4512-4520.

[29] Taylor, P. R. and Gordon, S., Monocyte heterogeneity and innate immunity. *Immunity* 2003. 19: 2-4.

[30] Geissmann, F., Auffray, C., Palframan, R., Wirrig, C., Ciocca, A., Campisi, L., Narni-Mancinelli, E. and Lauvau, G., Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. *Immunol Cell Biol* 2008. 86: 398-408.

[31] Fogg, D. K., Sibon, C., Miled, C., Jung, S., Aucouturier, P., Littman, D. R., Cumano, A. and Geissmann, F., A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* 2006. 311: 83-87.

[32] Varol, C., Landsman, L., Fogg, D. K., Greenshtein, L., Gildor, B., Margalit, R., Kalchenko, V., Geissmann, F. and Jung, S., Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J Exp Med* 2007. 204: 171-180.

[33] Waskow, C., Liu, K., Darrasse-Jeze, G., Guermonprez, P., Ginhoux, F., Merad, M., Shengelia, T., Yao, K. and Nussenzweig, M., The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. *Nat Immunol* 2008. 9: 676-683.

[34] Becker, S., Warren, M. K. and Haskill, S., Colony-stimulating factor-induced monocyte survival and differentiation into macrophages in serum-free cultures. *J Immunol* 1987. 139: 3703-3709.

[35] Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S. and Steinman, R. M., Generation of large numbers of dendritic cells from mouse bone

marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992. 176: 1693-1702.

[36] Inaba, K., Steinman, R. M., Pack, M. W., Aya, H., Inaba, M., Sudo, T., Wolpe, S. and Schuler, G., Identification of proliferating dendritic cell precursors in mouse blood. *J Exp Med* 1992. 175: 1157-1167.

[37] Lu, L., McCaslin, D., Starzl, T. E. and Thomson, A. W., Bone marrow-derived dendritic cell progenitors (NLDC 145+, MHC class II+, B7-1dim, B7-2-) induce alloantigen-specific hyporesponsiveness in murine T lymphocytes. *Transplantation* 1995. 60: 1539-1545.

[38] Peters, J. H., Xu, H., Ruppert, J., Ostermeier, D., Friedrichs, D. and Gieseler, R. K., Signals required for differentiating dendritic cells from human monocytes in vitro. *Adv Exp Med Biol* 1993. 329: 275-280.

[39] Sallusto, F. and Lanzavecchia, A., Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 1994. 179: 1109-1118.

[40] Sanchez-Torres, C., Garcia-Romo, G. S., Cornejo-Cortes, M. A., Rivas-Carvalho, A. and Sanchez-Schmitz, G., CD16+ and CD16- human blood monocyte subsets differentiate in vitro to dendritic cells with different abilities to stimulate CD4+ T cells. *Int Immunol* 2001. 13: 1571-1581.

[41] Scheicher, C., Mehlig, M., Zecher, R. and Reske, K., Dendritic cells from mouse bone marrow: in vitro differentiation using low doses of recombinant granulocyte-macrophage colony-stimulating factor. *J Immunol Methods* 1992. 154: 253-264.

[42] Zhou, L. J. and Tedder, T. F., CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells. *Proc Natl Acad Sci U S A* 1996. 93: 2588-2592.

[43] Ginhoux, F., Tacke, F., Angeli, V., Bogunovic, M., Loubeau, M., Dai, X. M., Stanley, E. R., Randolph, G. J. and Merad, M., Langerhans cells arise from monocytes in vivo. *Nat Immunol* 2006.

[44] Leon, B., Martinez del Hoyo, G., Parrillas, V., Vargas, H. H., Sanchez-Mateos, P., Longo, N., Lopez-Bravo, M. and Ardavin, C., Dendritic cell differentiation potential of mouse monocytes: monocytes represent immediate precursors of CD8- and CD8+ splenic dendritic cells. *Blood* 2004. 103: 2668-2676.

[45] Randolph, G. J., Inaba, K., Robbiani, D. F., Steinman, R. M. and Muller, W. A., Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity* 1999. 11: 753-761.

[46] Liu, Y. J., IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* 2005. 23: 275-306.

[47] Lian, Z. X., Okada, T., He, X. S., Kita, H., Liu, Y. J., Ansari, A. A., Kikuchi, K., Ikehara, S. and Gershwin, M. E., Heterogeneity of dendritic cells in the mouse liver: identification and characterization of four distinct populations. *J Immunol* 2003. 170: 2323-2330.

[48] Okada, T., Lian, Z. X., Naiki, M., Ansari, A. A., Ikehara, S. and Gershwin, M. E., Murine thymic plasmacytoid dendritic cells. *Eur J Immunol* 2003. 33: 1012-1019.

[49] O'Keeffe, M., Hochrein, H., Vremec, D., Caminschi, I., Miller, J. L., Anders, E. M., Wu, L., Lahoud, M. H., Henri, S., Scott, B., Hertzog, P., Tatarczuch, L. and Shortman, K., Mouse plasmacytoid cells: long-lived cells, heterogeneous in surface phenotype and function, that differentiate into CD8(+) dendritic cells only after microbial stimulus. *J Exp Med* 2002. 196: 1307-1319.

[50] Martin, P., Del Hoyo, G. M., Anjuere, F., Arias, C. F., Vargas, H. H., Fernandez, L. A., Parrillas, V. and Ardavin, C., Characterization of a new subpopulation of mouse CD8alpha+ B220+ dendritic cells endowed with type 1 interferon production capacity and tolerogenic potential. *Blood* 2002. 100: 383-390.

[51] Nakano, H., Yanagita, M. and Gunn, M. D., CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J Exp Med* 2001. 194: 1171-1178.

[52] Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Briere, F. and Trinchieri, G., Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat Immunol* 2001. 2: 1144-1150.

[53] Nikolic, T., Dingjan, G. M., Leenen, P. J. and Hendriks, R. W., A subfraction of B220(+) cells in murine bone marrow and spleen does not belong to the B cell lineage but has dendritic cell characteristics. *Eur J Immunol* 2002. 32: 686-692.

[54] Siegal, F. P., Kadowaki, N., Shodell, M., Fitzgerald-Bocarsly, P. A., Shah, K., Ho, S., Antonenko, S. and Liu, Y. J., The nature of the principal type 1 interferon-producing cells in human blood. *Science* 1999. 284: 1835-1837.

[55] Bjorck, P., Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor- treated mice. *Blood* 2001. 98: 3520-3526.

[56] Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R. W., Kastelein, R. A., Bazan, F. and Liu, Y. J., Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med* 2001. 194: 863-869.

[57] Jarrossay, D., Napolitani, G., Colonna, M., Sallusto, F. and Lanzavecchia, A., Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. *Eur J Immunol* 2001. 31: 3388-3393.

[58] Krug, A., Towarowski, A., Britsch, S., Rothenfusser, S., Hornung, V., Bals, R., Giese, T., Engelmann, H., Endres, S., Krieg, A. M. and Hartmann, G., Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *Eur J Immunol* 2001. 31: 3026-3037.

[59] Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S. and Reis e Sousa, C., Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 2004. 303: 1529-1531.

[60] Proietto, A. I., O'Keeffe, M., Gartlan, K., Wright, M. D., Shortman, K., Wu, L. and Lahoud, M. H., Differential production of inflammatory chemokines by murine dendritic cell subsets. *Immunobiology* 2004. 209: 163-172.

[61] Grouard, G., Rissoan, M. C., Filgueira, L., Durand, I., Banchereau, J. and Liu, Y. J., The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J Exp Med* 1997. 185: 1101-1111.

[62] Salio, M., Palmowski, M. J., Atzberger, A., Hermans, I. F. and Cerundolo, V., CpG-matured murine plasmacytoid dendritic cells are capable of in vivo priming of functional CD8 T cell responses to endogenous but not exogenous antigens. *J Exp Med* 2004. 199: 567-579.

[63] Krug, A., Veeraswamy, R., Pekosz, A., Kanagawa, O., Unanue, E. R., Colonna, M. and Cella, M., Interferon-producing cells fail to induce proliferation of naive T cells but can promote expansion and T helper 1 differentiation of antigen-experienced unpolarized T cells. *J Exp Med* 2003. 197: 899-906.

[64] Gilliet, M., Cao, W. and Liu, Y. J., Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol* 2008. 8: 594-606.

[65] Villadangos, J. A. and Young, L., Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity* 2008. 29: 352-361.

[66] Langerhans, P., *Über die Nerven der menschlichen Haut*: 1868.

[67] Stingl, G., Wolff-Schreiner, E. C., Pichler, W. J., Gschnait, F., Knapp, W. and Wolff, K., Epidermal Langerhans cells bear Fc and C3 receptors. *Nature* 1977. 268: 245-246.

[68] Rowden, G., Lewis, M. G. and Sullivan, A. K., Ia antigen expression on human epidermal Langerhans cells. *Nature* 1977. 268: 247-248.

[69] Klareskog, L., Tjernlund, U., Forsum, U. and Peterson, P. A., Epidermal Langerhans cells express Ia antigens. *Nature* 1977. 268: 248-250.

[70] Silberberg, I., Baer, R. L. and Rosenthal, S. A., The role of Langerhans cells in allergic contact hypersensitivity. A review of findings in man and guinea pigs. *J Invest Dermatol* 1976. 66: 210-217.

[71] Stingl, G., Katz, S. I., Clement, L., Green, I. and Shevach, E. M., Immunologic functions of Ia-bearing epidermal Langerhans cells. *J Immunol* 1978. 121: 2005-2013.

[72] Stingl, G., Katz, S. I., Shevach, E. M., Rosenthal, A. S. and Green, I., Analogous functions of macrophages and Langerhans cells in the initiation in the immune response. *J Invest Dermatol* 1978. 71: 59-64.

[73] Green, I., Stingl, G., Shevach, E. M. and Katz, S. I., Antigen presentation and allogeneic stimulation by Langerhans cells. *J Invest Dermatol* 1980. 75: 44-45.

[74] Schuler, G. and Steinman, R. M., Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J Exp Med* 1985. 161: 526-546.

[75] Allan, R. S., Smith, C. M., Belz, G. T., van Lint, A. L., Wakim, L. M., Heath, W. R. and Carbone, F. R., Epidermal viral immunity induced by CD8alpha+ dendritic cells but not by Langerhans cells. *Science* 2003. 301: 1925-1928.

[76] Zhao, X., Deak, E., Soderberg, K., Linehan, M., Spezzano, D., Zhu, J., Knipe, D. M. and Iwasaki, A., Vaginal submucosal dendritic cells, but not Langerhans cells, induce protective Th1 responses to herpes simplex virus-2. *J Exp Med* 2003. 197: 153-162.

[77] de Witte, L., Nabatov, A., Pion, M., Fluitsma, D., de Jong, M. A., de Gruijl, T., Piguet, V., van Kooyk, Y. and Geijtenbeek, T. B., Langerin is a natural barrier to HIV-1 transmission by Langerhans cells. *Nat Med* 2007. 13: 367-371.

[78] Birbeck, M. S., Breathnach, A.S., Everall, J.D., An electron microscopic study of basal melanocytes and high level clear cells (Langerhans cell) in vitiligo. *J Invest Dermatol* 1961. 37: 51-64.

[79] Valladeau, J., Ravel, O., Dezutter-Dambuyant, C., Moore, K., Kleijmeer, M., Liu, Y., Duvert-Frances, V., Vincent, C., Schmitt, D., Davoust, J., Caux, C., Lebecque, S. and Saeland, S., Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity* 2000. 12: 71-81.

[80] Mayerova, D., Parke, E. A., Bursch, L. S., Odumade, O. A. and Hogquist, K. A., Langerhans cells activate naive self-antigen-specific CD8 T cells in the steady state. *Immunity* 2004. 21: 391-400.

[81] Shibaki, A., Sato, A., Vogel, J. C., Miyagawa, F. and Katz, S. I., Induction of GVHD-like skin disease by passively transferred CD8(+) T-cell receptor transgenic T cells into keratin 14-ovalbumin transgenic mice. *J Invest Dermatol* 2004. 123: 109-115.

[82] Waithman, J., Allan, R. S., Kosaka, H., Azukizawa, H., Shortman, K., Lutz, M. B., Heath, W. R., Carbone, F. R. and Belz, G. T., Skin-derived dendritic cells can mediate deletional tolerance of class I-restricted self-reactive T cells. *J Immunol* 2007. 179: 4535-4541.

[83] van der Aar, A. M., Sylva-Steenland, R. M., Bos, J. D., Kapsenberg, M. L., de Jong, E. C. and Teunissen, M. B., Loss of TLR2, TLR4, and TLR5 on Langerhans cells abolishes bacterial recognition. *J Immunol* 2007. 178: 1986-1990.

[84] Krueger, G. G., Daynes, R. A. and Emam, M., Biology of Langerhans cells: selective migration of Langerhans cells into allogeneic and xenogeneic grafts on nude mice. *Proc Natl Acad Sci U S A* 1983. 80: 1650-1654.

[85] Giacometti, L. and Montagna, W., Langerhans cells: uptake of tritiated thymidine. *Science* 1967. 157: 439-440.

[86] Merad, M., Manz, M. G., Karsunky, H., Wagers, A., Peters, W., Charo, I., Weissman, I. L., Cyster, J. G. and Engleman, E. G., Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat Immunol* 2002. 3: 1135-1141.

[87] Ginhoux, F., Collin, M. P., Bogunovic, M., Abel, M., Leboeuf, M., Helft, J., Ochando, J., Kissenpfennig, A., Malissen, B., Grisotto, M., Snoeck, H., Randolph, G. and Merad, M., Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. *J Exp Med* 2007. 204: 3133-3146.

[88] de Fraissinette, A., Schmitt, D., Dezutter-Dambuyant, C., Guyotat, D., Zabot, M. T. and Thivolet, J., Culture of putative Langerhans cell bone marrow precursors: characterization of their phenotype. *Exp Hematol* 1988. 16: 764-768.

[89] Berman, A. and Winkelmann, R. K., Inflammatory seborrheic keratoses with mononuclear cell infiltration. *J Cutan Pathol* 1978. 5: 353-360.

[90] Stingl, G., Romani, N. and Wolff, K., Langerhans' cells: a distinctive member of bone marrow-derived dendritic cells. *Adv Dermatol* 1987. 2: 269-281.

[91] Cecchini, M. G., Dominguez, M. G., Mocci, S., Wetterwald, A., Felix, R., Fleisch, H., Chisholm, O., Hofstetter, W., Pollard, J. W. and Stanley, E. R., Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. *Development* 1994. 120: 1357-1372.

[92] Caux, C., Dezutter-Dambuyant, C., Schmitt, D. and Banchereau, J., GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature* 1992. 360: 258-261.

[93] Larregina, A. T., Morelli, A. E., Spencer, L. A., Logar, A. J., Watkins, S. C., Thomson, A. W. and Falo, L. D., Jr., Dermal-resident CD14+ cells differentiate into Langerhans cells. *Nat Immunol* 2001. 2: 1151-1158.

[94] Nestle, F. O., Zheng, X. G., Thompson, C. B., Turka, L. A. and Nickoloff, B. J., Characterization of dermal dendritic cells obtained from normal human skin reveals phenotypic and functionally distinctive subsets. *J Immunol* 1993. 151: 6535-6545.

[95] MacPherson, G. G. and Liu, L. M., Dendritic cells and Langerhans cells in the uptake of mucosal antigens. *Curr Top Microbiol Immunol* 1999. 236: 33-53.

[96] Iwasaki, A., Mucosal dendritic cells. *Annu Rev Immunol* 2007. 25: 381-418.

[97] Valladeau, J. and Saeland, S., Cutaneous dendritic cells. *Semin Immunol* 2005. 17: 273-283.

[98] Dupasquier, M., Stoitzner, P., van Oudenaren, A., Romani, N. and Leenen, P. J., Macrophages and dendritic cells constitute a major subpopulation of cells in the mouse dermis. *J Invest Dermatol* 2004. 123: 876-879.

[99] Duraiswamy, N., Tse, Y., Hammerberg, C., Kang, S. and Cooper, K. D., Distinction of class II MHC+ Langerhans cell-like interstitial dendritic antigen-presenting cells in murine dermis from dermal macrophages. *J Invest Dermatol* 1994. 103: 678-683.

[100] Bennett, C. L., van Rijn, E., Jung, S., Inaba, K., Steinman, R. M., Kapsenberg, M. L. and Clausen, B. E., Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. *J Cell Biol* 2005. 169: 569-576.

[101] Itano, A. A., McSorley, S. J., Reinhardt, R. L., Ehst, B. D., Ingulli, E., Rudensky, A. Y. and Jenkins, M. K., Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. *Immunity* 2003. 19: 47-57.

[102] von Stebut, E., Belkaid, Y., Nguyen, B. V., Cushing, M., Sacks, D. L. and Udey, M. C., Leishmania major-infected murine langerhans cell-like dendritic cells from susceptible mice release IL-12 after infection and vaccinate against experimental cutaneous Leishmaniasis. *Eur J Immunol* 2000. 30: 3498-3506.

[103] Filippi, C., Hugues, S., Cazareth, J., Julia, V., Glaichenhaus, N. and Ugolini, S., CD4+ T cell polarization in mice is modulated by strain-specific major histocompatibility complex-independent differences within dendritic cells. *J Exp Med* 2003. 198: 201-209.

[104] Bogunovic, M., Ginhoux, F., Wagers, A., Loubeau, M., Isola, L. M., Lubrano, L., Najfeld, V., Phelps, R. G., Grosskreutz, C., Scigliano, E., Frenette, P. S. and Merad, M., Identification of a radio-resistant and cycling dermal dendritic cell population in mice and men. *J Exp Med* 2006. 203: 2627-2638.

[105] McLellan, A. D., Kapp, M., Eggert, A., Linden, C., Bommhardt, U., Brocker, E. B., Kammerer, U. and Kampgen, E., Anatomic location and T-cell stimulatory functions of mouse dendritic cell subsets defined by CD4 and CD8 expression. *Blood* 2002. 99: 2084-2093.

[106] Sung, S. S., Fu, S. M., Rose, C. E., Jr., Gaskin, F., Ju, S. T. and Beaty, S. R., A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. *J Immunol* 2006. 176: 2161-2172.

[107] Johansson-Lindbom, B., Svensson, M., Pabst, O., Palmqvist, C., Marquez, G., Forster, R. and Agace, W. W., Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *J Exp Med* 2005. 202: 1063-1073.

[108] del Rio, M. L., Rodriguez-Barbosa, J. I., Kremmer, E. and Forster, R., CD103- and CD103+ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to CD4+ and CD8+ T cells. *J Immunol* 2007. 178: 6861-6866.

[109] Poulin, L. F., Henri, S., de Bovis, B., Devilard, E., Kissenpfennig, A. and Malissen, B., The dermis contains langerin+ dendritic cells that develop and function independently of epidermal Langerhans cells. *J Exp Med* 2007. 204: 3119-3131.

[110] Bursch, L. S., Wang, L., Igyarto, B., Kissenpfennig, A., Malissen, B., Kaplan, D. H. and Hogquist, K. A., Identification of a novel population of Langerin+ dendritic cells. *J Exp Med* 2007. 204: 3147-3156.

[111] Belz, G. T., Smith, C. M., Kleinert, L., Reading, P., Brooks, A., Shortman, K., Carbone, F. R. and Heath, W. R., Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proc Natl Acad Sci U S A* 2004. 101: 8670-8675.

[112] Jakubzick, C., Tacke, F., Ginhoux, F., Wagers, A. J., van Rooijen, N., Mack, M., Merad, M. and Randolph, G. J., Blood monocyte subsets differentially give rise to CD103+ and CD103- pulmonary dendritic cell populations. *J Immunol* 2008. 180: 3019-3027.

[113] Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B. U., Unanue, E. R., Diamond, M. S., Schreiber, R. D., Murphy, T. L. and Murphy, K. M., Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* 2008. 322: 1097-1100.

[114] De Smedt, T., Pajak, B., Muraille, E., Lespagnard, L., Heinen, E., De Baetselier, P., Urbain, J., Leo, O. and Moser, M., Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J Exp Med* 1996. 184: 1413-1424.

[115] Crowley, M., Inaba, K., Witmer-Pack, M. and Steinman, R. M., The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell Immunol* 1989. 118: 108-125.

[116] Agger, R., Witmer-Pack, M., Romani, N., Stossel, H., Swiggard, W. J., Metlay, J. P., Storozynsky, E., Freimuth, P. and Steinman, R. M., Two populations of splenic dendritic cells detected with M342, a new monoclonal to an intracellular antigen of interdigitating dendritic cells and some B lymphocytes. *J Leukoc Biol* 1992. 52: 34-42.

[117] Veerman, A. J. and van Ewijk, W., White pulp compartments in the spleen of rats and mice. A light and electron microscopic study of lymphoid and non-lymphoid celltypes in T- and B-areas. *Cell Tissue Res* 1975. 156: 417-441.

[118] Villadangos, J. A. and Schnorrer, P., Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nat Rev Immunol* 2007. 7: 543-555.

[119] Caminschi, I., Lucas, K. M., O'Keeffe, M. A., Hochrein, H., Laabi, Y., Brodnicki, T. C., Lew, A. M., Shortman, K. and Wright, M. D., Molecular cloning of a C-type lectin superfamily protein differentially expressed by CD8alpha(-) splenic dendritic cells. *Mol Immunol* 2001. 38: 365-373.

[120] Galibert, L., Diemer, G. S., Liu, Z., Johnson, R. S., Smith, J. L., Walzer, T., Comeau, M. R., Rauch, C. T., Wolfson, M. F., Sorensen, R. A., Van der Vuurst de Vries, A. R., Branstetter, D. G., Koelling, R. M., Scholler, J., Fanslow, W. C., Baum, P. R., Derry, J. M. and Yan, W., Nectin-like protein 2 defines a subset of T-cell zone dendritic cells and is a ligand for class-I-restricted T-cell-associated molecule. *J Biol Chem* 2005. 280: 21955-21964.

[121] Huysamen, C., Willment, J. A., Dennehy, K. M. and Brown, G. D., CLEC9A is a novel activation C-type lectin-like receptor expressed on BDCA3+ dendritic cells and a subset of monocytes. *J Biol Chem* 2008. 283: 16693-16701.

[122] Robbins, S. H., Walzer, T., Dembele, D., Thibault, C., Defays, A., Bessou, G., Xu, H., Vivier, E., Sellars, M., Pierre, P., Sharp, F. R., Chan, S., Kastner, P. and Dalod, M., Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome Biol* 2008. 9: R17.

[123] Sancho, D., Mourao-Sa, D., Joffre, O. P., Schulz, O., Rogers, N. C., Pennington, D. J., Carlyle, J. R. and Reis e Sousa, C., Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. *J Clin Invest* 2008. 118: 2098-2110.

[124] Metlay, J. P., Witmer-Pack, M. D., Agger, R., Crowley, M. T., Lawless, D. and Steinman, R. M., The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J Exp Med* 1990. 171: 1753-1771.

[125] Iwasaki, A. and Kelsall, B., Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary ymphoid organ chemokine. *J Exp Med* 2000. 191: 1381-1394.

[126] Naik, S. H., Proietto, A. I., Wilson, N. S., Dakic, A., Schnorrer, P., Fuchsberger, M., Lahoud, M. H., O'Keeffe, M., Shao, Q. X., Chen, W. F., Villadangos, J. A., Shortman, K. and Wu, L., Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J Immunol* 2005. 174: 6592-6597.

[127] Reis e Sousa, C., Hieny, S., Scharton-Kersten, T., Jankovic, D., Charest, H., Germain, R. N. and Sher, A., In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. *J Exp Med* 1997. 186: 1819-1829.

[128] Backer, R., van Leeuwen, F., Kraal, G. and den Haan, J. M., CD8(-) dendritic cells preferentially cross-present Saccharomyces cerevisiae antigens. *Eur J Immunol* 2008. 38: 370-380.

[129] Dudziak, D., Kamphorst, A. O., Heidkamp, G. F., Buchholz, V. R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H. W., Park, C. G., Steinman, R. M. and Nussenzweig, M. C., Differential antigen processing by dendritic cell subsets in vivo. *Science* 2007. 315: 107-111.

[130] Kronin, V., Vremec, D., Winkel, K., Classon, B. J., Miller, R. G., Mak, T. W., Shortman, K. and Suss, G., Are CD8+ dendritic cells (DC) veto cells? The role of CD8 on DC in DC development and in the regulation of CD4 and CD8 T cell responses. *Int Immunol* 1997. 9: 1061-1064.

[131] Rizzitelli, A., Hawkins, E., Todd, H., Hodgkin, P. D. and Shortman, K., The proliferative response of CD4 T cells to steady-state CD8+ dendritic cells is restricted by post-activation death. *Int Immunol* 2006. 18: 415-423.

[132] Schnorrer, P., Behrens, G. M., Wilson, N. S., Pooley, J. L., Smith, C. M., El-Sukkari, D., Davey, G., Kupresanin, F., Li, M., Maraskovsky, E., Belz, G. T., Carbone, F. R., Shortman, K., Heath, W. R. and Villadangos, J. A., The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc Natl Acad Sci U S A* 2006. 103: 10729-10734.

[133] Mackarehtschian, K., Hardin, J. D., Moore, K. A., Boast, S., Goff, S. P. and Lemischka, I. R., Targeted disruption of the flk2/flt3 gene leads to deficiencies in primitive hematopoietic progenitors. *Immunity* 1995. 3: 147-161.

[134] Rasko, J. E., Metcalf, D., Rossner, M. T., Begley, C. G. and Nicola, N. A., The flt3/flk-2 ligand: receptor distribution and action on murine haemopoietic cell survival and proliferation. *Leukemia* 1995. 9: 2058-2066.

[135] McKenna, H. J., Stocking, K. L., Miller, R. E., Brasel, K., De Smedt, T., Maraskovsky, E., Maliszewski, C. R., Lynch, D. H., Smith, J., Pulendran, B., Roux, E. R., Teepe, M., Lyman, S. D. and Peschon, J. J., Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood* 2000. 95: 3489-3497.

[136] Brawand, P., Fitzpatrick, D. R., Greenfield, B. W., Brasel, K., Maliszewski, C. R. and De Smedt, T., Murine plasmacytoid pre-dendritic cells generated from Flt3 ligand-supplemented bone marrow cultures are immature APCs. *J Immunol* 2002. 169: 6711-6719.

[137] Tussiwand, R., Onai, N., Mazzucchelli, L. and Manz, M. G., Inhibition of natural type I IFN-producing and dendritic cell development by a small molecule receptor tyrosine kinase inhibitor with Flt3 affinity. *J Immunol* 2005. 175: 3674-3680.

[138] Maraskovsky, E., Brasel, K., Teepe, M., Roux, E. R., Lyman, S. D., Shortman, K. and McKenna, H. J., Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J Exp Med* 1996. 184: 1953-1962.

[139] Pulendran, B., Banchereau, J., Burkeholder, S., Kraus, E., Guinet, E., Chalouni, C., Caron, D., Maliszewski, C., Davoust, J., Fay, J. and Palucka, K., Flt3-ligand and granulocyte colony-stimulating factor mobilize distinct human dendritic cell subsets in vivo. *J Immunol* 2000. 165: 566-572.

[140] Brasel, K., De Smedt, T., Smith, J. L. and Maliszewski, C. R., Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood* 2000. 96: 3029-3039.

[141] Gilliet, M., Boonstra, A., Paturel, C., Antonenko, S., Xu, X. L., Trinchieri, G., O'Garra, A. and Liu, Y. J., The development of murine plasmacytoid dendritic cell precursors is

differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *J Exp Med* 2002. 195: 953-958.

[142] Burgess, A. W., Camakaris, J. and Metcalf, D., Purification and properties of colony-stimulating factor from mouse lung-conditioned medium. *J Biol Chem* 1977. 252: 1998-2003.

[143] Vremec, D., Lieschke, G. J., Dunn, A. R., Robb, L., Metcalf, D. and Shortman, K., The influence of granulocyte/macrophage colony-stimulating factor on dendritic cell levels in mouse lymphoid organs. *Eur J Immunol* 1997. 27: 40-44.

[144] Hikino, H., Miyagi, T., Hua, Y., Hirohisa, S., Gold, D. P., Li, X. K., Fujino, M., Tetsuya, T., Amemiya, H., Suzuki, S., Robb, L., Miyata, M. and Kimura, H., GM-CSF-independent development of dendritic cells from bone marrow cells in the GM-CSF-receptor-deficient mouse. *Transplant Proc* 2000. 32: 2458-2459.

[145] Laouar, Y., Welte, T., Fu, X. Y. and Flavell, R. A., STAT3 is required for Flt3L-dependent dendritic cell differentiation. *Immunity* 2003. 19: 903-912.

[146] Esashi, E., Wang, Y. H., Perng, O., Qin, X. F., Liu, Y. J. and Watowich, S. S., The signal transducer STAT5 inhibits plasmacytoid dendritic cell development by suppressing transcription factor IRF8. *Immunity* 2008. 28: 509-520.

[147] Cohen, P. A., Koski, G. K., Czerniecki, B. J., Bunting, K. D., Fu, X. Y., Wang, Z., Zhang, W. J., Carter, C. S., Awad, M., Distel, C. A., Nagem, H., Paustian, C. C., Johnson, T. D., Tisdale, J. F. and Shu, S., STAT3- and STAT5-dependent pathways competitively regulate the pan-differentiation of CD34pos cells into tumor-competent dendritic cells. *Blood* 2008. 112: 1832-1843.

[148] Hirano, T., Interleukin 6 and its receptor: ten years later. *Int Rev Immunol* 1998. 16: 249-284.

[149] Chomarat, P., Banchereau, J., Davoust, J. and Palucka, A. K., IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol* 2000. 1: 510-514.

[150] Park, S. J., Nakagawa, T., Kitamura, H., Atsumi, T., Kamon, H., Sawa, S., Kamimura, D., Ueda, N., Iwakura, Y., Ishihara, K., Murakami, M. and Hirano, T., IL-6 regulates in vivo dendritic cell differentiation through STAT3 activation. *J Immunol* 2004. 173: 3844-3854.

[151] Koopman, G., Niphuis, H., Haaksma, A. G., Farese, A. M., Casey, D. B., Kahn, L. E., Mann, D., MacVittie, T. J., Woulfe, S. L. and Heeney, J. L., Increase in plasmacytoid and myeloid dendritic cells by progenipoietin-1, a chimeric Flt-3 and G-CSF receptor agonist, in SIV-Infected rhesus macaques. *Hum Immunol* 2004. 65: 303-316.

[152] Kared, H., Masson, A., Adle-Biassette, H., Bach, J. F., Chatenoud, L. and Zavala, F., Treatment with granulocyte colony-stimulating factor prevents diabetes in NOD mice by recruiting plasmacytoid dendritic cells and functional CD4(+)CD25(+) regulatory T-cells. *Diabetes* 2005. 54: 78-84.

[153] Chen, W., Antonenko, S., Sederstrom, J. M., Liang, X., Chan, A. S., Kanzler, H., Blom, B., Blazar, B. R. and Liu, Y. J., Thrombopoietin cooperates with FLT3-ligand in the generation of plasmacytoid dendritic cell precursors from human hematopoietic progenitors. *Blood* 2004. 103: 2547-2553.

[154] Wiktor-Jedrzejczak, W., Bartocci, A., Ferrante, A. W., Jr., Ahmed-Ansari, A., Sell, K. W., Pollard, J. W. and Stanley, E. R., Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proc Natl Acad Sci U S A* 1990. 87: 4828-4832.

[155] Wiktor-Jedrzejczak, W. W., Ahmed, A., Szczylik, C. and Skelly, R. R., Hematological characterization of congenital osteopetrosis in op/op mouse. Possible mechanism for abnormal macrophage differentiation. *J Exp Med* 1982. 156: 1516-1527.

[156] Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T. and Shultz, L. D., The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* 1990. 345: 442-444.

[157] MacDonald, K. P., Rowe, V., Bofinger, H. M., Thomas, R., Sasmono, T., Hume, D. A. and Hill, G. R., The colony-stimulating factor 1 receptor is expressed on dendritic cells during differentiation and regulates their expansion. *J Immunol* 2005. 175: 1399-1405.

[158] 158 Witmer-Pack, M. D., Hughes, D. A., Schuler, G., Lawson, L., McWilliam, A., Inaba, K., Steinman, R. M. and Gordon, S., Identification of macrophages and dendritic cells in the osteopetrotic (op/op) mouse. *J Cell Sci* 1993. 104 (Pt 4): 1021-1029.

[159] Onai, N., Obata-Onai, A., Schmid, M. A., Ohteki, T., Jarrossay, D. and Manz, M. G., Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nat Immunol* 2007. 8: 1207-1216.

[160] Fancke, B., Suter, M., Hochrein, H. and O'Keeffe, M., M-CSF: a novel plasmacytoid and conventional dendritic cell poietin. *Blood* 2008. 111: 150-159.

[161] Wu, L. and Liu, Y. J., Development of dendritic-cell lineages. *Immunity* 2007. 26: 741-750.

[162] Taniguchi, T., Ogasawara, K., Takaoka, A. and Tanaka, N., IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol* 2001. 19: 623-655.

[163] Driggers, P. H., Ennist, D. L., Gleason, S. L., Mak, W. H., Marks, M. S., Levi, B. Z., Flanagan, J. R., Appella, E. and Ozato, K., An interferon gamma-regulated protein that binds the interferon-inducible enhancer element of major histocompatibility complex class I genes. *Proc Natl Acad Sci U S A* 1990. 87: 3743-3747.

[164] Holtschke, T., Lohler, J., Kanno, Y., Fehr, T., Giese, N., Rosenbauer, F., Lou, J., Knobeloch, K. P., Gabriele, L., Waring, J. F., Bachmann, M. F., Zinkernagel, R. M., Morse, H. C., 3rd, Ozato, K. and Horak, I., Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. *Cell* 1996. 87: 307-317.

[165] Schiavoni, G., Mattei, F., Borghi, P., Sestili, P., Venditti, M., Morse, H. C., 3rd, Belardelli, F. and Gabriele, L., ICSBP is critically involved in the normal development and trafficking of Langerhans cells and dermal dendritic cells. *Blood* 2004. 103: 2221-2228.

[166] Tsujimura, H., Tamura, T. and Ozato, K., Cutting edge: IFN consensus sequence binding protein/IFN regulatory factor 8 drives the development of type I IFN-producing plasmacytoid dendritic cells. *J Immunol* 2003. 170: 1131-1135.

[167] Schiavoni, G., Mattei, F., Sestili, P., Borghi, P., Venditti, M., Morse, H. C., 3rd, Belardelli, F. and Gabriele, L., ICSBP is essential for the development of mouse type I

interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells. *J Exp Med* 2002. 196: 1415-1425.

[168] Aliberti, J., Schulz, O., Pennington, D. J., Tsujimura, H., Reis e Sousa, C., Ozato, K. and Sher, A., Essential role for ICSBP in the in vivo development of murine CD8alpha + dendritic cells. *Blood* 2003. 101: 305-310.

[169] Suzuki, S., Honma, K., Matsuyama, T., Suzuki, K., Toriyama, K., Akitoyo, I., Yamamoto, K., Suematsu, T., Nakamura, M., Yui, K. and Kumatori, A., Critical roles of interferon regulatory factor 4 in CD11bhighCD8alpha- dendritic cell development. *Proc Natl Acad Sci U S A* 2004. 101: 8981-8986.

[170] Tamura, T., Tailor, P., Yamaoka, K., Kong, H. J., Tsujimura, H., O'Shea, J. J., Singh, H. and Ozato, K., IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. *J Immunol* 2005. 174: 2573-2581.

[171] Honda, K., Mizutani, T. and Taniguchi, T., Negative regulation of IFN-alpha/beta signaling by IFN regulatory factor 2 for homeostatic development of dendritic cells. *Proc Natl Acad Sci U S A* 2004. 101: 2416-2421.

[172] Baeuerle, P. A. and Henkel, T., Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol* 1994. 12: 141-179.

[173] Gilmore, T. D., The Rel/NF-kappaB signal transduction pathway: introduction. *Oncogene* 1999. 18: 6842-6844.

[174] O'Keeffe, M., Grumont, R. J., Hochrein, H., Fuchsberger, M., Gugasyan, R., Vremec, D., Shortman, K. and Gerondakis, S., Distinct roles for the NF-kappaB1 and c-Rel transcription factors in the differentiation and survival of plasmacytoid and conventional dendritic cells activated by TLR-9 signals. *Blood* 2005. 106: 3457-3464.

[175] Hacker, C., Kirsch, R. D., Ju, X. S., Hieronymus, T., Gust, T. C., Kuhl, C., Jorgas, T., Kurz, S. M., Rose-John, S., Yokota, Y. and Zenke, M., Transcriptional profiling identifies Id2 function in dendritic cell development. *Nat Immunol* 2003. 4: 380-386.

[176] Spits, H., Couwenberg, F., Bakker, A. Q., Weijer, K. and Uittenbogaart, C. H., Id2 and Id3 inhibit development of CD34(+) stem cells into predendritic cell (pre-DC)2 but not into pre-DC1. Evidence for a lymphoid origin of pre-DC2. *J Exp Med* 2000. 192: 1775-1784.

[177] Schotte, R., Nagasawa, M., Weijer, K., Spits, H. and Blom, B., The ETS transcription factor Spi-B is required for human plasmacytoid dendritic cell development. *J Exp Med* 2004. 200: 1503-1509.

[178] Schotte, R., Rissoan, M. C., Bendriss-Vermare, N., Bridon, J. M., Duhen, T., Weijer, K., Briere, F. and Spits, H., The transcription factor Spi-B is expressed in plasmacytoid DC precursors and inhibits T-, B-, and NK-cell development. *Blood* 2003. 101: 1015-1023.

[179] Cisse, B., Caton, M. L., Lehner, M., Maeda, T., Scheu, S., Locksley, R., Holmberg, D., Zweier, C., den Hollander, N. S., Kant, S. G., Holter, W., Rauch, A., Zhuang, Y. and Reizis, B., Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. *Cell* 2008. 135: 37-48.

[180] Nagasawa, M., Schmidlin, H., Hazekamp, M. G., Schotte, R. and Blom, B., Development of human plasmacytoid dendritic cells depends on the combined action of

the basic helix-loop-helix factor E2-2 and the Ets factor Spi-B. *Eur J Immunol* 2008. 38: 2389-2400.

[181] Burnham, K., Robb, L., Scott, C. L., O'Keeffe, M. and Shortman, K., Effect of granulocyte-macrophage colony-stimulating factor on the generation of epidermal Langerhans cells. *J Interferon Cytokine Res* 2000. 20: 1071-1076.

[182] Maraskovsky, E., Pulendran, B., Brasel, K., Teepe, M., Roux, E. R., Shortman, K., Lyman, S. D. and McKenna, H. J., Dramatic numerical increase of functionally mature dendritic cells in FLT3 ligand-treated mice. *Adv Exp Med Biol* 1997. 417: 33-40.

[183] Maraskovsky, E., Daro, E., Roux, E., Teepe, M., Maliszewski, C. R., Hoek, J., Caron, D., Lebsack, M. E. and McKenna, H. J., In vivo generation of human dendritic cell subsets by Flt3 ligand. *Blood* 2000. 96: 878-884.

[184] Pulendran, B., Lingappa, J., Kennedy, M. K., Smith, J., Teepe, M., Rudensky, A., Maliszewski, C. R. and Maraskovsky, E., Developmental pathways of dendritic cells in vivo: distinct function, phenotype, and localization of dendritic cell subsets in FLT3 ligand-treated mice. *J Immunol* 1997. 159: 2222-2231.

[185] D'Amico, A. and Wu, L., The early progenitors of mouse dendritic cells and plasmacytoid predendritic cells are within the bone marrow hemopoietic precursors expressing Flt3. *J Exp Med* 2003. 198: 293-303.

[186] O'Keeffe, M., Hochrein, H., Vremec, D., Pooley, J., Evans, R., Woulfe, S. and Shortman, K., Effects of administration of progenipoietin 1, Flt-3 ligand, granulocyte colony-stimulating factor, and pegylated granulocyte- macrophage colony-stimulating factor on dendritic cell subsets in mice. *Blood* 2002. 99: 2122-2130.

[187] Miller, G., Pillarisetty, V. G., Shah, A. B., Lahrs, S., Xing, Z. and DeMatteo, R. P., Endogenous granulocyte-macrophage colony-stimulating factor overexpression in vivo results in the long-term recruitment of a distinct dendritic cell population with enhanced immunostimulatory function. *J Immunol* 2002. 169: 2875-2885.

[188] Daro, E., Pulendran, B., Brasel, K., Teepe, M., Pettit, D., Lynch, D. H., Vremec, D., Robb, L., Shortman, K., McKenna, H. J., Maliszewski, C. R. and Maraskovsky, E., Polyethylene glycol-modified GM-CSF expands CD11b(high)CD11c(high) but notCD11b(low)CD11c(high) murine dendritic cells in vivo: a comparative analysis with Flt3 ligand. *J Immunol* 2000. 165: 49-58.

[189] Borkowski, T. A., Letterio, J. J., Farr, A. G. and Udey, M. C., A role for endogenous transforming growth factor beta 1 in Langerhans cell biology: the skin of transforming growth factor beta 1 null mice is devoid of epidermal Langerhans cells. *J Exp Med* 1996. 184: 2417-2422.

[190] Geissmann, F., Prost, C., Monnet, J. P., Dy, M., Brousse, N. and Hermine, O., Transforming growth factor beta1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *J Exp Med* 1998. 187: 961-966.

[191] Tsujimura, H., Tamura, T., Gongora, C., Aliberti, J., Reis e Sousa, C., Sher, A. and Ozato, K., ICSBP/IRF-8 retrovirus transduction rescues dendritic cell development in vitro. *Blood* 2003. 101: 961-969.

[192] Ichikawa, E., Hida, S., Omatsu, Y., Shimoyama, S., Takahara, K., Miyagawa, S., Inaba, K. and Taki, S., Defective development of splenic and epidermal CD4+ dendritic cells

in mice deficient for IFN regulatory factor-2. *Proc Natl Acad Sci U S A* 2004. 101: 3909-3914.

[193] Wu, L., D'Amico, A., Winkel, K. D., Suter, M., Lo, D. and Shortman, K., RelB is essential for the development of myeloid-related CD8alpha- dendritic cells but not of lymphoid-related CD8alpha+ dendritic cells. *Immunity* 1998. 9: 839-847.

[194] Weih, F., Carrasco, D., Durham, S. K., Barton, D. S., Rizzo, C. A., Ryseck, R. P., Lira, S. A. and Bravo, R., Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kappa B/Rel family. *Cell* 1995. 80: 331-340.

[195] Burkly, L., Hession, C., Ogata, L., Reilly, C., Marconi, L. A., Olson, D., Tizard, R., Cate, R. and Lo, D., Expression of relB is required for the development of thymic medulla and dendritic cells. *Nature* 1995. 373: 531-536.

[196] Kusunoki, T., Sugai, M., Katakai, T., Omatsu, Y., Iyoda, T., Inaba, K., Nakahata, T., Shimizu, A. and Yokota, Y., TH2 dominance and defective development of a CD8+ dendritic cell subset in Id2-deficient mice. *J Allergy Clin Immunol* 2003. 111: 136-142.

[197] Rathinam, C., Geffers, R., Yucel, R., Buer, J., Welte, K., Moroy, T. and Klein, C., The transcriptional repressor Gfi1 controls STAT3-dependent dendritic cell development and function. *Immunity* 2005. 22: 717-728.

[198] Fainaru, O., Woolf, E., Lotem, J., Yarmus, M., Brenner, O., Goldenberg, D., Negreanu, V., Bernstein, Y., Levanon, D., Jung, S. and Groner, Y., Runx3 regulates mouse TGF-beta-mediated dendritic cell function and its absence results in airway inflammation. *Embo J* 2004. 23: 969-979.

[199] Wu, L., Nichogiannopoulou, A., Shortman, K. and Georgopoulos, K., Cell-autonomous defects in dendritic cell populations of Ikaros mutant mice point to a developmental relationship with the lymphoid lineage. *Immunity* 1997. 7: 483-492.

[200] Naik, S. H., Metcalf, D., van Nieuwenhuijze, A., Wicks, I., Wu, L., O'Keeffe, M. and Shortman, K., Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat Immunol* 2006. 7: 663-671.

[201] Randolph, G. J., Beaulieu, S., Lebecque, S., Steinman, R. M. and Muller, W. A., Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science* 1998. 282: 480-483.

[202] Randolph, G. J., Sanchez-Schmitz, G., Liebman, R. M. and Schakel, K., The CD16(+) (FcgammaRIII(+)) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. *J Exp Med* 2002. 196: 517-527.

[203] Yrlid, U., Jenkins, C. D. and MacPherson, G. G., Relationships between distinct blood monocyte subsets and migrating intestinal lymph dendritic cells in vivo under steady-state conditions. *J Immunol* 2006. 176: 4155-4162.

[204] Xu, Y., Zhan, Y., Lew, A. M., Naik, S. H. and Kershaw, M. H., Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. *J Immunol* 2007. 179: 7577-7584.

[205] Baiocchi, G., Scambia, G., Benedetti, P., Menichella, G., Testa, U., Pierelli, L., Martucci, R., Foddai, M. L., Bizzi, B., Mancuso, S. and et al., Autologous stem cell transplantation: sequential production of hematopoietic cytokines underlying granulocyte recovery. *Cancer Res* 1993. 53: 1297-1303.

[206] Barak, V., Levi-Schaffer, F., Nisman, B. and Nagler, A., Cytokine dysregulation in chronic graft versus host disease. *Leuk Lymphoma* 1995. 17: 169-173.

[207] Cebon, J., Layton, J. E., Maher, D. and Morstyn, G., Endogenous haemopoietic growth factors in neutropenia and infection. *Br J Haematol* 1994. 86: 265-274.

[208] Cheers, C., Haigh, A. M., Kelso, A., Metcalf, D., Stanley, E. R. and Young, A. M., Production of colony-stimulating factors (CSFs) during infection: separate determinations of macrophage-, granulocyte-, granulocyte-macrophage-, and multi-CSFs. *Infect Immun* 1988. 56: 247-251.

[209] Mtcalf, D., Mechanisms contributing to the sex difference in levels of granulocyte-macrophage colony-stimulating factor in the urine of GM-CSF transgenic mice. *Exp Hematol* 1988. 16: 794-800.

[210] Zwierzina, H., Schollenberger, S., Herold, M., Schmalzl, F. and Besemer, J., Endogenous serum levels and surface receptor expression of GM-CSF and IL-3 in patients with myelodysplastic syndromes. *Leuk Res* 1992. 16: 1181-1186.

[211] Walzer, T., Brawand, P., Swart, D., Tocker, J. and De Smedt, T., No defect in T-cell priming, secondary response, or tolerance induction in response to inhaled antigens in Fms-like tyrosine kinase 3 ligand-deficient mice. *J Allergy Clin Immunol* 2005. 115: 192-199.

[212] Naik, S. H., Sathe, P., Park, H. Y., Metcalf, D., Proietto, A. I., Dakic, A., Carotta, S., O'Keeffe, M., Bahlo, M., Papenfuss, A., Kwak, J. Y., Wu, L. and Shortman, K., Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat Immunol* 2007. 8: 1217-1226.

[213] Nicola, N. A. and Metcalf, D., *The Hemopoietic Colony-Stimulating Factors*. Cambridge University Press: 1995.

[214] Serbina, N. V., Salazar-Mather, T. P., Biron, C. A., Kuziel, W. A. and Pamer, E. G., TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* 2003. 19: 59-70.

[215] Serbina, N. V. and Pamer, E. G., Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol* 2006. 7: 311-317.

[216] Pulendran, B., Smith, J. L., Caspary, G., Brasel, K., Pettit, D., Maraskovsky, E. and Maliszewski, C. R., Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc Natl Acad Sci U S A* 1999. 96: 1036-1041.

[217] Weigel, B. J., Nath, N., Taylor, P. A., Panoskaltsis-Mortari, A., Chen, W., Krieg, A. M., Brasel, K. and Blazar, B. R., Comparative analysis of murine marrow-derived dendritic cells generated by Flt3L or GM-CSF/IL-4 and matured with immune stimulatory agents on the in vivo induction of antileukemia responses. *Blood* 2002. 100: 4169-4176.

[218] Martinez del Hoyo, G., Martin, P., Arias, C. F., Marin, A. R. and Ardavin, C., CD8alpha+ dendritic cells originate from the CD8alpha- dendritic cell subset by a maturation process involving CD8alpha, DEC-205, and CD24 up- regulation. *Blood* 2002. 99: 999-1004.

[219] Naik, S., Vremec, D., Wu, L., O'Keeffe, M. and Shortman, K., CD8alpha+ mouse spleen dendritic cells do not originate from the CD8alpha- dendritic cell subset. *Blood* 2003. 102: 601-604.

[220] Landsman, L., Varol, C. and Jung, S., Distinct differentiation potential of blood monocyte subsets in the lung. *J Immunol* 2007. 178: 2000-2007.

[221] Kamath, A. T., Henri, S., Battye, F., Tough, D. F. and Shortman, K., Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs. *Blood* 2002. 100: 1734-1741.

[222] Kamath, A. T., Pooley, J., O'Keeffe, M. A., Vremec, D., Zhan, Y., Lew, A. M., D'Amico, A., Wu, L., Tough, D. F. and Shortman, K., The development, maturation, and turnover rate of mouse spleen dendritic cell populations. *J Immunol* 2000. 165: 6762-6770.

[223] Diao, J., Winter, E., Chen, W., Cantin, C. and Cattral, M. S., Characterization of distinct conventional and plasmacytoid dendritic cell-committed precursors in murine bone marrow. *J Immunol* 2004. 173: 1826-1833.

[224] Bruno, L., Seidl, T. and Lanzavecchia, A., Mouse pre-immunocytes as non-proliferating multipotent precursors of macrophages, interferon-producing cells, CD8alpha(+) and CD8alpha(-) dendritic cells. *Eur J Immunol* 2001. 31: 3403-3412.

[225] del Hoyo, G. M., Martin, P., Hernandez Vargas, H., Ruiz, S., Fernandez Arias, C. and Ardavin, C., corrigendum: Characterization of a common precursor population for dendritic cells. *Nature* 2004. 429: 205.

[226] del Hoyo, G. M., Martin, P., Vargas, H. H., Ruiz, S., Arias, C. F. and Ardavin, C., Characterization of a common precursor population for dendritic cells. *Nature* 2002. 415: 1043-1047.

[227] Traver, D., Akashi, K., Manz, M., Merad, M., Miyamoto, T., Engleman, E. G. and Weissman, I. L., Development of CD8alpha-positive dendritic cells from a common myeloid progenitor. *Science* 2000. 290: 2152-2154.

[228] Wu, L., D'Amico, A., Hochrein, H., O'Keeffe, M., Shortman, K. and Lucas, K., Development of thymic and splenic dendritic cell populations from different hemopoietic precursors. *Blood* 2001. 98: 3376-3382.

[229] Manz, M. G., Traver, D., Miyamoto, T., Weissman, I. L. and Akashi, K., Dendritic cell potentials of early lymphoid and myeloid progenitors. *Blood* 2001. 97: 3333-3341.

[230] Karsunky, H., Merad, M., Cozzio, A., Weissman, I. L. and Manz, M. G., Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and myeloid-committed progenitors to Flt3+ dendritic cells in vivo. *J Exp Med* 2003. 198: 305-313.

[231] Auffray, C., Emre, Y. and Geissmann, F., Homeostasis of dendritic cell pool in lymphoid organs. *Nat Immunol* 2008. 9: 584-586.

[232] Naik, S. H., Corcoran, L. M. and Wu, L., Development of murine plasmacytoid dendritic cell subsets. *Immunol Cell Biol* 2005. 83: 563-570.

[233] Corcoran, L., Ferrero, I., Vremec, D., Lucas, K., Waithman, J., O'Keeffe, M., Wu, L., Wilson, A. and Shortman, K., The lymphoid past of mouse plasmacytoid cells and thymic dendritic cells. *J Immunol* 2003. 170: 4926-4932.

[234] Yoneyama, H., Matsuno, K., Zhang, Y., Nishiwaki, T., Kitabatake, M., Ueha, S., Narumi, S., Morikawa, S., Ezaki, T., Lu, B., Gerard, C., Ishikawa, S. and Matsushima,

K., Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules. *Int Immunol* 2004. 16: 915-928.

[235] Penna, G., Sozzani, S. and Adorini, L., Cutting edge: selective usage of chemokine receptors by plasmacytoid dendritic cells. *J Immunol* 2001. 167: 1862-1866.

[236] Omatsu, Y., Iyoda, T., Kimura, Y., Maki, A., Ishimori, M., Toyama-Sorimachi, N. and Inaba, K., Development of murine plasmacytoid dendritic cells defined by increased expression of an inhibitory NK receptor, Ly49Q. *J Immunol* 2005. 174: 6657-6662.

[237] Toyama-Sorimachi, N., Omatsu, Y., Onoda, A., Tsujimura, Y., Iyoda, T., Kikuchi-Maki, A., Sorimachi, H., Dohi, T., Taki, S., Inaba, K. and Karasuyama, H., Inhibitory NK receptor Ly49Q is expressed on subsets of dendritic cells in a cellular maturation- and cytokine stimulation-dependent manner. *J Immunol* 2005. 174: 4621-4629.

[238] Kamogawa-Schifter, Y., Ohkawa, J., Namiki, S., Arai, N., Arai, K. and Liu, Y., Ly49Q defines 2 pDC subsets in mice. *Blood* 2005. 105: 2787-2792.

[239] Bendriss-Vermare, N., Barthelemy, C., Durand, I., Bruand, C., Dezutter-Dambuyant, C., Moulian, N., Berrih-Aknin, S., Caux, C., Trinchieri, G. and Briere, F., Human thymus contains IFN-alpha-producing CD11c(-), myeloid CD11c(+), and mature interdigitating dendritic cells. *J Clin Invest* 2001. 107: 835-844.

[240] Wang, Y. H. and Liu, Y. J., Mysterious origin of plasmacytoid dendritic cell precursors. *Immunity* 2004. 21: 1-5.

[241] Shigematsu, H., Reizis, B., Iwasaki, H., Mizuno, S. I., Hu, D., Traver, D., Leder, P., Sakaguchi, N. and Akashi, K., Plasmacytoid dendritic cells activate lymphoid-specific genetic programs irrespective of their cellular origin. *Imunity* 2004. 21: 43-53.

[242] Res, P. C., Couwenberg, F., Vyth-Dreese, F. A. and Spits, H., Expression of pTa mRNA in a committed dendritic cell precursor in the human thymus. *Blood* 1999. 94: 2647-2657.

[243] Cavanagh, L. L., Bonasio, R., Mazo, I. B., Halin, C., Cheng, G., van der Velden, A. W., Cariappa, A., Chase, C., Russell, P., Starnbach, M. N., Koni, P. A., Pillai, S., Weninger, W. and von Andrian, U. H., Activation of bone marrow-resident memory T cells by circulating, antigen-bearing dendritic cells. *Nat Immunol* 2005. 6: 1029-1037.

[244] Donskoy, E. and Goldschneider, I., Two developmentally distinct populations of dendritic cells inhabit the adult mouse thymus: demonstration by differential importation of hematogenous precursors under steady state conditions. *J Immunol* 2003. 170: 3514-3521.

[245] Liu, K., Waskow, C., Liu, X., Yao, K., Hoh, J. and Nussenzweig, M., Origin of dendritic cells in peripheral lymphoid organs of mice. *Nat Immunol* 2007. 8: 578-583.

[246] Heng, T. S. and Painter, M. W., The Immunological Genome Project: networks of gene expression in immune cells. *Nat Immunol* 2008. 9: 1091-1094.

[247] Naik, S., Distinct precursors of the dendritic cell subtypes. *Uni Melb eprints* 2006. http://eprints.infodiv.unimelb.edu.au/archive/00001885/.

[248] Karsunky, H., Merad, M., Mende, I., Manz, M. G., Engleman, E. G. and Weissman, I. L., Developmental origin of interferon-alpha-producing dendritic cells from hematopoietic precursors. *Exp Hematol* 2005. 33: 173-181.

[249] Ishikawa, F., Niiro, H., Iino, T., Yoshida, S., Saito, N., Onohara, S., Miyamoto, T., Minagawa, H., Fujii, S., Shultz, L. D., Harada, M. and Akashi, K., The developmental

program of human dendritic cells is operated independently of conventional myeloid and lymphoid pathways. *Blood* 2007. 110: 3591-3660.

[250] Akashi, K., Cartography of hematopoietic stem cell commitment dependent upon a reporter for transcription factor activation. *Ann N Y Acad Sci* 2007. 1106: 76-81.

[251] Arinobu, Y., Mizuno, S., Chong, Y., Shigematsu, H., Iino, T., Iwasaki, H., Graf, T., Mayfield, R., Chan, S., Kastner, P. and Akashi, K., Reciprocal activation of GATA-1 and PU.1 marks initial specification of hematopoietic stem cells into myeloerythroid and myelolymphoid lineages. *Cell Stem Cell* 2007. 1: 416-427.

[252] Iwasaki, H. and Akashi, K., Myeloid lineage commitment from the hematopoietic stem cell. *Immunity* 2007. 26: 726-740.

[253] Onai, N., Obata-Onai, A., Schmid, M. A. and Manz, M. G., Flt3 in regulation of type I interferon-producing cell and dendritic cell development. *Ann N Y Acad Sci* 2007. 1106: 253-261.

[254] Onai, N., Obata-Onai, A., Tussiwand, R., Lanzavecchia, A. and Manz, M. G., Activation of the Flt3 signal transduction cascade rescues and enhances type I interferon-producing and dendritic cell development. *J Exp Med* 2006. 203: 227-238.

[255] Welner, R. S., Pelayo, R. and Kincade, P. W., Evolving views on the genealogy of B cells. *Nat Rev Immunol* 2008. 8: 95-106.

[256] McCulloch, E. A. and Till, J. E., Perspectives on the properties of stem cells. *Nat Med* 2005. 11: 1026-1028.

[257] Osawa, M., Hanada, K., Hamada, H. and Nakauchi, H., Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* 1996. 273: 242-245.

[258] Dick, J. E., Magli, M. C., Huszar, D., Phillips, R. A. and Bernstein, A., Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/Wv mice. *Cell* 1985. 42: 71-79.

[259] Dykstra, B., Kent, D., Bowie, M., McCaffrey, L., Hamilton, M., Lyons, K., Lee, S. J., Brinkman, R. and Eaves, C., Long-term propagation of distinct hematopoietic differentiation programs in vivo. *Cell Stem Cell* 2007. 1: 218-229.

[260] Sieburg, H. B., Cho, R. H., Dykstra, B., Uchida, N., Eaves, C. J. and Muller-Sieburg, C. E., The hematopoietic stem compartment consists of a limited number of discrete stem cell subsets. *Blood* 2006. 107: 2311-2316.

[261] Schepers, K., Swart, E., van Heijst, J. W., Gerlach, C., Castrucci, M., Sie, D., Heimerikx, M., Velds, A., Kerkhoven, R. M., Arens, R. and Schumacher, T. N., Dissecting T cell lineage relationships by cellular barcoding. *J Exp Med* 2008. 205: 2309-2318.

[262] Keller, P. J., Schmidt, A. D., Wittbrodt, J. and Stelzer, E. H., Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. *Science* 2008. 322: 1065-1069.

[263] Schroeder, T., Imaging stem-cell-driven regeneration in mammals. *Nature* 2008. 453: 345-351.

[264] Bedoui, S., Whitney, P. G., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., Allan, R. S., Wojtasiak, M., Shortman, K., Carbone, F. R., Brooks, A. G. and Heath, W. R.

Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. *Nat Immunol* 2009 10, 488-495

[265] del Rio, M. L., Rodriguez-Barbosa, J. I., Bolter, J., Ballmaier, M., Dittrich-Breiholz, O., Kracht, M., Jung, S. and Forster, R. CX3CR1+ c-kit+ bone marrow cells give rise to CD103+ and CD103- dendritic cells with distinct functional properties. *J Immunol* 2008 181, 6178-6188

[266] Jaensson, E., Uronen-Hansson, H., Pabst, O., Eksteen, B., Tian, J., Coombes, J. L., Berg, P. L., Davidsson, T., Powrie, F., Johansson-Lindbom, B. and Agace, W. W. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J Exp Med* 2008 205, 2139-2149

[267] Lukens, M. V., Kruijsen, D., Coenjaerts, F. E., Kimpen, J. L. and van Bleek, G. M. Respiratory Syncytial Virus induced activation and migration of respiratory dendritic cells and subsequent antigen presentation in the lung draining lymph node. *J Virol* 2009

[268] Qiu, C. H., Miyake, Y., Kaise, H., Kitamura, H., Ohara, O. and Tanaka, M. Novel subset of CD8{alpha}+ dendritic cells localized in the marginal zone is responsible for tolerance to cell-associated antigens. *J Immunol* 2009 182, 4127-4136

[269] Kingston, D., Schmid, M. A., Onai, N., Obata-Onai, A., Baumjohann, D. and Manz, M. G. The concerted action of GM-CSF and Flt3-ligand on in vivo dendritic cell homeostasis. *Blood* 2009

[270] Liu, K., Victora, G. D., Schwickert, T. A., Guermonprez, P., Meredith, M. M., Yao, K., Chu, F. F., Randolph, G. J., Rudensky, A. Y. and Nussenzweig, M. In vivo analysis of dendritic cell development and homeostasis. *Science* 2009 324, 392-397

[271] Auffray, C., Fogg, D. K., Narni-Mancinelli, E., Senechal, B., Trouillet, C., Saederup, N., Leemput, J., Bigot, K., Campisi, L., Abitbol, M., Molina, T., Charo, I., Hume, D. A., Cumano, A., Lauvau, G. and Geissmann, F. CX3CR1+ CD115+ CD135+ common macrophage/DC precursors and the role of CX3CR1 in their response to inflammation. *J Exp Med* 2009 206, 595-606

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Chapter 5

The Making and Breaking of a B Lymphocyte*

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Abstract

The progressive differentiation and ultimate commitment of hematopoietic progenitors to the B lymphocyte lineage requires both the activation of the B lymphocyte transcriptional program and the repression of other lineage-inappropriate genes, such as those favoring macrophage development. This process is controlled by the coordinated activity of a small number of transcription factors. One class of factors including PU.1, Ikaros and E2A appears to be first required at the level of the differentiation of the Lymphoid-Primed Multipotent Progenitor (LMPP), although in all cases the molecular targets through which these genes act are largely unknown. Correct specification of the LMPP results in the formation of the more lymphoid-restricted Common Lymphoid Progenitor (CLP). CLP require signaling through the interleukin-7 receptor (IL-7R), and to some degree Flt-3, as well as the transcription factor EBF1. EBF1 has the ability to promote the B lymphocyte fate in multipotent progenitors as well as activate the transcription of Pax5. Pax5, EBF1 and potentially Ikaros are required to maintain lineage commitment in early B lymphocyte progenitors. Pax5 then sustains the B lymphoid transcriptional program in committed cells until its physiological down-regulation during plasma cell differentiation. Thus, lineage commitment appears to be an active process that is maintained throughout the lifespan of a B lymphocyte.

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Lineage Commitment in the Hematopoietic System

A fundamental question in biology is how multiple genetically identical, yet functionally distinct, cell types are generated from a common progenitor. This process can be divided into two steps: the commitment of the progenitor to a given lineage and the subsequent differentiation of the committed cell to establish the gene expression profile characteristic of a specific cell type. This question is particularly pertinent within the hematopoietic system, as a single self-renewing hematopoietic stem cell (HSC) is capable of generating all blood cell lineages throughout the life of the individual. A large body of research has shown that the commitment and differentiation of HSC towards mature cell types is controlled by the interplay of intrinsic and extrinsic factors that produce the initially more restricted and ultimately lineage-committed progeny (reviewed by [1]). The extrinsic factors include cytokines, hormones and the interaction with non-hematopoietic cell types, such as stromal cells, that provide signals to, such as adhesion, which maintain the progenitor's 'niche' [2-4]. The predominant intrinsic components that control early hematopoiesis are transcription factors; sequence-specific DNA binding proteins that are able to both promote and repress gene expression depending on the cellular context. These transcription factors ensure that appropriate lineage-specific genes are activated and that the potential responsiveness to signals for other lineages is suppressed, thereby, fixing the fate of a cell to one lineage [5-7].

A general rule in cellular and developmental biology is that lineage commitment, through the collaborative action of transcription factors and epigenetic regulators, such as chromatin-modifying proteins, is irreversible. While this dogma holds up well in normal circumstances, in certain situations committed cells can show remarkable lineage plasticity. For example, the enforced expression of transcription factors and cytokine receptors has shown that hematopoietic cells can be re-programmed by the direct trans-differentiation from one lineage to another [8,9]. Examples of trans-differentiation within the lymphoid lineages include the conversion of B lymphocytes into macrophages by the over-expression of CCAAT/enhancer binding protein α (C/EBPα) [10], whereas T cell progenitors can be converted into dendritic cells and mast cells by the over-expression of PU.1 and GATA3 respectively [9,11].

While in the cases mentioned above re-programming appears to occur by direct trans-differentiation to a distinct cell type, other strategies have shown that re-programming can also occur by de-differentiation to a more immature and multi-potent state. Examples of de-differentiation include nuclear cloning, where committed nuclei can be re-programmed into immature cells by placing the nucleus of the committed cell into the cytoplasmic environment of a pluripotent oocyte [12]. Similarly, the introduction of only a few transcription factors into mature cell types, such as fibroblasts, is sufficient to re-program these cells into embryonic stem cell-like cells (termed induced pluripotent stem cells (iPS)) that are capable of differentiating into multiple cell types in the culture dish, as well as during mouse development [12]. It is important to note that while these studies highlight the plasticity of certain mature cells, all of these experiments relied on the ectopic expression of transcription factors, or other non-physiological conditions, and hence their relevance to normal lineage determination needs to be rigorously addressed.

B Lymphocyte Development

The differentiation of B lymphocytes from HSC occurs in a step-wise process through a series of intermediate progenitors (reviewed by [6], Figure 1). The earliest progeny of HSC are multipotent progenitors (MPP), which have lost extensive self-renewal capacity but retain multi-lineage differentiation potential [13]. A subset of MPP with high levels of the FMS-like tyrosine kinase-3 receptor (Flt-3) have little erythroid and megakaryocytic potential but retain lymphoid and other myeloid differentiation capacity. These cells are termed lymphoid-primed multipotent progenitors (LMPP) [14]. The LMPP population contains early lymphoid progenitors (ELP), which are lymphoid restricted cells defined by expression of a Rag1-GFP reporter [15]. ELP appear to be the precursors of CLP, which are lymphoid-restricted when tested *in vivo*, but retain myeloid potential *in vitro* [16-18]. It is currently controversial whether CLP are the physiological lymphoid progenitors, with some studies suggesting that they are primarily progenitors of B lymphocytes and natural killer cells [19,20]. Recent studies have attempted to address these controversies and suggested that expression of Flt-3 can distinguish true CLP (Flt-3^{+ve}) from a more B lymphocyte restricted (Flt-3^{-ve}) fraction [21,22]. Moreover a small proportion (5%) of Flt-3^{+ve} CLP that express a human CD25 reporter under the control of *λ5* (a transcriptional target of EBF1, see below) have been proposed to represent the first B lymphocyte-restricted progenitors [22]. It should be noted, however, that variations in the identification schemes used by the different investigators, as well as the transient nature of the populations and the difficulty of analyzing gene expression of non-cell surface proteins, such as transcription factors on a single cell level, mean that overlap in these populations is inevitable.

The first unequivocally identified B lymphocyte-specified progenitors arise from CLP in the bone marrow are termed pre-pro B cells. These cells can be identified by expression of the B lymphocyte associated marker B220, D-J rearrangements of the *Immunoglobulin heavy chain* (*Igh)* gene and activation of many B lymphocyte-specific genes [18,23]. Whilst in normal circumstances pre-pro-B cells are fated to become B lymphocytes, they are not irreversibly committed to this lineage, a process that requires the up-regulation of *Pax5* at the pro-B cell stage [24]. Successful rearrangement of the *Igh* locus at the pro-B cell stage causes cells to proceed to the pre-B cell stage. Pre-B cells proliferate rapidly and initiate recombination of the *Immunoglobulin light chain* (*Igl*) locus [25]. Productive *Igl* recombination leads to progression to the immature B lymphocyte stage and exit from the bone marrow [26]. Mature B lymphocytes are relatively long-lived and quiescent, however stimulation with their appropriate antigen initiates rapid proliferation and maturation into antibody-secreting plasma cells (reviewed in [27]).

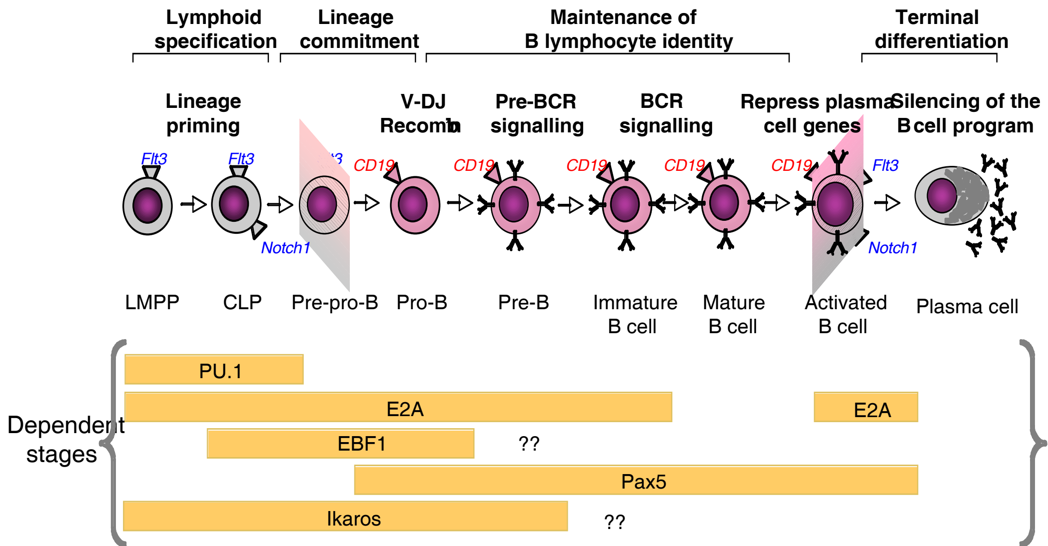

Figure 1. The transcriptional control of B lymphocyte commitment.

Shown is a simplified model of the stages of B lymphocyte development. Uncommitted progenitors and plasma cell are in grey; committed B lymphocyte stages are indicated in pink. Major phases of the commitment process and events during B lymphopoiesis are indicated on the upper portion of the figure. The lower portion of the figure indicates the stages of B lymphopoiesis have to date been shown to be dependent on the function of the indicated transcription factors. Note that the function(s) of EBF1 and Ikaros in the later stages of B lymphocyte differentiation have not been determined.

PU.1 is required for the formation of LMPP and CLP, but inactivation beyond that point in development results in relatively normal B lymphocyte numbers and function. E2A is also required for normal development of LMPP and CLP and is essential for the production of pre-pro-B, pro-B, pre-B and immature B cells. E2A is dispensable for mature B cell and plasma cell survival, but plays an as yet undefined role in activated B cells. Loss of EBF1 results in a block in B cell development at the CLP to pre-pro-B cell transition, although transient EBF1 expression can overcome this block and allow the formation of EBF1-deficient pro-B cells that share many characteristics with Pax5-deficient pro-B cells, including multi-lineage differentiation capacity. Pax5 is required for differentiation beyond the pro-B cell stage in adult bone marrow. Pax5 is also essential to maintain B lineage throughout B-lymphopoiesis, before being silenced during plasma cell development. The cell surface expression of the protein products of two Pax5-repressed genes, *Flt3* and *Notch1*, and one activated gene, *CD19* are indicated, and provide markers of Pax5 function and B lineage commitment in wild type cells. *Flt3* and *Notch1* are re-expressed upon the inhibition of Pax5 function during plasma cell differentiation. Ikaros is required for the formation of LMPP and, in its absence, no B lymphocytes are formed. Ectopic EBF1 expression can overcome this block and allow the formation of Ikaros-deficient pro-B cells that - despite the expression of EBF1 and Pax5 - retain the capacity to differentiate into macrophages in vitro. Mice harboring a hypomorphic allele of Ikaros show defects IL-7 in responses and in the transition from pro- to pre-B cells.

The Early Events in B Lymphocyte-Specification

Early B lymphocyte development and lineage commitment critically depends on the activity of two cytokine receptors, the interleukin-7 receptor (IL-7R) and Flt-3, as well as a number of transcription factors, including Ikaros, PU.1, E2A, Early B cell factor 1 (EBF1) and Pax5 that function in a transcriptional network [28].

The emergence of CLP from LMPP is demarcated by increased expression of the IL-7R, composed of a dimer between the common γ chain (*Il2rg*) and IL-7Rα (*Il7r*). While IL-7R expression marks CLP, IL-7R signaling is not absolutely required for generation of these cells. IL-7- and IL-7Rα-deficient mice show a 3-fold decrease in CLP numbers, but are profoundly impaired in their ability to differentiate into pre-pro-B lymphocytes and to undergo cytokine-induced proliferation [29,30]. As described in the next section, this decreased B lymphocyte differentiation suggests a role for IL-7R signaling in the induction of the B lymphocyte lineage-specification factor EBF1 [29-31]. Importantly, mice lacking the signals from both Flt-3 and IL-7R fail to develop any B lymphocytes, demonstrating that together these receptors and their ligands are essential for virtually all B lymphocyte development [32,33].

The Ikaros gene (*Ikzf1*) encodes multiple Zinc finger proteins that can function as transcriptional activators or repressors and may antagonize other Ikaros or related transcription factors [34]. *Ikzf1*$^{-/-}$ mice lack B lymphocytes from the earliest detectable stage [35], while a hypomorphic allele of Ikaros results in impaired ability to undergo the pro- to pre-B cell transition and to form IL-7-dependent pro-B cell colonies *in vitro* (Figure 1 [36]). Ikaros-deficient hematopoietic progenitors lack Flt-3 leading to the apparent loss of LMPP. However, by tracing expression of an *Ikzf1* promoter/enhancer- driven GFP reporter it was demonstrated that LMPP develop in Ikaros-deficient mice [37]. *Ikzf1*$^{-/-}$ LMPP are impaired in lymphoid developmental potential and displayed reduced expression of *Il7r* and *Rag1* suggesting that Ikaros is required for the further specification of LMPP into the lymphoid pathway [37]. As a consequence of the early block in lymphopoiesis, it has proven difficult to determine if Ikaros plays a role in committed B lymphocytes. However, the Singh laboratory has recently shown that Ikaros is also required for B lineage commitment at the pro-B cell stage (see next section) [38].

Another candidate control to the specification of LMPP is PU.1. Mice lacking *Sfpi1* (the gene encoding PU.1) die during late embryogenesis or shortly after birth and lack identifiable B lymphocytes and myeloid cells in the liver, as well as T cells in the thymus, suggesting a block in development at or before the LMPP stage [39-41]. Early studies suggested that PU.1 was absolutely required for pro-B cell development, potentially because of its role in regulating *Il7r* and *Ebf1* expression [41,42]. However, *Sfp1*$^{-/-}$ B lymphocyte colonies can be derived from fetal liver progenitors, albeit at a low frequency and with slower kinetics than from wild-type embryos [43]. As is the case for Ikaros deficiency, Flt-3 is not expressed at wild-type levels in *Sfpi1*$^{-/-}$ embryos, making it difficult to ascertain whether PU.1 is required for development of LMPP, or for their further differentiation. Nonetheless, PU.1 functions very early in the stepwise progression toward B lymphocyte specification (Figure 1).

A role for PU.1 in LMPP would agree with previous studies that showed that PU.1 dose determines B lymphocyte versus macrophage specification [44]. This conclusion was based

on the observation that PU.1-deficient fetal liver cells transduced with a PU.1-producing retrovirus formed macrophages with high PU.1 expression, while B lymphocytes expressed significantly lower amounts of PU.1 [44]. This finding parallels the known differential expression of PU.1 in B lymphocytes and macrophages, and the high levels of PU.1 in LMPP [45,46]. The examination of the function of PU.1 in adult bone marrow lymphopoiesis has been examined using both PU.1 conditional mutant mice and hypomorphic mutations that knock down PU.1 expression but still result in viable adult mice (reviewed by [47]). The conditional inactivation of PU.1 throughout adult bone marrow results in a greatly perturbed hematopoiesis and, in contrast to fetal development, markedly excess granulopoiesis [48]. Adult PU.1-deficient mice lack identifiable LMPP and CLP populations and are not able to contribute to the lymphoid lineages in competitive reconstitution assays. Inactivation of PU.1 in sorted CLP, as in committed B lymphocytes using CD19-Cre, allows normal B lymphocyte differentiation, suggesting that PU.1 functions primarily to specify lymphoid progenitors but is not required for further B lymphocyte differentiation [43,49,50].

A second genetic model of PU.1 function in adults was produced by deleting an upstream regulatory element in the *Sfpi1* gene resulting in reduced PU.1 expression and a profound block in B lymphopoiesis [51]. Recently an alternative hypomorphic allele of *Sfpi1* was described in a study that reached a similar conclusion [52]. Lowering PU.1 expression preferentially allows B1 B lymphocyte development [51]. A similar expansion of B1 cells was reported in aged mice after conditionally inactivating *Sfpi1* with CD19-Cre suggesting a role for PU.1 in specifying or maintaining the B1 versus conventional (B2) B lymphocyte fate [43]. In summary, ample data demonstrate that PU.1 is essential for early lymphopoiesis; the molecular targets of PU.1 involved in initial lymphoid specification remain to be determined.

The basic helix-loop-helix proteins, E12 and E47, known collectively as E2A, are essential for B lymphocyte formation and have very recently been shown to be required for the development of both LMPP and CLP (Figure 1 [53,54]). E2A promotes the development of LMPP where it primes the expression of lymphoid genes and, at some level, suppresses myelopoiesis [55]. E2A-deficient CLP fail to initiate *Igh* recombination because of the lack of *Rag1* expression and transcribe only low amounts of *Ebf1* and lack B lymphocyte-specific transcripts such as *Pax5, Cd79a* and *λ5* [31]. The development of a conditional *E2a* allele has allowed the function of E2A in committed B lymphocytes to be assessed. Inactivation of *E2a* in pre-pro-B, pro-B, pre-B or immature B lymphocytes results in the loss of deleted cells, and the down-regulation of the B lymphocyte-specific gene expression profile, demonstrating that E2A is essential for bone marrow B lymphopoiesis [56]. This finding contrasts with the failure to find a critical role for E2A in immortalized pre-B cell lines, and highlights the need to analyze lineage commitment and development in primary cells [57]. Surprisingly, the ectopic expression of Pax5 on an E2A-deficient background *in vivo* allows the formation of pro-B cells, suggesting that a major function of E2A is to induce *Pax5* [56].

B lymphocyte Commitment: Roles for Pax5, EBF1 and Ikaros

From the information above, it is apparent that one of the critical events in specification of the B lymphocyte developmental program is induction of *Ebf1*. Mice lacking EBF1 fail to express most B lymphocyte genes including *Cd79a*, *Cd79b, λ5*, and *VpreB1* and do not undergo *Igh* recombination (Figure 1[58]), whereas over-expression of EBF1 in HSC biases differentiation toward B lymphocytes [59]. Recent studies suggest that EBF1 is required for priming the B lymphoid gene expression program as early as the CLP stage [31]. Enforced expression of EBF1 is also able to rescue B lymphocyte differentiation from multipotent progenitors blocked at earlier stages of development due to targeted deletion of key lymphoid genes, including E2A, IL-7R, Ikaros and PU.1 [29,30,38,42,60]. Thus EBF1 is an essential specification factor for B lymphocytes.

Genetic and biochemical approaches have demonstrated that *Ebf1* expression requires both E2A and IL-7. In the absence of IL-7-signalling *in vivo*, pre-pro-B cells form but have low levels of *Ebf1* mRNA and are unable to maintain B lymphocyte potential, even if IL-7 is added to the cultures [61]. *Ebf1* is controlled through two promoters, a distal and a proximal promoter [62]. The distal promoter is regulated by E2A and indirectly STAT5 [62]. Since STAT5 is activated by IL-7R signaling, this finding provides a possible explanation for the dependence of *Ebf1* expression on both E2A and IL-7R [29,30,60,61]. EBF1 binding sites are also present in the distal promoter, suggesting an auto-regulatory function for EBF1, whereas the proximal promoter is regulated by Ets1, PU.1 and Pax5 [62]. A role for Pax5 in promoting *Ebf1* expression is supported by the findings that ectopic expression of Pax5 in T cell progenitors induced *Ebf1* [63] and that *Pax5*$^{-/-}$ pro-B cells have reduced *Ebf1* mRNA [62,64,65]. As *Pax5* is also dependent on EBF1, EBF1 regulates its own expression directly through the regulation of its promoter and indirectly through up-regulation of *Pax5*. This EBF1-Pax5 regulatory loop could function to amplify B lymphocyte-specific gene expression and solidify commitment to the B lymphocyte pathway.

These studies highlighted the essential functions of several transcription factors in the specification of lymphoid progenitors and ultimately B lymphopoiesis. However the simple lack of B lineage cells in mice deficient for Ikaros, PU.1, E2A and EBF1 made investigations into their role in lineage commitment problematic. This limitation was not the case for one factor, Pax5, that has been clearly shown to be required for B lineage commitment. Pax5 is expressed at a stable level throughout the B lymphocyte lineage, initiating in the pre-pro-B cell stage, until its down-regulation in plasma cells (Figure 1 [24]). In the absence of Pax5, development is arrested at the early pro-B cell stage of differentiation characterized by expression of B lymphocyte-specific transcripts and *D*-J_H rearrangements at the *Igh* locus [66,67]. Intriguingly, while *Pax5*$^{-/-}$ pro-B cells are unable to differentiate into mature B lymphocytes, they can be cultivated indefinitely in the presence of IL-7 and stroma. Most surprisingly, however, is that these pro-B cells are not committed to the B lymphocyte lineage but instead are capable of differentiating into a broad spectrum of hematopoietic cell types [68-71]. Restoration of Pax5 expression in *Pax5*$^{-/-}$ pro-B cells suppresses this multi-lineage potential, whereas conditional inactivation of *Pax5* in pro-B cells reverts lineage commitment and allows reacquisition of multi-lineage potential [72]. E2A-deficient lymphoid

cell lines also possess a similar capacity for multi-lineage differentiation, although in this case the cells lack high-level expression of markers of B lineage specification, suggesting that they represent an earlier stage of differentiation [73].

Interestingly, while Pax5 is required for B lymphocyte commitment, it does not possess the potent trans-differentiation potential of factors such as EBF1 [59], PU.1 [9] or C/EBPα [10]. Enforced expression of Pax5 in hematopoietic progenitors *in vivo*, using either transgenic or retroviral approaches, does not interfere with HSC function or myelopoiesis, but promotes B lymphocyte development at the expense of T lymphopoiesis [74,75]. *In vitro* over-expression of Pax5 in myeloid cell lines [76,77] and in hematopoietic progenitors [78] results in the inhibition of myeloid growth factor receptor expression and some co-expression of myeloid and B lymphoid associated genes, but not lineage conversion. Pax5 was also able to promote B lymphocyte differentiation in uncommitted CLP that express a myelopoiesis-promoting cytokine receptor [79]. Thus, Pax5 appears to be only capable of promoting the B lymphocyte fate in already lymphoid committed progenitors. Moreover, in contrast to EBF1, ectopic expression of Pax5 is unable to rescue B lymphocyte differentiation from IL-7R [30], EBF1 [80] and PU.1 [42] -deficient progenitors. These studies should be interpreted with care however, as recent studies have demonstrated that while Pax5 cannot rescue E2A-deficient pro-B cell development *in vitro*, it can do so *in vivo* [56].

The requirement for Pax5 in lineage commitment has generated intense interest in understanding the mechanisms by which Pax5 controls gene expression. As Pax5 has the ability to both activate and repress genes [66], it was initially hypothesized that Pax5 may promote lineage commitment by repressing the expression of non-B lymphocyte genes [68]. In keeping with this concept, *Pax5*$^{-/-}$ pro-B cells express many genes associated with MPP or non-B lineage cells [64,68,81]. The genes encoding M-CSF-R and Notch1, factors that promote macrophage and T cell development respectively, are directly repressed by Pax5 and provide a molecular explanation of the lineage plasticity of the *Pax5*$^{-/-}$ pro-B cells [75,82]. Pax5 also directly represses *Flt-3* [81,83]. This repression is crucial for B lymphopoiesis as enforced expression of Flt-3 throughout hematopoiesis or injection of Flt-3L blocks B lymphocyte formation [83,84].

Global transcriptional profiling has been employed to identify several hundred Pax5-regulated genes [64,65,81]. Interestingly, the majority of Pax5-repressed genes are normally expressed in non-B lymphocyte lineages. Surprisingly, the conditional inactivation of Pax5 in committed pro-B cells or mature B lymphocytes resulted in the re-activation of many of these repressed genes, a process that also occurs to some degree following the physiological down-regulation of Pax5 during plasma cell differentiation (See below) [81,85,86]. Pax5 activates many B lymphocyte-specific genes, including those coding for components of the pre-B cell receptor and associated signaling molecules such as CD19, Blnk, CD79a and λ5 (reviewed by [87]). Pax5 also positively regulates the genes that encode a number of transcription factors important for B lymphocyte differentiation, including SpiB, Aiolos, Lef1, IRF4 and IRF8, demonstrating that Pax5 acts to reinforce B cell commitment and subsequent differentiation [64,65]. Interestingly, *Ebf1* that, as outlined above, acts upstream of *Pax5*, is also up-regulated by Pax5 [62], demonstrating that the transcriptional network controlling B lymphocyte-specification and commitment is not a simple linear cascade but involves multiple combinatorial inputs and feedback loops.

In keeping with the importance of the EBF1-Pax5 regulatory loop, recent studies from the Singh laboratory have shown that EBF1 is also essential for B lymphocyte commitment [80]. Similarly to the Pax5-deficient pro-B cell lines, *Ebf1*$^{-/-}$ lymphoid progenitor cell lines express B lineage transcripts such as B220 and can be maintained in IL-7 and stromal cell co-cultures. These EBF1-deficient progenitors are not restricted to the B lymphocyte lineage and can differentiate into T lymphocytes and macrophages. Most importantly the authors demonstrated that ectopic expression of EBF1 in *Pax5*$^{-/-}$ pro-B cells suppressed the myeloid differentiation capacity of the Pax5-deficient cells, suggesting that EBF1 promotes lineage commitment downstream of Pax5 [80]. As expected, EBF1 promoted the expression of B lineage genes, but interestingly, EBF1 also repressed a cohort of non-B lymphocyte genes, many of which are also repressed by Pax5 [80]. Thus EBF1 and Pax5 appear to synergistically mediate B lymphocyte commitment.

Our understanding of the regulation of B lymphocyte commitment has been further enhanced by a second finding from the Singh laboratory, that Ikaros is also a component of the regulatory network controlling the process [38]. The authors used retroviral transduction to over-express EBF1 in Ikaros-deficient progenitors. The rescued pro-B cells were CD19^{+ve} and expressed normal levels of both *Pax5* and endogenous *Ebf1*. Surprisingly, these Ikaros-deficient pro-B cells could trans-differentiate into macrophages in a manner similar to *Pax5*$^{-/-}$ pro-B cells [38]. Thus, expression of neither EBF1 nor Pax5 results in lineage specification in the absence of a third factor, Ikaros.

The Maintenance of B Lymphocyte Commitment

The studies outlined above have concentrated on the initial commitment to the B lymphoid lineage. The Busslinger laboratory has subsequently made use of a mouse strain that allows the inducible deletion of *Pax5*, to demonstrate that Pax5 also actively maintains lineage identity in committed pro-B cells. Inactivation of *Pax5* in pro-B cell cultures results in the cells acquiring a multi-lineage potential and gene expression program identical to *Pax5*$^{-/-}$ pro-B cells and unequivocally established that lineage commitment is an ongoing process, not a discrete event [72].

One question that arose from these studies was whether the loss of Pax5 permits the direct trans-differentiation of pro-B cells into other lineages, or whether, as for the iPS pathway, de-differentiation of pro-B cells into uncommitted progenitors occurs. The de-differentiated pro-B cells would then proceed along a conventional pathway to, for example, T lymphocytes . This question has been recently addressed by inactivating *Pax5* in peripheral B lymphocytes (Figure 2 [88]). This was accomplished by either inducing the deletion of *Pax5* in isolated mature B lymphocytes in culture, or by sorting *Pax5*-deleted mature peripheral B lymphocytes. In both cases the Pax5-deficient mature B lymphocytes were transferred into *Rag1*$^{-/-}$ mice, that lack lymphocytes, and the progeny of the manipulated B lymphocytes monitored. Loss of Pax5 in mature B lymphocytes led to the de-differentiation of a small proportion of the cells into a population identical to *Pax5*$^{-/-}$ pro-B cells [88]. These de-differentiated pro-B cells displayed productively rearranged *Igh* and *Igl* genes that definitively marked them as having derived from mature B lymphocytes. The de-

differentiated pro-B cells were able to generate T lymphocytes and in some cases myeloid cells in the host mice. These B lymphocyte-derived T lymphocytes were also functional as they could successfully contribute to an immune response after immunization. In the future it will be interesting to examine if the conditional inactivation of EBF1 or Ikaros results in a loss of commitment similar to that observed in the absence of Pax5.

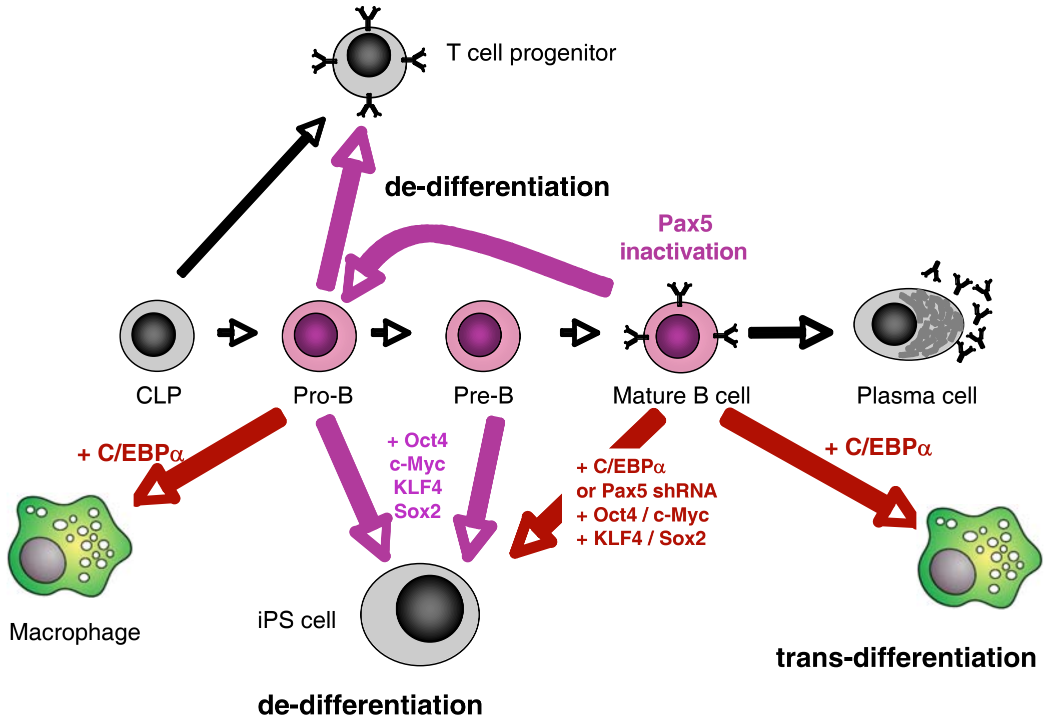

Figure 2. Re-programming of B lymphocytes through the loss of Pax5.

The schematic diagram of B lymphocyte differentiation shows the various differentiation stages and experimental manipulations that result in the re-programming of B lymphocytes into other cell types. Pax5 expressing B lymphocytes are shown in pink, with black arrows indicating normal B cell differentiation. The ectopic expression of C/EBPα represses *Pax5* expression and results in trans-differentiation of pro-B and mature B cells to macrophages (red arrows). Purple arrows indicate the de-differentiation pathways, either in the absence of Pax5 (above) or in the presence of induced pluripotent stem (iPS) cell promoting transcription factors (Oct4, c-Myc, KLF4, Sox2). The conditional inactivation of *Pax5* in committed B cells results in the de-differentiation of mature B cells to the pro-B cell stage, that then generate lymphocyte-derived T lymphocytes. Enforced expression of Oct4, c-Myc, KLF4 and Sox2 results in the de-differentiation of pro-B or pre-B cells, but not mature B cells, to iPS cells. Mature B cells instead require antagonism of the B lymphocyte gene expression program, either by over-expression of C/EBPα or knock-down of Pax5 using shRNA technology, followed by expression of the iPS cell promoting factors.

While these experiments demonstrate de-differentiation as the mechanism of re-programming, they also raise a number of questions. For example, a common feature of re-programming in multiple systems is that de-differentiation is a slow (taking several days to

weeks) and inefficient process. While the reason(s) for this delay is unclear, it does suggest that there are several rate limiting steps. The de-differentiation of mature B lymphocytes relied on ectopic expression of *Bcl2* to block apoptosis, suggesting that under normal circumstances mechanisms are in place to prevent de-differentiation [88]. If so, it will be interesting to determine what these checkpoints are. One reason why such check-points might exist is highlighted by the finding that the de-differentiation of B lymphocytes resulted in the formation of aggressive lymphomas with a phenotype resembling *Pax5*$^{-/-}$ pro-B cells [88].

The fact that B lineage commitment is an ongoing and active process was further highlighted by studies that examined the ability of B lymphocytes to be re-programmed to iPS cells (Figure 2 [89]). In the last couple of years it has become apparent that the ectopic expression of four transcription factors (Oct4, Sox2, Klf4, and c-Myc) is sufficient to reprogram mouse and human cells to a pluripotent state [12]. Using B lymphocytes as a model system, the Jaenisch laboratory has shown that inducible expression of these four transcription factors was sufficient to re-program pro- and pre-B cells, but not mature B lymphocytes [89]. The authors reasoned that the re-programming of mature B lymphocytes might require prior inhibition of the B lineage commitment program, and thus inhibited B lymphocyte-identity by over-expressing C/EBPα that, as mentioned earlier, is sufficient to antagonize Pax5 expression in B lymphocytes and allow trans-differentiation to macrophages (Figure 2). Strikingly, these experiments led to a relatively high frequency of re-programming to iPS cells, an outcome that was also achieved simply by knocking-down *Pax5* [89]. How the re-programming works is still unclear. Whether Pax5 insufficiency results in the de-differentiation of mature B lymphocytes into a progenitor akin to *Pax5*$^{-/-}$ pro-B cells, which is then re-programmed to iPS cells, remains to be determined. An alternative possibility is that C/EBPα over-expression or Pax5 elimination results in the formation of myeloid cells *in vitro*, which are potentially more amenable to iPS re-programming. Either way it appears that inhibition of Pax5-mediated lineage commitment must be overcome to allow the cellular plasticity of mature B lymphocytes to be revealed.

The Down-Regulation of the B Lymphocyte Program during Plasma Cell Differentiation

The terminal differentiation of B lymphocytes to antibody-secreting plasma cells is accompanied by a dramatic shift in the gene expression profiles between the two cell types (reviewed in [27]). While Pax5 function is essential throughout B lymphopoiesis, its expression is rapidly down-regulated as cells commit to plasma cell differentiation. This is thought to be required because Pax5 represses a number of genes that are important in plasma cell biology. The Pax5-repressed genes include known plasma cell genes such as *Xbp1* [90], *J chain* [91], high-level *Igh* expression and potentially the master regulator of plasma cells, *Blimp1* [86,92]. Interestingly, many of those B lineage-inappropriate genes that are silenced by Pax5 upon initial B lymphocyte commitment are also re-expressed during plasma cell differentiation [81,85]. In case of two targets, CD28 and CCR2, it appears that they play a role in normal plasma cell biology, demonstrating how the appropriate silencing of Pax5

expression during terminal differentiation contributes to the plasma cell transcription program [81].

In keeping with this concept, the inactivation of *Pax5* in a chicken mature B lymphocyte line results in premature expression of genes involved in plasma cell differentiation and increased IgM secretion [86]. However this does not appear to be the case after the deletion of *Pax5* in mature mouse B lymphocytes, which as outlined in the previous section, instead undergo de-differentiation to a more immature stage of lymphopoiesis [88]. One explanation for this could be that the strategy used to inactivate *Pax5* in mature B lymphocytes did not use antigen stimulation, whereas the normal down-regulation of *Pax5* that accompanies terminal differentiation is induced by encounter of a B lymphocyte with its cognate antigen, the resulting B cell receptor (BCR) signaling may set in motion a process that favors the forward differentiation to the plasma cell fate. BCR signaling also induces anti-apoptotic regulators of the Bcl2 family, whereas the de-differentiation phenomenon requires ectopic *Bcl2* expression to occur [88]. These observations suggest that encounter with an appropriate antigen, enables plasma cell differentiation by co-coordinatingco-ordinating the silencing of *Pax5* with the expression of survival promoting genes.

The molecular events that inhibit Pax5 expression and thus initiate plasma cell differentiation are still emerging. It is proposed that the mutual repression between the B lymphocyte program, mediated by Pax5, and the plasma cell program, controlled by Blimp1, Irf4 and Xbp1 separates these two radically distinct transcriptional profiles [93]. This antagonism is expected to be direct, as Pax5 has been shown to directly repress *Blimp1* [94] and *Xbp1* [90] transcription and the inactivation of Pax5 results in premature *Blimp1* expression [81,86]. However, in contrast to the studies that show that *Pax5* is directly repressed by Blimp1 [92], *Pax5* expression is down-regulated in pre-plasmablasts even in the absence of functional Blimp1 [85]. Analysis of the regulation of transcription by Pax5 in differentiating B lymphocytes showed that known target genes, both activated and repressed genes, were deregulated prior to Blimp1 expression [85]. This occurs in the absence of any change in Pax5 protein levels and DNA-binding function, suggesting that the initiation of plasma cell differentiation is triggered by altered Pax5 function, although the mechanism by which this occurs remains to be determined.

Conclusion

The last decade has seen great advances in our understanding of the control of B lymphocyte lineage commitment. These advances have relied on the synergistic benefit derived from pursuing a variety of approaches, including the prospective isolation of lymphoid progenitors by flow cytometry, the development of highly efficient culture systems to assay cell fate decisions and the precise genomic and biochemical analyses of single transcription factors. More recently, global analyses of gene transcription and DNA-binding have brought in new insights to aid in understanding the complex interplay between gene regulation and cellular differentiation.

The recognition of the role of Pax5 in early lymphopoiesis has provided the first clear molecular description of the lineage commitment process in the hematopoietic system and

pointed to the role played by the repression of alternative fates, and their corresponding genes, in the process. The recent reports that both EBF1 and Ikaros are also obligate components of the lineage commitment program raise the challenge of integrating these findings into those already established for Pax5. In all cases, the further identification of the molecular targets of the transcription factors involved in early B lymphocyte development will be crucial in unlocking the transcriptional networks that control the process. Finally, an understanding of the factors that regulate the loss of B lymphocyte identity, either physiologically during plasma cell differentiation or in leukemic cells, has important clinical implications for modulating the immune response in situations such as vaccination or treating autoimmunity and cancer.

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References

[1] Buza-Vidas, N., Luc, S. and Jacobsen, S.E. (2007) Delineation of the earliest lineage commitment steps of haematopoietic stem cells: new developments, controversies and major challenges. *Curr Opin Hematol* 14, 315-321.

[2] Martinez-Agosto, J.A., Mikkola, H.K., Hartenstein, V. and Banerjee, U. (2007) The hematopoietic stem cell and its niche: a comparative view. *Genes Dev* 21, 3044-3060.

[3] Iwasaki-Arai, J., Iwasaki, H., Miyamoto, T., Watanabe, S. and Akashi, K. (2003) Enforced granulocyte/macrophage colony-stimulating factor signals do not support lymphopoiesis, but instruct lymphoid to myelomonocytic lineage conversion. *J Exp Med* 197, 1311-1322.

[4] Kondo, M., Scherer, D.C., Miyamoto, T., King, A.G., Akashi, K., Sugamura, K. and Weissman, I.L. (2000) Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. *Nature* 407, 383-386.

[5] Rothenberg, E.V. (2007) Negotiation of the T lineage fate decision by transcription-factor interplay and microenvironmental signals. *Immunity* 26, 690-702.

[6] Nutt, S.L. and Kee, B.L. (2007) The transcriptional regulation of B cell lineage commitment. *Immunity* 26, 715-725.

[7] Iwasaki, H. and Akashi, K. (2007) Myeloid lineage commitment from the hematopoietic stem cell. *Immunity* 26, 726-740.

[8] Laiosa, C.V., Stadtfeld, M. and Graf, T. (2006) Determinants of lymphoid-myeloid lineage diversification. *Annu Rev Immunol* 24, 705-738.

[9] Laiosa, C.V., Stadtfeld, M., Xie, H., de Andres-Aguayo, L. and Graf, T. (2006) Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBP alpha and PU.1 transcription factors. *Immunity* 25, 731-744.

[10] Xie, H., Ye, M., Feng, R. and Graf, T. (2004) Stepwise reprogramming of B cells into macrophages. *Cell* 117, 663-676.

[11] Taghon, T., Yui, M.A. and Rothenberg, E.V. (2007) Mast cell lineage diversion of T lineage precursors by the essential T cell transcription factor GATA-3. *Nat Immunol* 8, 845-855.

[12] Jaenisch, R. and Young, R. (2008) Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell* 132, 567-582.

[13] Adolfsson, J., Borge, O.J., Bryder, D., Theilgaard-Monch, K., Astrand-Grundstrom, I., Sitnicka, E., Sasaki, Y. and Jacobsen, S.E. (2001) Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. *Immunity* 15, 659-669.

[14] Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C.T., Bryder, D., Yang, L., Borge, O.J., Thoren, L.A., Anderson, K., Sitnicka, E., Sasaki, Y., Sigvardsson, M. and Jacobsen, S.E. (2005) Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell* 121, 295-306.

[15] Igarashi, H., Gregory, S.C., Yokota, T., Sakaguchi, N. and Kincade, P.W. (2002) Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity* 17, 117-130.

[16] Kondo, M., Weissman, I.L. and Akashi, K. (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 91, 661-672.

[17] Balciunaite, G., Ceredig, R., Massa, S. and Rolink, A.G. (2005) A B220+ CD117+ CD19- hematopoietic progenitor with potent lymphoid and myeloid developmental potential. *Eur J Immunol* 35, 2019-2030.

[18] Rumfelt, L.L., Zhou, Y., Rowley, B.M., Shinton, S.A. and Hardy, R.R. (2006) Lineage specification and plasticity in CD19- early B cell precursors. *J Exp Med* 203, 675-687.

[19] Bhandoola, A. and Sambandam, A. (2006) From stem cell to T cell: one route or many? *Nat Rev Immunol* 6, 117-126.

[20] Allman, D., Sambandam, A., Kim, S., Miller, J.P., Pagan, A., Well, D., Meraz, A. and Bhandoola, A. (2003) Thymopoiesis independent of common lymphoid progenitors. *Nat Immunol* 4, 168-174.

[21] Karsunky, H., Inlay, M.A., Serwold, T., Bhattacharya, D. and Weissman, I.L. (2008) Flk2+ common lymphoid progenitors possess equivalent differentiation potential for the B and T lineages. *Blood* 111, 5562-5570.

[22] Mansson, R., Zandi, S., Anderson, K., Martensson, I.L., Jacobsen, S.E., Bryder, D. and Sigvardsson, M. (2008) B-lineage commitment prior to surface expression of B220 and CD19 on hematopoietic progenitor cells. *Blood* 112, 1048-1055.

[23] Gounari, F., Aifantis, I., Martin, C., Fehling, H.J., Hoeflinger, S., Leder, P., von Boehmer, H. and Reizis, B. (2002) Tracing lymphopoiesis with the aid of a pTalpha-controlled reporter gene. *Nat Immunol* 3, 489-496.

[24] Fuxa, M. and Busslinger, M. (2007) Reporter gene insertions reveal a strictly B lymphoid-specific expression pattern of Pax5 in support of its B cell identity function. *J Immunol* 178, 3031-3037.

[25] Martensson, I.L., Keenan, R.A. and Licence, S. (2007) The pre-B-cell receptor. *Curr Opin Immunol* 19, 137-142.

[26] Rolink, A.G., Andersson, J. and Melchers, F. (2004) Molecular mechanisms guiding late stages of B-cell development. *Immunol Rev* 197, 41-50.

[27] Fairfax, K.A., Kallies, A., Nutt, S.L. and Tarlinton, D.M. (2008) Plasma cell development: from B-cell subsets to long-term survival niches. *Semin Immunol* 20, 49-58.

[28] **28**Singh, H., Medina, K.L. and Pongubala, J.M. (2005) Contingent gene regulatory networks and B cell fate specification. *Proc Natl Acad Sci U S A* 102, 4949-4953.

[29] Dias, S., Silva, H., Jr., Cumano, A. and Vieira, P. (2005) Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors. *J Exp Med* 201, 971-979.

[30] Kikuchi, K., Lai, A.Y., Hsu, C.L. and Kondo, M. (2005) IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF. *J Exp Med* 201, 1197-1203.

[31] Zandi, S., Mansson, R., Tsapogas, P., Zetterblad, J., Bryder, D. and Sigvardsson, M. (2008) EBF1 is essential for B-lineage priming and establishment of a transcription factor network in common lymphoid progenitors. *J Immunol* 181, 3364-3372.

[32] Sitnicka, E., Bryder, D., Theilgaard-Monch, K., Buza-Vidas, N., Adolfsson, J. and Jacobsen, S.E. (2002) Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. *Immunity* 17, 463-472.

[33] Vosshenrich, C.A., Cumano, A., Muller, W., Di Santo, J.P. and Vieira, P. (2003) Thymic stromal-derived lymphopoietin distinguishes fetal from adult B cell development. *Nat Immunol* 4, 773-779.

[34] Ng, S.Y., Yoshida, T. and Georgopoulos, K. (2007) Ikaros and chromatin regulation in early hematopoiesis. *Curr Opin Immunol* 19, 116-122.

[35] Wang, J.H., Nichogiannopoulou, A., Wu, L., Sun, L., Sharpe, A.H., Bigby, M. and Georgopoulos, K. (1996) Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity* 5, 537-549.

[36] Kirstetter, P., Thomas, M., Dierich, A., Kastner, P. and Chan, S. (2002) Ikaros is critical for B cell differentiation and function. *Eur J Immunol* 32, 720-730.

[37] Yoshida, T., Ng, S.Y., Zuniga-Pflucker, J.C. and Georgopoulos, K. (2006) Early hematopoietic lineage restrictions directed by Ikaros. *Nat Immunol* 7, 382-391.

[38] Reynaud, D., Demarco, I.A., Reddy, K.L., Schjerven, H., Bertolino, E., Chen, Z., Smale, S.T., Winandy, S. and Singh, H. (2008) Regulation of B cell fate commitment and immunoglobulin heavy-chain gene rearrangements by Ikaros. *Nat Immunol* 9, 927-936.

[39] Scott, E.W., Simon, M.C., Anastasi, J. and Singh, H. (1994) Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 265, 1573-1577.

[40] McKercher, S.R., Torbett, B.E., Anderson, K.L., Henkel, G.W., Vestal, D.J., Baribault, H., Klemsz, M., Feeney, A.J., Wu, G.E., Paige, C.J. and Maki, R.A. (1996) Targeted

disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *Embo J* 15, 5647-5658.

[41] DeKoter, R.P., Lee, H.J. and Singh, H. (2002) PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors. *Immunity* 16, 297-309.

[42] Medina, K.L., Pongubala, J.M., Reddy, K.L., Lancki, D.W., Dekoter, R., Kieslinger, M., Grosschedl, R. and Singh, H. (2004) Assembling a gene regulatory network for specification of the B cell fate. *Dev Cell* 7, 607-617.

[43] Ye, M., Ermakova, O. and Graf, T. (2005) PU.1 is not strictly required for B cell development and its absence induces a B-2 to B-1 cell switch. *J Exp Med* 202, 1411-1422.

[44] DeKoter, R.P. and Singh, H. (2000) Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science* 288, 1439-1441.

[45] Arinobu, Y., Mizuno, S., Chong, Y., Shigematsu, H., Iino, T., Iwasaki, H., Graf, T., Mayfield, R., Chan, S., Kastner, P. and Akashi, K. (2007) Reciprocal activation of GATA-1 and PU.1 marks initial specification of hematopoietic stem cells into myeloerythroid and myelolymphoid lineages. *Cell Stem Cell* 1, 416-427.

[46] Nutt, S.L., Metcalf, D., D'Amico, A., Polli, M. and Wu, L. (2005) Dynamic regulation of PU.1 expression in multipotent hematopoietic progenitors. *J Exp Med* 201, 221-231.

[47] Dakic, A., Wu, L. and Nutt, S.L. (2007) Is PU.1 a dosage-sensitive regulator of haemopoietic lineage commitment and leukaemogenesis? *Trends Immunol* 28, 108-114.

[48] Dakic, A., Metcalf, D., Di Rago, L., Mifsud, S., Wu, L. and Nutt, S.L. (2005) PU.1 regulates the commitment of adult hematopoietic progenitors and restricts granulopoiesis. *J Exp Med* 201, 1487-1502.

[49] Iwasaki, H., Somoza, C., Shigematsu, H., Duprez, E.A., Iwasaki-Arai, J., Mizuno, S., Arinobu, Y., Geary, K., Zhang, P., Dayaram, T., Fenyus, M.L., Elf, S., Chan, S., Kastner, P., Huettner, C.S., Murray, R., Tenen, D.G. and Akashi, K. (2005) Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood* 106, 1590-1600.

[50] Polli, M., Dakic, A., Light, A., Wu, L., Tarlinton, D.M. and Nutt, S.L. (2005) The development of functional B lymphocytes in conditional PU.1 knock-out mice. *Blood* 106, 2083-2090.

[51] Rosenbauer, F., Owens, B.M., Yu, L., Tumang, J.R., Steidl, U., Kutok, J.L., Clayton, L.K., Wagner, K., Scheller, M., Iwasaki, H., Liu, C., Hackanson, B., Akashi, K., Leutz, A., Rothstein, T.L., Plass, C. and Tenen, D.G. (2006) Lymphoid cell growth and transformation are suppressed by a key regulatory element of the gene encoding PU.1. *Nat Genet* 38, 27-37.

[52] Houston, I.B., Kamath, M., Schweitzer, B.L., Chlon, T.M. and DeKoter, R.P. (2007) Reduction in PU.1 activity results in a block to B cell development, abnormal myeloid proliferation and neonatal lethality. *Exp Hematol* in press35, 1056-1068.

[53] Bain, G., Robanus Maandag, E.C., te Riele, H.P., Feeney, A.J., Sheehy, A., Schlissel, M., Shinton, S.A., Hardy, R.R. and Murre, C. (1997) Both E12 and E47 allow commitment to the B cell lineage. *Immunity* 6, 145-154.

[54] Borghesi, L., Aites, J., Nelson, S., Lefterov, P., James, P. and Gerstein, R. (2005) E47 is required for V(D)J recombinase activity in common lymphoid progenitors. *J Exp Med* 202, 1669-1677.

[55] Dias, S., Mansson, R., Gurbuxani, S., Sigvardsson, M. and Kee, B.L. (2008) E2A Proteins Promote Development of Lymphoid-Primed Multipotent Progenitors. *Immunity* 29, 217-227.

[56] Kwon, K., Hutter, C., Sun, Q., Bilic, I., Cobaleda, C., Malin, S. and Busslinger, M. (2008) Instructive role of the transcription factor E2A in early B lymphopoiesis and germinal center B cell development. *Immunity* 28, 751-762.

[57] Lazorchak, A.S., Wojciechowski, J., Dai, M. and Zhuang, Y. (2006) E2A promotes the survival of precursor and mature B lymphocytes. *J Immunol* 177, 2495-2504.

[58] Lin, H. and Grosschedl, R. (1995) Failure of B-cell differentiation in mice lacking the transcription factor EBF. *Nature* 376, 263-267.

[59] Zhang, Z., Cotta, C.V., Stephan, R.P., deGuzman, C.G. and Klug, C.A. (2003) Enforced expression of EBF in hematopoietic stem cells restricts lymphopoiesis to the B cell lineage. *Embo J* 22, 4759-4769.

[60] Seet, C.S., Brumbaugh, R.L. and Kee, B.L. (2004) Early B cell factor promotes B lymphopoiesis with reduced interleukin 7 responsiveness in the absence of E2A. *J Exp Med* 199, 1689-1700.

[61] Kikuchi, K., Kasai, H., Watanabe, A., Lai, A.Y. and Kondo, M. (2008) IL-7 specifies B cell fate at the common lymphoid progenitor to pre-proB transition stage by maintaining early B cell factor expression. *J Immunol* 181, 383-392.

[62] Roessler, S., Gyory, I., Imhof, S., Spivakov, M., Williams, R.R., Busslinger, M., Fisher, A.G. and Grosschedl, R. (2007) Distinct promoters mediate the regulation of Ebf1 gene expression by interleukin-7 and Pax5. *Mol Cell Biol* 27, 579-594.

[63] Fuxa, M., Skok, J., Souabni, A., Salvagiotto, G., Roldan, E. and Busslinger, M. (2004) Pax5 induces V-to-DJ rearrangements and locus contraction of the immunoglobulin heavy-chain gene. *Genes Dev* 18, 411-422.

[64] Pridans, C., Holmes, M.L., Polli, M., Wettenhall, J.M., Dakic, A., Corcoran, L.M., Smyth, G.K. and Nutt, S.L. (20072008) Identification of Pax5 Target genes in early B cell differentiation. *J Immunol* in press180, 1719-1728.

[65] Schebesta, A., McManus, S., Salvagiotto, G., Delogu, A., Busslinger, G.A. and Busslinger, M. (2007) Transcription factor pax5 activates the chromatin of key genes involved in B cell signaling, adhesion, migration, and immune function. *Immunity* 27, 49-63.

[66] Nutt, S.L., Morrison, A.M., Dorfler, P., Rolink, A. and Busslinger, M. (1998) Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. *Embo J* 17, 2319-2333.

[67] Nutt, S.L., Urbanek, P., Rolink, A. and Busslinger, M. (1997) Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes Dev* 11, 476-491.

[68] Nutt, S.L., Heavey, B., Rolink, A.G. and Busslinger, M. (1999) Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature* 401, 556-562.

[69] Rolink, A.G., Nutt, S.L., Melchers, F. and Busslinger, M. (1999) Long-term in vivo reconstitution of T-cell development by Pax5-deficient B-cell progenitors. *Nature* 401, 603-606.

[70] Schaniel, C., Bruno, L., Melchers, F. and Rolink, A.G. (2002) Multiple hematopoietic cell lineages develop in vivo from transplanted Pax5-deficient pre-B I-cell clones. *Blood* 99, 472-478.

[71] Schaniel, C., Gottar, M., Roosnek, E., Melchers, F. and Rolink, A.G. (2002) Extensive in vivo self-renewal, long-term reconstitution capacity, and hematopoietic multipotency of Pax5-deficient precursor B-cell clones. *Blood* 99, 2760-2766.

[72] Mikkola, I., Heavey, B., Horcher, M. and Busslinger, M. (2002) Reversion of B cell commitment upon loss of Pax5 expression. *Science* 297, 110-113.

[73] Ikawa, T., Kawamoto, H., Wright, L.Y. and Murre, C. (2004) Long-term cultured E2A-deficient hematopoietic progenitor cells are pluripotent. *Immunity* 20, 349-360.

[74] Cotta, C.V., Zhang, Z., Kim, H.G. and Klug, C.A. (2003) Pax5 determines B- versus T-cell fate and does not block early myeloid-lineage development. *Blood* 101, 4342-4346.

[75] Souabni, A., Cobaleda, C., Schebesta, M. and Busslinger, M. (2002) Pax5 promotes B lymphopoiesis and blocks T cell development by repressing Notch1. *Immunity* 17, 781-793.

[76] Chiang, M.Y. and Monroe, J.G. (1999) BSAP/Pax5A expression blocks survival and expansion of early myeloid cells implicating its involvement in maintaining commitment to the B-lymphocyte lineage. *Blood* 94, 3621-3632.

[77] Chiang, M.Y. and Monroe, J.G. (2001) Role for transcription Pax5A factor in maintaining commitment to the B cell lineage by selective inhibition of granulocyte-macrophage colony-stimulating factor receptor expression. *J Immunol* 166, 6091-6098.

[78] Aderson, K., Rusterholz, C., Mansson, R., Jensen, C.T., Bacos, K., Zandi, S., Sasaki, Y., Nerlov, C., Sigvardsson, M. and Jacobsen, S.E. (2007) Ectopic expression of PAX5 promotes maintenance of biphenotypic myeloid progenitors coexpressing myeloid and B-cell lineage-associated genes. *Blood* 109, 3697-3705.

[79] Hsu, C.L., King-Fleischman, A.G., Lai, A.Y., Matsumoto, Y., Weissman, I.L. and Kondo, M. (2006) Antagonistic effect of CCAAT enhancer-binding protein-alpha and Pax5 in myeloid or lymphoid lineage choice in common lymphoid progenitors. *Proc Natl Acad Sci U S A* 103, 672-677.

[80] Pongubala, J.M., Northrup, D.L., Lancki, D.W., Medina, K.L., Treiber, T., Bertolino, E., Thomas, M., Grosschedl, R., Allman, D. and Singh, H. (2008) Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5. *Nat Immunol* 9, 203-215.

[81] Delogu, A., Schebesta, A., Sun, Q., Aschenbrenner, K., Perlot, T. and Busslinger, M. (2006) Gene repression by Pax5 in B cells is essential for blood cell homeostasis and is reversed in plasma cells. *Immunity* 24, 269-281.

[82] Tagoh, H., Ingram, R., Wilson, N., Salvagiotto, G., Warren, A.J., Clarke, D., Busslinger, M. and Bonifer, C. (2006) The mechanism of repression of the myeloid-specific c-fms gene by Pax5 during B lineage restriction. *Embo J* 25, 1070-1080.

[83] Holmes, M.L., Carotta, S., Corcoran, L.M. and Nutt, S.L. (2006) Repression of Flt3 by Pax5 is crucial for B-cell lineage commitment. *Genes Dev* 20, 933-938.

[84] Ceredig, R., Rauch, M., Balciunaite, G. and Rolink, A.G. (2006) Increasing Flt3L availability alters composition of a novel bone marrow lymphoid progenitor compartment. *Blood* 108, 1216-1222.

[85] Kallies, A., Hasbold, J., Fairfax, K., Pridans, C., Emslie, D., McKenzie, B.S., Lew, A.M., Corcoran, L.M., Hodgkin, P.D., Tarlinton, D.M. and Nutt, S.L. (2007) Initiation of plasma cell differentiation is independent of the transcription factor Blimp-1. *Immunity* i26, 555-566.n press

[86] Nera, K.P., Kohonen, P., Narvi, E., Peippo, A., Mustonen, L., Terho, P., Koskela, K., Buerstedde, J.M. and Lassila, O. (2006) Loss of Pax5 promotes plasma cell differentiation. *Immunity* 24, 283-293.

[87] Cobaleda, C., Schebesta, A., Delogu, A. and Busslinger, M. (2007) Pax5: the guardian of B cell identity and function. *Nat Immunol* 8, 463-470.

[88] Cobaleda, C., Jochum, W. and Busslinger, M. (2007) Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors. *Nature* 449, 473-477.

[89] Hanna, J., Markoulaki, S., Schorderet, P., Carey, B.W., Beard, C., Wernig, M., Creyghton, M.P., Steine, E.J., Cassady, J.P., Foreman, R., Lengner, C.J., Dausman, J.A. and Jaenisch, R. (2008) Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. *Cell* 133, 250-264.

[90] Reimold, A.M., Ponath, P.D., Li, Y.S., Hardy, R.R., David, C.S., Strominger, J.L. and Glimcher, L.H. (1996) Transcription factor B cell lineage-specific activator protein regulates the gene for human X-box binding protein 1. *J Exp Med* 183, 393-401.

[91] Rinkenberger, J.L., Wallin, J.J., Johnson, K.W. and Koshland, M.E. (1996) An interleukin-2 signal relieves BSAP (Pax5)-mediated repression of the immunoglobulin J chain gene. *Immunity* 5, 377-386.

[92] Lin, K.I., Angelin-Duclos, C., Kuo, T.C. and Calame, K. (2002) Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. *Mol Cell Biol* 22, 4771-4780.

[93] Calame, K.L., Lin, K.I. and Tunyaplin, C. (2003) Regulatory Mechanisms that Determine the Development and Function of Plasma Cells. *Annu Rev Immunol* 8, 8.

[94] Mora-Lopez, F., Reales, E., Brieva, J.A. and Campos-Caro, A. (2007) Human BSAP and BLIMP1 conform an autoregulatory feedback loop. *Blood* 110, 3150-3157.

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Chapter 6

Developmental Pathways and Molecular Regulation of Early T Cell Development in Mouse and Human*

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Abstract

In contrast to other hematopoietic lineages that develop in the bone marrow, T cells differentiate in an alternative anatomical site, the thymus, where multiple T-lineage cell types with specific immune functions are generated as a result of unique developmental programs. T cell development depends on the migration of hematopoietic precursor cells with multi-lineage potential from the bone marrow towards the thymus. There, precursor cell interactions with the thymic microenvironment gradually limit the alternative developmental options that are initially available and initiate the T-cell differentiation program. The availability of such a broad range of lineage choices, both before and after commitment to the T cell lineage, is reflected by the complexity of the molecular network that controls the T cell differentiation program. In contrast to many other hematopoietic lineages, the T-cell program lacks a simple instructive component that initiates a straightforward molecular cascade that drives further differentiation. Instead, T cell development depends on the input of a broad range of environmental factors that change continuously as the cells migrate through the different microenvironments of the thymus, as well as on multiple intrinsic transcriptional regulators that differ in time, resulting in the generation of stage-specific molecular networks. In this chapter, we describe the recent advances in both mouse and human T cell biology that have provided fundamental insights into these aspects of early T cell differentiation and commitment. While illuminating T cell development itself, the characterization of the robust lymphoid and

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myeloid lineage potential of early T cell precursors has made a large impact on the current models of hematopoietic stem cell differentiation as the initially proposed segregation between lymphoid and myeloid lineages no longer seems supported. The molecular mechanisms that regulate early T cell differentiation through transformation of early multi-lineage precursors into T-lineage committed cells are discussed.

Introduction

T cells comprise a population of blood lymphocytes that is critical in the regulation of the immune system. While some T cells have the potential to kill malignant tumor cells as well as virally infected cells, others are essential to aid non T-lineage immune cells in exerting their function or to dampen the immune response. Unlike other hematopoietic lineages that differentiate from hematopoietic stem cells in the bone marrow, the majority of circulating T cells undergoes development at a specific anatomical site, the thymus, located in the upper part of the chest cavity, in the anterior side behind the sternum. Each *de novo* generated T cell expresses a unique T cell receptor (TCR) that associates with CD3 molecules and this TCR-complex is the hallmark of T cells. The generation of this receptor is a critical element in the characterization of the successive stages of T cell development, as well as in the determination of the different T cell lineages. Since T cells must be able to recognize the endless variety of potential pathogens, the T cell repertoire must be as broad and diverse as possible. This is achieved through a series of recombination events in which different TCR encoding pieces of genomic DNA are - to a certain extent - randomly combined so that each newly generated T cell expresses a unique TCR-complex. It is this highly variable process that ensures the recognition of the wide variety of possible foreign and malignant structures by this receptor. Two types of TCRs exist: one is composed of a γδ heterodimer, the other of a αβ heterodimer. This developmental divergence is the earliest lineage choice that occurs within the T cell pathway. While the TCR-γδ pathway results in a rapid end-maturation, multiple developmental options remain available for the αβ-lineage cells. Thus, in parallel with the many lineage choices that are available to hematopoietic stem cells (HSCs) in the bone marrow, T cell precursors have multiple lineage potentials within the thymus (see Box 1). In contrast with the microenvironment of the bone marrow, the thymus is unable to maintain and expand HSCs and, therefore, precursors with T-lineage potential must migrate from the bone marrow into the thymus throughout life and do so via the blood. While several extrathymic progenitor populations with T cell potential have been characterized, it is clear that the most immature thymocytes still have the potential to differentiate into other hematopoietic lineages. T cell commitment is only complete after entry into the thymus and is the result of the unique combination of environmental signals that is provided by the thymic microenvironment. This chapter will focus on the developmental and molecular changes that occur during early T cell differentiation and commitment in both mouse and man and aims at clarifying the complexity of the molecular network that controls these processes in light of the many developmental options that are available to early T cell precursors.

Box 1. Similarities between Bone Marrow Hematopoiesis and Thymopoiesis

During adult life, hematopoietic stem cells reside in the bone marrow where they can multiply and differentiate into different hematopoietic lineages. One of the most recent models in bone marrow hematopoiesis is depicted in Figure 1. For comparison with other models, see chapters 1, 2 & 10. Long-term reconstituting HSCs (LT-HSCs) give rise to short-term reconstituting HSCs (SC-HSCs) that have lost self-renewing capacity but that still have the potential to differentiate into all hematopoietic lineages. It is currently thought that the erythroid and megakaryocytic lineages segregate first from the other blood cell lineages, resulting in the lymphoid-primed multipotent progenitor (LMPP) that generates lymphoid, monocytic and granulocytic lineages, as discussed in other chapters (see chapters 1, 2, 10 and 11). During thymopoiesis, thymus seeding precursor cells, most likely the (LMPP) from the bone marrow as discussed further in this chapter, first commit to the T-cell lineage by losing alternative lineage potentials (not depicted, see further in this chapter). Shortly after this commitment step, the first of the T-cell lineage choices occurs : the TCR-αβ versus TCR-γδ lineage choice (reviewed in [1]). While the TCR-γδ developmental choice results in a rapid end-maturation, αβ-lineage cells extensively proliferate and progress towards an intermediate $CD4^{+}CD8^{+}$ double positive (DP) stage from which cytotoxic $CD8\alpha\beta^{+}$ T cells, $CD4^{+}$ T helper cells, regulatory T cells, natural killer (NK) T cells and CD8αα 'innate-like' T cells develop.

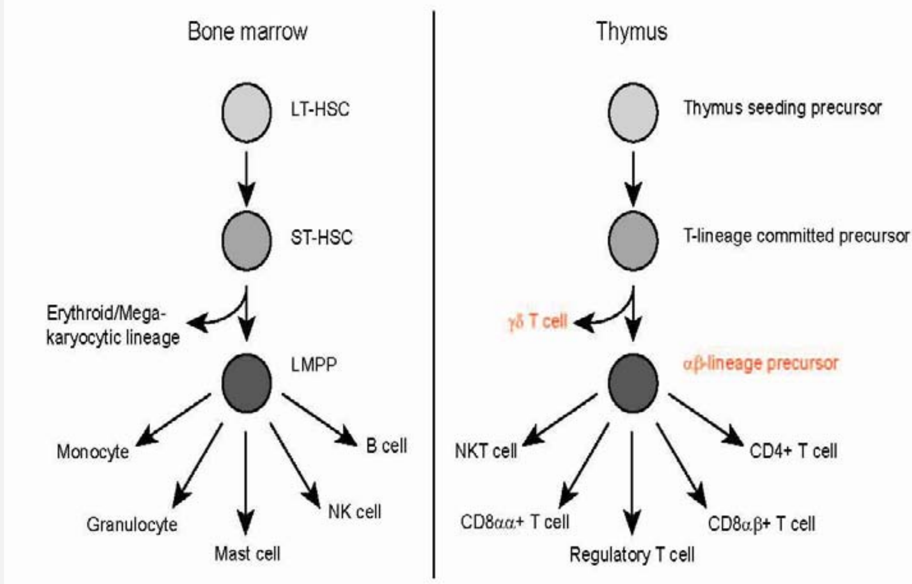


Figure 1. One of the most recent models in bone marrow matopoiesis.

Thus, the segregation of T cell development and bone marrow hematopoiesis may have provided T cell precursors with alternative and novel developmental options and as such, additional complexity to the T cell differentiation program. For the simplicity of this comparison, we have omitted the alternative, non T-lineage potential of the earliest intrathymic T cell precursors. This critical aspect with respect to the induction of T cell commitment is discussed further in detail.

Early stages of T Cell Development

T cell development is a complex, multi-stage process in which several intermediate stages of development have been characterized through several critical surface markers. In both human and mouse, the majority of thymocytes belongs to the αβ-lineage and are double positive for the co-receptors CD4 and CD8 while expressing low levels of CD3 and TCR-αβ. They form an intermediate stage during development as these are the cells that await positive and negative selection. These processes ensure that both useless and auto-reactive T cells are eliminated and prevented from further differentiation into fully mature and functional T cells. Prior to this DP stage, newly arrived thymic immigrants are gradually transformed as they enter the T-lineage developmental program. This is necessary as thymopoiesis is dependent on the continuous influx of bone marrow derived precursor cells that still possess multiple lineage potentials. The transition from uncommitted precursor cell into an irreversibly committed T cell precursor can be monitored as the discrete developmental stages are characterized by the expression of several different surface markers. As shown in detail in Box 2, these phenotypes differ between mouse and human. One major difference between both species, with respect to developmental progression as defined by the TCR protein expression, is the earlier expression of the CD4 and CD8α co-receptors during human T cell development (Box 2). This has made it difficult to compare these early developmental stages between mouse and human. However, due to some recent novel insights into early human T cell development, it is now more feasible to compare similar developmental stages between mouse and human, if we take a new approach with respect to the conventionally used human markers of early T cell differentiation and use a more general nomenclature for early T cell precursors [2;3] (details in Box 2).

Box 2. Early Stages of T Cell Development

Figure 2 presents a detailed comparison of the early stages of mouse and human intrathymic T cell development. The most immature mouse thymocytes are generally further subdivided using c-Kit, CD44, CD25 and CD27 as additional markers. Within the heterogeneous $CD44^{+}CD25^{-}$ double negative (DN)1 subset [4], it is generally accepted that the $c\text{-}Kit^{+}$ early thymocyte progenitor (ETP) population contains the 'canonical' T cell precursor [5-7] that still has multi-lineage potential. Under the influence of the thymic microenvironment, T cell precursors up-regulate CD25 as the cells become T-lineage specified at the DN2 stage of T cell development, which still represents a heterogeneous

population [8-10]. Commitment is complete at the DN3a stage, which is characterized by down-regulation of CD44 and low levels of CD27 are expressed on these cells [11]. Up-regulation of this latter marker indentifies both β and γδ-selected thymocytes [11], while CD28 has also been used to identify TCR-β^+ DN3 thymocytes [12]. Further differentiation along the TCR-αβ lineage results in rapid down-regulation of CD25 (DN4 stage), up-regulation of CD8α (for most mouse strains, ISP stage) and then CD4, resulting in DP thymocytes. Thus, the co-receptors CD4 and CD8 are only expressed after passage through the β-selection checkpoint (transition from DN3a to DN3b) during mouse T cell development. γδ-selected thymocytes mainly remain DN and may also pass through the DN4 stage as they will also down regulate CD25 expression during the end maturation into $CD3^+$TCR-$\gamma\delta^+$ T cells [11;13].

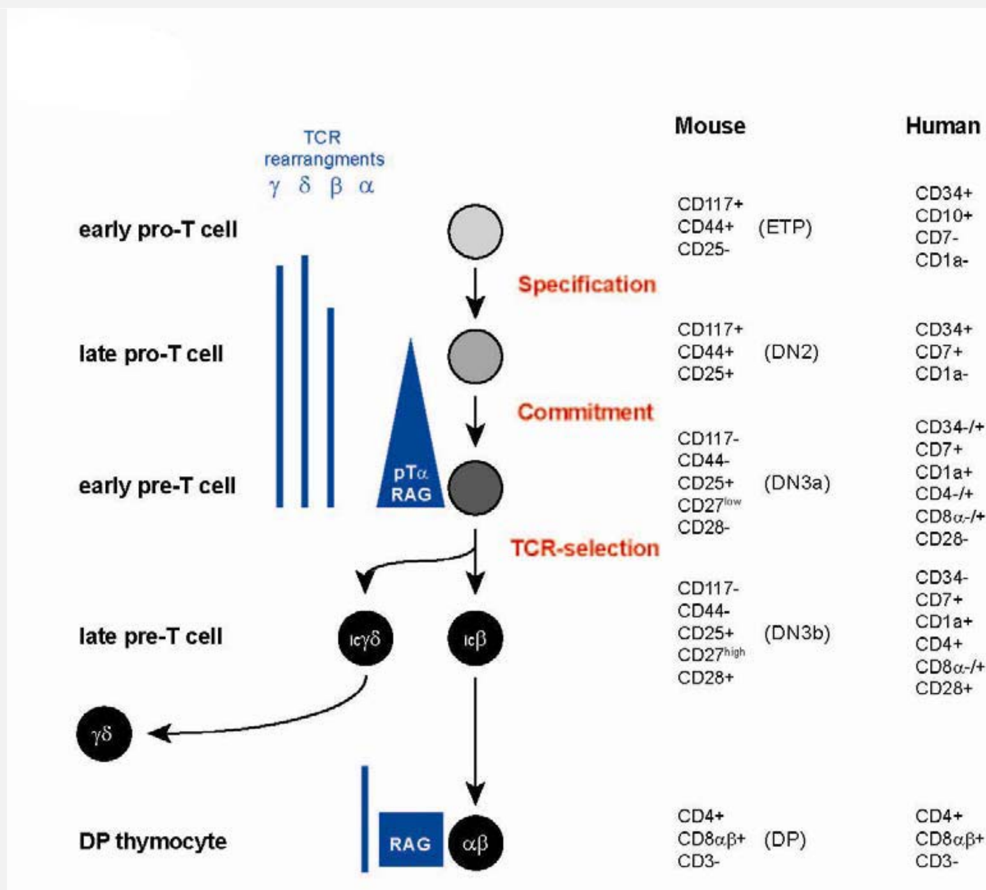


Figure 2. Comparison of the early stages of mouse and human intrathymic T cell development.

In humans, recent data has provided strong evidence that the earliest thymocyte subset is characterized as $CD34^+CD10^+CD1a^-CD7^-$ [14;15] (also Taghon et al, unpublished data). Specification towards the T-cell lineage occurs as CD7 expression is up-regulated [14] (also Taghon et al, unpublished data). Previously, CD38 has been used as a marker to identify the most immature thymocyte subset, defined as $CD34^+CD38^-$ [16], similar to the most primitive

human hematopoietic progenitors in the bone marrow [17]. However, in contrast to CD7 [14] (also Taghon et al, unpublished data), there is no evidence that differential CD38 expression correlates with subsets that posses distinct developmental potentials, although $CD34^+CD38^-$ thymocytes seem more primitive based on quantitative TCR rearrangement analysis [18]. Commitment to the T cell pathway is virtually complete when the immature T cell marker CD1a is expressed [19]. The further subdivision of human postnatal thymocytes has been difficult as the TCR-dependent checkpoint has not been well defined. As a result, various subpopulations have been characterized, including the $CD34^+CD1^+$, $CD34^{+/-}CD4^+CD8\alpha^-CD8\beta^-CD3^-$ and $CD4^+CD8\alpha^+CD8\beta^-CD3^-$ subsets [16;19] that fail to segregate distinct populations based on TCR protein expression. Although they represent a certain degree of developmental progression as shown by the progressive increase in the frequency of intracellular TCR-β^+ thymocytes [20], these populations remain heterogeneous with respect to the status of successful in frame TCR-rearrangements, a critical denominator to characterize the developmental progression of a differentiating T cell precursor. Recently, we have shown that, as in the mouse, CD28 can be used as an additional marker in these stages to identify thymocytes that express a TCR-β chain intracellularly [21], as earlier suggested [19]. Although not yet formally proven, CD28 presumably also marks γδ-selected human thymocytes at these stages. Thus, CD28 now allows us to separate human thymocytes, post T-cell lineage commitment and prior to the $CD4^+CD8\alpha\beta^+$ DP stage, in correlation with their TCR-dependent developmental progression. As a result, the $CD28^-$ fraction includes the intracellular TCR-β^- fraction of $CD34^+CD1^+$, $CD34^+CD4^+CD8\alpha^-CD8\beta^-CD3^-$, $CD34^-CD4^+CD8\alpha^-CD8\beta^-CD3^-$ and $CD34^-CD4^+CD8\alpha^+CD8\beta^-CD3^-$ thymocytes, while the $CD28^+$ subsets contains the TCR-β^+ fraction of these subsets that will then differentiate into $CD4^+CD8\alpha^+CD8\beta^+CD3^-$ DP cells.

To overcome the different nomenclature and surface markers that are currently used to define mouse and human stages of T cell development, an older nomenclature is reintroduced to define early thymocyte subsets [2;3]. In sequential order of developmental progression, these are early pro-T cells, late pro-T cells, early pre-T cells and late pre-T cells, as indicated in Figure 2. TCR rearrangements, mediated by the recombinase-activating-gene (RAG) proteins are initiated as the cells become T-lineage specified during the transition from early to late pro-T cell. There is evidence from a detailed and quantitative study in human that there is a certain sequence in the order of induction of rearrangements at the specific loci with TCR-δ rearrangements being earlier than TCR-γ rearrangements, and these in turn being earlier than recombination events at the TCR-β locus [18]. The events that mediate and determine the transition from one developmental stage to the next are discussed in the main text.

The most immature early pro-T cells, that still posses the potential to develop into alternative hematopoietic lineages, differentiate into the T-cell lineage under the influence of the thymic micro-environment. During the initial specification process, the precursor cells undergo a first wave of T-cell specific changes without fully committing to the T-cell lineage. During this transition, TCR rearrangements at the TCR-γ, TCR-δ and TCR-β loci are initiated in the late pro-T cells and the frequency of these processes increases as the cells differentiate further into early pre-T cells. While T-lineage commitment at this stage is complete, the

further differentiation into αβ- or γδ-lineage cells is mainly, but not solely (reviewed in [1]), dependent on the outcome of the TCR-rearrangements, as only thymocytes that were able to successfully rearrange their TCR genes will be selected for further differentiation. In frame rearrangement of both the TCR-γ and TCR-δ genes will result in the formation of a TCR-γδ receptor that mediates selection of these thymocytes, possibly through the receptor interacting with a ligand [22]. In case TCR-β chain rearrangements are successful, thymocytes will progress through the so-called β-selection checkpoint, thereby extensively proliferating and rapidly differentiating into DP thymocytes [11;23]. This process is dependent on the expression of a surrogate TCR-α chain, called pre-TCRα (pTα), which partners with the newly generated TCR-β chain to form the preTCR complex [24;25]. The formation of this preTCR will initiate the β-selection process, independent of ligand-interaction, and will induce differentiation as well as extensive proliferation. Subsequently, rearrangements at the TCR-α locus are induced to generate a TCR-α chain that will replace the surrogate pTα protein. Since the outcome of the TCR-α re-arrangements will be different for each daughter cell that is derived from the same β-selected cell, a high degree of diversity is generated as cells with a identical TCR-β chain will receive a different TCR-α chain, thereby generating a unique TCR-αβ heterodimer. These TCR-$\alpha\beta^{low}$CD3^{low} DP thymocytes await a second round of TCR-dependent selection, which depends on their ability to interact with self-MHC molecules (positive selection) but not with any other self-peptides (negative selection) [26]. Thus, early T cell precursors can be phenotypically separated into subsets that represent cells with different lineage potentials and different TCR rearrangement status.

T Cell Precursors in the Mouse

The most immature thymocyte subset has multiple lineage potential and the thymus needs to be continuously replenished by these immigrating progenitor cells that are derived from the bone marrow. As a result, a lot of effort has been invested to identify the elusive T-cell progenitor that migrates from the bone marrow, through the blood, into the thymus (for detailed review, see [27]). This precursor must possess several characteristics to qualify as the thymus seeding progenitor. Obviously, it should posses T-lineage potential. In the mouse, several types of precursor cells have been identified in the both the fetal liver and the adult bone marrow that generate T cells *in vivo* and *in vitro*. Although T cells are ultimately derived from HSCs [28], defined as CD135$^-$ LSK cells [29], several downstream progenitor populations have been described that also posses T-lineage potential. Details for adult bone marrow precursor cells, which have been most extensively studied, are provided in box 3. Since each of these subsets express different marker genes, identified through either transgenesis or through direct surface marker staining, it has been difficult to establish the developmental relationship between these precursor subsets. This is of critical importance to identify a possible homogeneous population of thymus-seeding progenitor cells, as it seems very likely that these subsets at least partially overlap with each other. The matter becomes even more complex when addressing fetal and adult T-lineage progenitor populations as it is obvious that prethymic precursors in the fetus seem to have different properties compared to those in adult life. Since it is clear that a variety of progenitor populations have T-lineage

potential, the identification of the precursor cells that seeds the thymus cannot be established using this sole criterion.

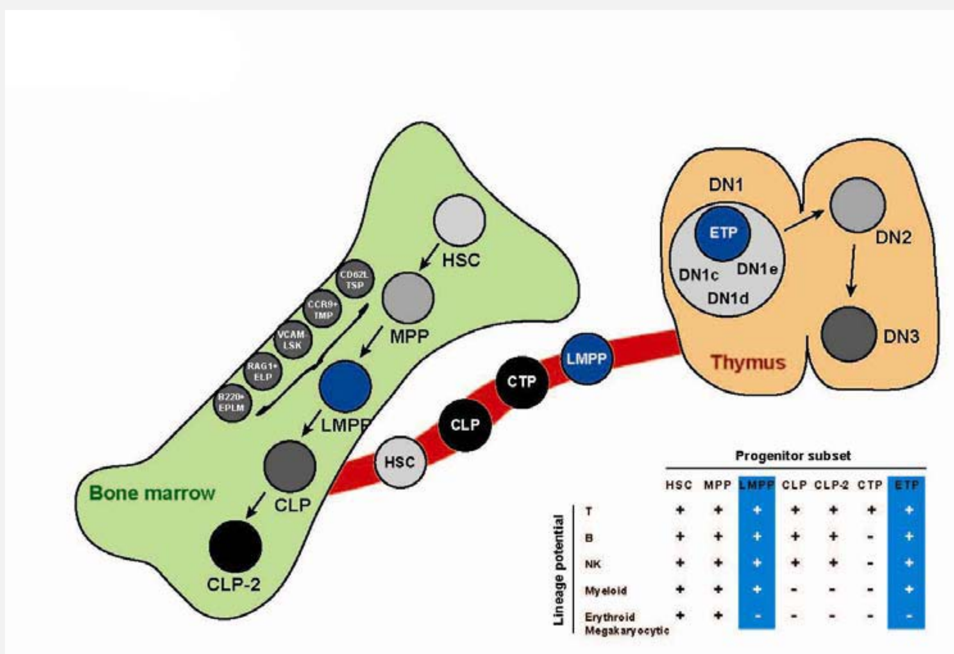
Thymus-seeding precursor cells must also have the ability to home towards the thymus. There is evidence that entry of precursor cells into the thymus is a well orchestrated phenomenon (reviewed in [30]). Thymus colonization seems to occur in waves as the entry is regulated by the opening of specific thymus 'gates' and this process may attract specific populations of precursor cells. This suggests the involvement of chemokines and adhesion molecules that regulate the specificity of thymic entry. One of these involved is the chemokine receptor CCR9. While its expression has been used as a marker to identify precursor cells with T-lineage potential, CCR9 is also functionally important for efficient thymic reconstitution by extra-thymic precursors [31]. Besides CCR9, CCR7 [32], P-selectin [33] and CD44 [34][33] have also been implicated in the homing mechanism of T cell precursors towards and into the thymus. The process of homing also implies that the thymus-seeding progenitors are detectable in the blood as this is the passageway for precursor cells from the bone marrow towards the thymus. While most bone marrow precursor populations with T-lineage potential have been detected in the peripheral blood (for details, see box 3), it is unlikely that HSCs effectively immigrate into the thymus since no HSC-activity can be detected among the most immature thymocyte subsets [35;36] and HSCs also fail to express the chemokine receptor CCR9, which is critical for efficient thymic reconstitution [31]. Thus, based on the criteria of T-lineage potential and homing capabilities, the LMPP and CLP subsets remain candidates for being the thymus-seeding progenitors.

Assuming that no drastic changes occur upon thymic entry, the progenitor that seeds the thymus must also be detectable in a very similar form within the thymus itself, and must have similar expression profiles as well as developmental potentials. Within the most immature $CD44^{+}CD25^{-}$ DN1 subset, the c-Kit^{high} subset, called ETP, is considered to contain the canonical T cell precursors as they have the most robust T cell potential and are capable of homing to the thymus when intravenously injected [2;4-7]. Mouse ETPs contain robust NK and dendritic cell potential. Whether or not they have any B-lineage potential has been a controversial issue. ETPs have been subdivided into 2 separate populations based on Flt3 expression and it was shown that the $Flt3^{+}$ subset still contains B cell potential, in contrast to the $Flt3^{-}$ subset [5;37]. While some reports confirm the B-lineage potential of ETPs [38;39], others have failed to do so [40-42]. These differences may relate to the level of c-Kit that is included in the sorting gates [4], but they may also be the result of age differences [43]. In each case, it is clear that very few ETPs have B-lineage potential and that this capacity is rapidly lost within the thymic immigrating precursors, presumably as a result of Notch signaling as discussed later in this chapter. In contrast, ETPs seem to have robust myeloid potential. Several studies had reported myeloid potential from bulk cultures [5;40;44] and recently, two independent groups showed, at a clonal level, that bipotent ETPs, containing T and myeloid potential, are clearly present in the adult mouse thymus, while bipotent B- and T-lineage precursors are not [41;42]. Thus, the most immature thymocyte subset clearly possesses more robust myeloid potential as compared to B-lineage potential and seemingly very little if any erythroid potential. Thus, based on all the above described evidence, a LMPP-like cell type seems the most likely candidate for receiving the title of 'thymus-seeding progenitor', since the CLP and CLP-2 subsets lack myeloid potential. However, this

does not necessarily imply that these are the only precursor cells that effectively enter the thymus [45].

This characterization of the canonical T-lineage precursor cell in the thymus also has important consequences with respect to the different models of hematopoiesis that are currently in use. The myeloid potential of ETPs strongly disfavors the classical dichotonomous common lymphoid progenitor (CLP)/common myeloid progenitor (CMP) model of hematopoiesis, since this model implies that T-lineage cells are derived from a lymphoid-restricted precursor. The effective myeloid potential of ETPs clearly does not support a segregated lymphoid- versus myeloid-lineage model, but rather fits into the hematopoietic scheme that proposes the initial segregation of erythroid and megakaryocytic lineages versus lymphoid and myeloid lineages (see chapters 2 and 10). Thus, early T cell precursors have been critical in obtaining more general insights into the hematopoietic system.

Box 3. T-Lineage Precursors Cells in the Bone Marrow, Blood and Thymus of the Adult Mouse



	HSC	MPP	LMPP	CLP	CLP-2	CTP	ETP
T	+	+	+	+	+	+	+
B	+	+	+	+	+	-	+
NK	+	+	+	+	+	-	+
Myeloid	+	+	+	-	-	-	+
Erythroid Megakaryocytic	+	+	-	-	-	-	-

Figure 3. Cells in the Bone Marrow, Blood and Thymus of Adult Mouse

In the adult mouse, several progenitors have been identified that contain T-lineage potential. In the bone marrow, there is the obvious HSC population, characterized as Lin$^-$ Sca1$^+$c-Kit$^+$(LSK)CD135$^-$, but several other, more differentiated downstream precursors have been characterized. These include the heterogeneous non-self renewing multipotent MPP (CD135$^+$ LSK) population; the downstream CD135^{high} LMPP [46] cells that are lymphoid

primed, still contain myeloid but lack robust erythroid/megakaryocytic potential and presumably bundle the individually identified RAG1$^+$ ELP [47], VCAM1$^-$CD135^{high} LSK [48], CD62L$^+$ TSP [49], CCR9$^+$ TMPs [38] and B220$^+$ EPLM [50] subsets that have similar properties; and the lymphoid-restricted CLP [51] and downstream CLP-2 populations [52].

Of the above precursor subsets, HSCs, MPPs, LMPPs, ELPs, TSPs and CLPs have been shown to circulate in the blood [53-55]. Intriguingly, the adult blood also seems to contain a T-lineage committed precursor (CTP) [56], similar to the T-cell specified precursor that was described earlier in the fetal blood [57;58]. Although the origin of this progenitor is still unclear, it is unlikely that it represents recirculating thymocytes that became T-lineage committed in the thymic microenvironment as athymic nude mice also contain this precursor [56].

In the adult mouse thymus, the most immature CD44$^+$CD25$^-$ DN1 subset is composed of several different subsets as defined by Petrie and colleagues [4]. The DN1a+DN1b subsets as defined in this paper [4], correspond with the ETPs as defined by the Bhandoola group [5], and are now generally accepted to be the mainstream early T cell precursors within the thymus [2;7], as recently also elegantly shown through lineage-tracing in a physiological experimental system [6]. ETPs will differentiate into DN2 and subsequently DN3a cells as they develop along the T-lineage pathway.

The table provides a schematic overview of the lineage potential of the different progenitor subsets that contain T-cell potential. The relevance of this aspect with respect to the identification of the potential thymus-seeding progenitor is discussed in the text.

Human T Cell Progenitors

In case of human T cell development, very little is known about the nature of the migrating precursor cells. As in the mouse, several types of precursor cells have been identified that contain T-lineage potential. Although a CD34$^-$ HSC population has been suggested [59], human HSCs are considered to be contained in the CD34$^+$CD38$^-$Lin$^-$ fraction [17], but seem to express lower levels of c-Kit [60] and higher levels of Flt3 [61] in comparison to mouse HSCs. Studies that report T-lineage potential of human precursors are limited, especially with respect to adult bone marrow derived precursors. This presumably reflects the limited availability of young bone marrow samples and the observation that T-lineage potential declines with increased age [62], but probably also the lack of T-cell differentiation models that are easy to handle *in vitro* and *in vivo*. Since the current models mainly depend on a murine microenvironment, the species difference might also account for a suboptimal setting to study human T cell development. Since new *in vitro* [63] and *in vivo* [64] models have been developed in recent years that seems more accessible and robust compared to previous models, one can expect significant advances within the next years, though some issues remain difficult to solve. Nevertheless, T cells have been shown to differentiate from the CD34$^+$Lin$^-$ bone marrow cells in various *in vitro* models, but mainly when cells were derived from children or young adults [62;65-67]. As in the mouse, a more lymphoid-restricted population has been identified in human bone marrow. This subset was characterized as CD34$^+$CD45RA$^+$CD10$^+$Lin$^-$ and was shown to contain T-lineage potential

when injected intrathymically in severe combined immunodeficient (SCID)-hu mice [68]. Recently, this population was further subdivided on the basis of CD24 expression and the multi-lymphoid potential was shown to reside in the $CD34^+CD10^+CD24^-Lin^-$ subset [69]. This population is also present in peripheral blood [69] and resembles the most immature $CD34^+CD7^-$ subset in the thymus of which the majority expresses CD10 [14;15]. Whether this is the actual precursor that migrates from the bone marrow to the thymus remains to be proven. Most of these cells lack expression of CCR9, but it is presently unclear if this chemokine receptor is also involved in the homing of human precursor cells towards the thymus. While *in vivo* homing of human precursors into the mouse thymus, followed by in situ T cell development has been achieved [70], this approach remains rather inefficient and thus it is unclear if this or other techniques will be sufficiently robust to study the thymus homing and T cell differentiation potential of human peripheral blood precursors.

Cord blood has been used as an extensive source for human hematopoietic progenitor cells and it has been shown that both the most primitive $CD34^+CD38^-Lin^-$ as well as the more differentiated $CD34^+CD38^+Lin^-$ fractions contain T cell precursors [66]. Within the $CD34^+CD38^-Lin^-$ fraction, Crooks and colleagues identified a $CD7^{low/+}CD45RA^+$ population that is highly enriched for lymphoid precursors [71], including robust T-lineage potential [72;73]. While this phenotype does not seem to correspond with the most immature $CD34^+CD7^-$ postnatal thymocyte population [14;15], there is evidence that the human fetal thymus is colonized by $CD34^+CD7^+$ progenitors derived from the fetal bone marrow, a population that declines after birth [74]. Furthermore, the thymus may be colonized by more than one type of progenitor cell as both $CD34^+CD7^-$ and $CD34^+CD7^{low/+}$ postnatal thymocytes can be detected that contain multi-lineage and lymphoid lineage potential, respectively [14].

Expression of pTα, which is associated with T-lineage differentiation, has been detected in $CD34^+$ cells from mobilized peripheral blood [75] and may be expressed in a subset of human precursor cells that is comparable to the mouse CTP or CLP-2 [52;56]. Also, TCR-β rearrangements have been detected in bone marrow $CD7^+CD10^+$ cells [76], as well as in $CD34^+CD7^+$ CB precursors [77]. Although these reports suggest that initiation of T-lineage differentiation can occur outside the human thymus, there is also evidence to suggest that these events are only initiated after entry of precursor cells into the thymus [14;15;18;78]. Similar as in mouse, the mainstream pathway for initiation of T-lineage differentiation most likely involves an intrathymic process. Besides recirculation of early thymocytes, a temporal induction of RAG expression, as also observed in mouse ELPs [47], may account for these early rearrangement events without being coupled to early T cell differentiation. More work in this field would be desirable to dissect this matter more carefully.

Human early pro-T cells contain multi-lineage potential. While the initial reports concerning myeloid differentiation potential were contradictory [79;80], the NK [81;82] and dendritic cell potential [81;83] of $CD34^+$ early human thymocytes has been well established. However, more recent reports from at least three independent groups have demonstrated that human early pro-T cells also contain myelo-monocytic potential [14;84;85], although it remains unclear if they represent individual precursors that contain both myeloid and lymphoid potential. More strikingly, two of these reports also suggest that erythroid potential is contained within human ETPs [14;85]. This would suggest that the human thymus is

colonized by more primitive precursors compared to the mouse, if the order of hematopoietic lineage segregation in the bone marrow is the same in human as in mouse. Since human thymocytes fail to repopulate non-obese diabetic (NOD)-SCID mice [85;86], it is unlikely that HSCs are present in the human postnatal thymus. Furthermore, it is important to exclude contaminating precursors from the thymic blood vessels, which is not as easy to control as in mouse experiments. Further work involving clonal studies will be necessary to clarify the progenitor-product relationship with respect to erythroid and T-lineage differentiation of human immature thymocytes. This is also the case with respect to B-lineage potential. While both Staal and colleagues [85] and Crooks and colleagues [14] have been able to generate $CD19^{+}$ B cells from $CD34^{+}CD1^{-}$ and $CD34^{+}CD7^{-/low}$ thymocyte progenitors, respectively, it is unclear whether these are generated from the same precursors that will also yield T cells. In each case, it is clear that also the human thymus is colonized by precursor cells that have multiple lineage potentials.

Initiation of T Cell Specification in Mouse and Human

The transition from multipotent progenitor into a T-lineage restricted precursor proceeds through a gradual sequence of events. Early pro-T cells differentiate into late pro-T cells under the influence of the thymic micro-environment and thereby gradually obtain the first characteristics of T-lineage cells. This so-called specification process is initiated by the activity of the Notch signaling pathway, which is triggered when Notch ligands, which are abundantly expressed on thymic epithelial cells, interact with Notch receptors, present on early T cell precursors (see box 4 for a detailed description of the Notch pathway).

Box 4. The Essential Role for Notch Signaling at the Initial Phase of T-Lineage Specification

The Notch pathway is a well conserved signaling pathway that is used in many developmental programs [87]. Within the hematopoietic system, Notch signaling is best known for its role in the B- versus T-cell lineage decision. The Notch pathway consists of 4 different Notch receptors (Notch1-4) that can interact with 5 different ligands, belonging to either the Delta (Delta-like-1, -3 and -4) or the Jagged (Jagged-1 and-2) families [88;89]. Activation of Notch signaling results in cleavage of the heterodimeric Notch receptor and as a result, the intracellular activated form of Notch (intracellular Notch, ICN) is released that migrates to the nucleus. There, it interacts with CSL, a transcription factor that acts as a repressor in the absence of ICN. The formation of the CSL-ICN complex results in the recruitment of other co-activator molecules such as proteins of the Mastermind family and this transforms CSL into a transcriptional activator, resulting in the expression of Notch target genes which include HES1 [90;91], Nrarp [92-94], Deltex1 [95;96], pTa [97] and cmyc [98;99].

Initiation of T cell development in hematopoietic precursors requires the interaction of the Notch1 receptor with Delta-like-1 or with Delta-like-4, with the latter being the most likely ligand that interacts in vivo [100]. While initiating T-lineage specification, Notch at the same time represses differentiation into alternative lineages, with the B-cell lineage being the most sensitive with respect to Notch mediated inhibition [101;102]. Since thymic epithelial cells express a high amount of Notch ligands, B-lineage potential in the early-pro T cells is presumably rapidly lost as a result of active Notch signaling. Notch1 is the critical receptor for the induction of T cell development since Notch1 deficient precursor cells fail to differentiate into the T-cell lineage and ectopically generate B cells in the thymus instead [103;104]. The essential role for Notch signaling at the earliest stage of T cell development has also been illustrated in experiments with human hematopoietic progenitor cells. Overexpression of the active form of Notch1 in such precursors cells imposes T-lineage differentiation *in vivo* in the bone marrow of mice that received the human manipulated precursor cells after intravenous transfer [105]. Moreover, inhibition of Notch signaling in $CD34^+$ CB progenitors, through pharmacological inhibition with γ-secretase inhibitors that prevent cleavage of the Notch receptor and thus the release of the activated form of Notch, results in B cell development instead of T cell differentiation in a fetal thymus organ culture, an *in vitro* model in which human precursors can differentiate into T-lineage cells in a murine fetal thymus lobe [101]. A similar result was obtained when Notch signaling was inhibited through overexpression of a dominant-negative mutant of Mastermind, which inhibits the transactivation potential of the ICN-CSL complex [21].

The essential role for Notch signaling during early T cell development, and especially its ability to induce T-lineage differentiation while repressing alternative cell fates, has led to the establishment of a novel *in vitro* co-culture system to assay the early stages of T cell differentiation, the OP9-DL1 coculture system [63;106]. This technique, which was elegantly developed in the laboratory of Zuniga-Pflucker and colleagues, is based on the use of the OP9 bone marrow stromal cell, derived from the op/op mouse, which is deficient in the production of M-SCF and as a result efficiently supports B cell development from mouse hematopoietic precursor cells. The introduction of the Notch ligand Delta-Like-1 resulted in the inhibition of B cell differentiation as a result of Notch activation. Surprisingly, this was also sufficient to induce and support T cell differentiation, which was until then only considered possible within the 3-dimensional structure of the thymus as 2-dimensional cultures with thymic epithelial cells failed to do so, a result of rapid in vitro loss of Delta-like ligands [107]. While the system may not be optimal for efficiently generating fully functional and conventional T cells, the simplicity of the system compared to previous *in vivo* and *in vitro* models has opened up many new avenues of research in the field of T cell biology. The system has been shown to support early T cell development from both mouse [106] and human [65;108] hematopoietic progenitor cells, and strikingly, also from mouse [109] and human [110] totipotent embryonic stem cells.

In the mouse, Notch signaling results in the rapid induction of CD25 expression when T cell precursors are cultured in vitro in the OP9-DL1 coculture system [106] (see box 4 for detailed description), thereby generating $CD44^+CD25^+$ DN2 thymocytes. *In vivo*, Notch activity already occurs in the early pro-T stage itself, thereby inducing the rapid loss of B-

lineage potential, and Notch in fact is required for the generation of ETPs [37]. During human T cell development, data from Crooks and colleagues has recently suggested that the T-cell specification process occurs at the CD34^{+}CD7$^{-/low}$ to CD34^{+}CD7^{high} transition [14], since the latter cells only contain robust NK- and T-lineage potential, largely comparable with mouse DN2 thymocytes. Such a CD7^{+}cyCD3^{+} T/NK restricted population is also generated when human CD34^{+}CD38^{-} fetal liver precursors are cultured in FTOC [111]. Consistent with the specification process, Notch signaling is more active in CD34^{+}CD7^{high} thymocytes than in CD34^{+}CD7$^{-/low}$ cells and also the expression of the T lineage specific transcription factors GATA-3 and TCF-1 is upregulated in CD34^{+}CD7^{high} T cell precursors (Taghon et al, unpublished data). Furthermore, CD7 is most likely the earliest T-lineage specific marker that human cord blood derived CD34^{+}CD38^{-} precursor cells acquire when cultured on OP9-DL1 stromal cells, and this is a Notch dependent process [108;112-114].

Specification results in the initiation of TCR rearrangements which depend on the activity of enzymes encoded by RAG-1 and RAG-2, the expression of which now becomes detectable. Also the expression of pTα and components of the CD3 TCR coreceptor complex is turned on (Figure 2), as well as that of the signaling molecules Lck, ZAP70 and LAT that are critical for transmitting TCR activation signals [9;10]. Thus, as soon as functional, in-frame TCR rearrangements are generated, a full TCR complex can be generated, capable of signaling which is required to induce further T cell maturation. While in theory possible at the late pro-T cell stage, this almost exclusively occurs after T cell commitment at the early pre-T cell stage [11;115]. During these early specification events, thymocytes are strongly proliferating as a result of SCF and IL-7 signaling, growth factors that are provided by the thymic microenvironment. Yet, despite this initiation of T cell development, late pro-T cells still maintain the option to divert into other hematopoietic lineages, although this may also reflect heterogeneity of the DN2 population that could be composed of a non-specified multi-lineage potent early DN2 subset on the one hand and a more restricted T-lineage specified population on the other hand [8-10].

Transcriptional Regulators that Mediate Early T Cell Specification

As a result of their multi-lineage potential, early pro-T cells initially express a broad range of transcriptional regulators that reflect this capacity and developmental plasticity, a property shared with HSCs and LMPPs [10;116]. Some of these factors are turned off during the initial stages of T cell development as they have no further roles during T-lineage differentiation. Examples include the GATA family member GATA-2 [117], the CCAAT-enhancer binding protein α (C/EBPα) [118] and the stem-cell leukemia protein SCL [119]. This downregulation is critical as they may induce differentiation towards other hematopoietic lineages [120] or even be antagonistic when they continue to be expressed at later stages of T cell differentiation, as observed for Ets family factor PU.1 [121;122]. Other factors that are expressed in these early pro-T cells have critical recurrent roles at later stages of T cell development and as such, these factors continue to be expressed at later stages of the T-lineage differentiation program. These include the runt-related transcription factor 1 (Runx1) that forms a complex with the core binding factor β (CBFβ) [123-128], Ikaros and

its family members Helios and Aiolos [10;11;129-132], the Avian myeloblastosis viral (v-myb) oncogene homolog c-Myb [133-135], the Growth factor independent transcription repressor Gfi-1 [136-139] and the Notch signaling pathway [88;103;104;109;131]. These factors are part of a core group of transcriptional regulators that control the T-lineage developmental program. Yet, they are not sufficient by themselves.

During the specification process, a novel set of transcriptional regulators becomes active that is specifically required for T cell development, in addition to those expressed at the early pro-T cell stage. These include HEBalt, the alternative splice variant of the E protein family member HEB [140], the Zinc-finger containing protein Bcl11b [10;116;141] and, temporarily, the Hedgehog signaling activated Gli-2 protein [142]. At the same time, expression of the high mobility group factor TCF1 [10;143-145] and its relative, the lymphoid-enhancer-binding factor LEF1 [10;146] is up-regulated, but these factors only reach their maximum expression levels at the subsequent early pre-T cell stage [10;147]. A similar sequence of events occurs for the Ets family proteins Ets1 [148] and Ets2 [10]. Finally, in contrast to the downregulation of its family member GATA-2, GATA-3 is also up-regulated at the late pro-T cell stage. GATA-3 is already expressed at a reasonably detectable level in the early T cell precursors, consistent with its very early requirement during T cell development [149;150], and perhaps even earlier at the stem cell stage [151;152], but its role with respect to the T cell differentiation program is presumably an event that occurs following Notch activation [131;153]. Thus, it is evident that at the early and late pro-T cell stages, multiple transcriptional regulators are expressed that each have critical roles in the early T cell differentiation process. One of the earliest events involves the activation of the Notch signaling pathway, a process that has been extensively studied.

Role of Notch Signaling during Early T Cell Differentiation

The induction of Notch signaling is presumably the first of the T-lineage specific events that occurs after entry of the thymus seeding precursors into the thymic microenvironment. However, while essential and required at multiple stages of T cell development [88;130], Notch signaling by itself is not sufficient for the specification and commitment of hematopoietic precursor cells into the T-cell lineage [131], nor to activate the expression of other T-lineage specific genes, in both mouse [131] and human [154] (I. VandeWalle and T. Taghon, unpublished results) extrathymic hematopoietic precursor cells. While the thymic microenvironment also provides the critical growth factors SCF and IL-7, as well as essential Wnt proteins [155], among others, early T cell development also depends on the activity of many intrinsic and stem cell ‘inherited’ transcriptional regulators as discussed above. Thus, in contrast to other hematopoietic lineages in which one factor can be dominant for the induction of a particular lineage choice (for example PAX5 [156;157] or EBF [158] for the induction of B-lineage differentiation or GATA-1 that induced erythroid-megakaryocytic differentiation [159;160]), T cell differentiation depends on the precise integration of multiple transcriptional regulators who’s expression levels are critical for proper specification and commitment into the T-cell lineage.

With respect to the role of Notch signaling itself, it has been particularly difficult to place the positive regulatory effects in the context of factors that negatively regulate the Notch pathway, particularly since some of these are induced by Notch activation itself. A first hurdle that needs to be taken involves counteracting the activity of the Leukemia/Lymphoma Related Factor (LRF). In the bone marrow, LRF is required for B cell development as it represses Notch signaling. As a result, in the absence of this factor, the hematopoietic precursor cells will abnormally initiate T cell differentiation in the bone marrow [161]. Thus, Notch activation in the thymus needs to reach a certain threshold in order to be able to neutralize LRF activity. This presumably depends on Delta-like ligands as these are less abundant in the bone marrow compared to in the thymus [89]. In the bone marrow, Jagged proteins are probably sufficient to activate Notch signaling and to induce T cell differentiation in the absence of LRF. Since the expression of the Zbtb7a gene (encoding for LRF) remains unchanged in the DN1 to DN3 stages of mouse T cell differentiation (Ellen V Rothenberg, personal communication) and is virtually unaffected in human hematopoietic precursor cells as they enter the thymus or when exposed to Delta-like ligands in OP9 co-culture (Taghon et al, unpublished results), LRF activity most likely is silenced through protein-protein interactions. The precise mechanism through which LRF controls the B versus T cell lineage decision remains to be elucidated but will probably also reveal its interaction partners.

Notch activation also induces the expression of two other genes, the products of which seem to have a negative impact on the activation status of the Notch pathway, namely Deltex1 and Nrarp. While Deltex proteins in general do not seem to have a critical role during T cell development [162], Deltex1 expression, which *in vivo* in the adult mouse increases from the ETP stage until the DN3a stage but declines thereafter [147], needs to be precisely timed as early activation represses T cell differentiation and results in intrathymic B cell development instead [163], mimicking a Notch deficiency. Similarly, the Notch regulated ankyrin-repeat protein Nrarp is induced upon Notch activation, but also seems part of a negative feedback loop [164]. Intriguingly, Nrarp expression is also influenced by the activity of the Msx2-interacting protein (MINT), which also negatively impacts Notch activity through interaction with CSL. Mice deficient for MINT display a defect in T-lineage specification, which is surprising as Notch activity should be increased in these animals [165]. However, the inhibition may result from the increase in Nrarp expression that is observed in MINT deficient cells [165]. Thus, it is obvious that the Notch signaling pathway is subject to many different levels of regulation that control the early T cell specification stages.

After Notch induced specification, Notch remains critical during mouse T cell differentiation to maintain T-lineage fidelity until commitment is complete. In the absence of Notch signaling, T-lineage specified mouse thymocytes will adopt a NK-cell fate when IL-15 is available for the cells, although Notch signaling is more permissive for NK cell differentiation than for B cell development [102;166]. In its role to maintain T-cell lineage fidelity, Notch also seems to serve as a gate-keeper to prevent other factors from initiating differentiation along other hematopoietic pathways [122;167]. Consistent with the continuous early T-lineage requirement for Notch, the expression of Notch target genes, which reflects the amount of Notch activity within the cells, increases in mouse thymocytes from the ETP

stage until the DN3a stage [11;147;168]. At this step of differentiation, signaling through the Notch1 receptor is critical for TCR-β chain rearrangements [169] and Notch signaling is also critical to support the metabolic activity of the rapidly dividing β-selected DN3b thymocytes [170-172]. Past the β-selection checkpoint, Notch signaling seems no longer as strictly required for the further differentiation of mouse thymocytes [11;168].

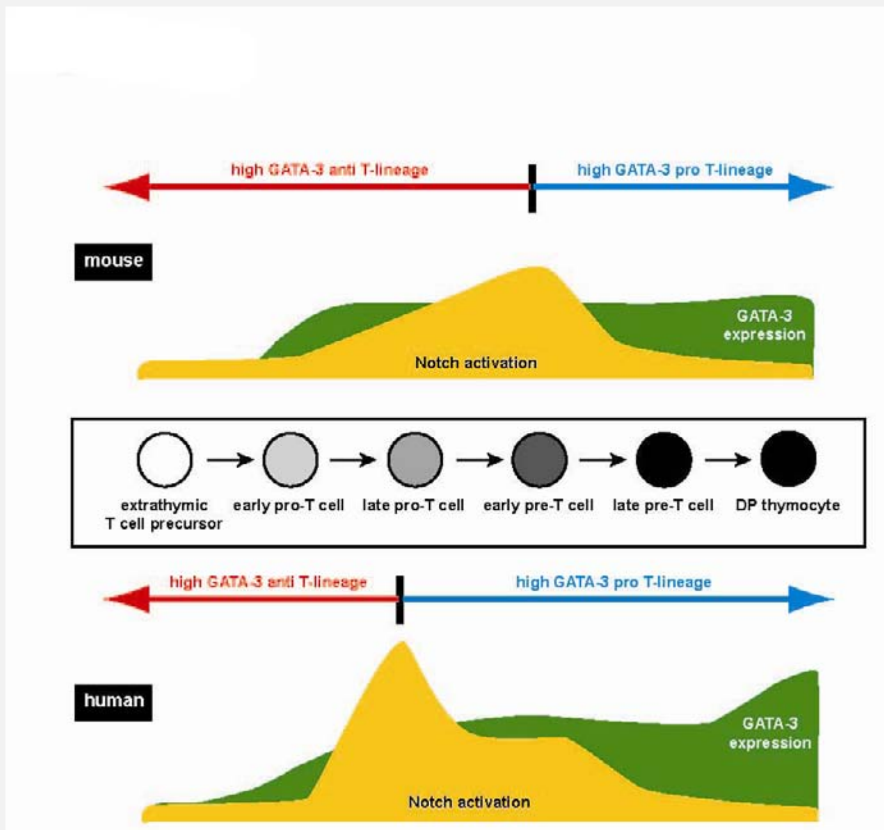
In human, the transition from a Notch-dependent to a Notch-independent state seems to occur at an earlier stage of T cell differentiation. Notch target gene expression already peaks during T-lineage specification in $CD34^{+}CD1^{-}$ thymocytes, and their expression levels are partially downregulated already at the $CD34^{+}CD1^{+}$ stage that represents T-lineage commitment. Consistent with this early activation pattern, Notch is permissive for human NK cell differentiation for which the lineage potential is present in these early T-lineage specified $CD34^{+}CD1^{-}$ thymocytes and Notch triggering during early NK cell development results in the generation of NK cells that contain characteristics of T cells [173]. Even more strikingly, human $CD34^{+}$ T cell precursors will spontaneously differentiate into $CD4^{+}CD8\beta^{+}$ DP thymocytes in the absence of Notch signaling, both in FTOC in the presence of γ-secretase inhibitors [101] or after transduction with a dominant-negative mutant of Mastermind-like-1, as well as on OP9 stromal cells in the absence of Delta-like ligands [21]. Furthermore, while Notch is absolutely required during mouse β-selection, we have recently shown that differentiation through this checkpoint in human can efficiently occur in the absence of active Notch signaling [21]. While these observations may seem in contrast with the insights obtained from mouse experiments, it does fit with the idea that Notch signaling by itself is not sufficient to drive the T-lineage program [131;154]. The fact that Notch signaling requires additional regulatory inputs to support T cell differentiation, or that other pathways become more dominant after the initial induction of T-lineage specification seems more pronounced in human compared to in mouse, as also illustrated by the difference in effect of GATA-3 activity between mouse and human as discussed further. These observations correlate with the earlier decrease in Notch activity during human T cell development in comparison with the mouse and may also be related to the earlier induction of expression of the CD4 and CD8 coreceptors.

Interplay between Notch and GATA during Early T Cell Development

One important aspect that has been difficult to clarify involves the modes of interaction between Notch and the other regulatory inputs that are critical in early T cell development. One of the most intriguing candidates for an additional positive T-lineage transcriptional regulator has been GATA-3. Mice deficient for this Zn-finger protein have a defect in fetal hematopoiesis [151] and lack T cells [150]. The developmental inhibition of T-cell lineage differentiation occurs at the earliest stages and very few thymocytes can be detected in the absence of GATA-3 [149], even in the presence of excessive Notch signaling [153]. Thus, GATA-3 has a crucial role for inducing T cell development. Yet, overexpression experiments in both mouse fetal liver [167;174] and human cord blood (Taghon et al, unpublished data) extrathymic hematopoietic precursor cells fail to induce T cell differentiation in the absence of Notch signaling and results in severe toxicity in its presence. Thus, Notch signaling seems

to have an essential role in preparing the T cell precursors for increased GATA-3 levels [153]. Yet, even after this initial Notch dependent specification, early thymocytes in mice remain highly sensitive to appropriate GATA-3 levels. In fetal thymocytes, increased GATA-3 levels even induce differentiation into a completely different hematopoietic lineage, namely mast cells, when Notch signaling is simultaneously removed [167]. Elevated GATA-3 activity therefore does not seem permissive during these early stages of mouse T cell differentiation (Figure 4). Later during development, GATA-3 has important roles at both TCR-signaling dependent stages of mouse T cell differentiation, namely the β-selection checkpoint [175] and positive selection where it is critical for driving CD4 T cell maturation [175-177]. The transition at β-selection correlates with the transition from Notch dependent to Notch-independent stages of mouse T cell differentiation [11] and only from this stage onward can one observe some clues for a positive regulatory role for GATA-3 in the T cell program, suggesting incompatible roles for Notch and GATA-3 during these early stages of T cell development (Figure 4). This GATA-3 mediated acceleration of T-lineage differentiation becomes even more apparent with the use of the hypo-acetylated GATA-3-KRR mutant that is less cytotoxic compared to the wild-type and acts as a partial GATA-3 agonist [11].

Box 5. Schematic overview of the interplay between Notch and GATA-3



During mouse T cell development, Notch activation is gradually upregulated and peaks at the DN3a early pre-T cell stage, followed by a sharp decline after TCR mediated selection at the late pre-T cell stage [11;168]. GATA-3 expression on the other hand is initially upregulated in DN1 early pro-T cells, increases during T-lineage specification at the late pro-T cell stage, but remains almost unaltered in the following DN and DP stages of mouse T cell differentiation [147;174;176]. Early GATA-3 activation, prior to the DN3 stage, induces inhibition of T cell differentiation and in specific culture conditions also developmental diversion into other hematopoietic lineages [167;178]. Positive effects for elevated GATA-3 expression in the mouse can only be observed from the DN3 stage onwards and are more apparent when a weaker mutant GATA-3 protein is overexpressed as observed for the partial agonist GATA-3-KRRm [167]. During human T cell development, Notch activation reaches its climax at the $CD34^{+}CD7^{+}CD1^{-}$ late pro-T cell stage and initially declines at the T-lineage committed early pre-T cell stage before a sharper decrease occurs at the late pre-T cell stage following TCR mediated selection [21;114]. GATA-3 expression is initially similar in comparison with during early mouse T cell differentiation but further displays a small increase at the later DP stages [114]. Enforced expression of GATA-3 in prethymic CB precursors severely inhibits T cell differentiation (Taghon et al, unpublished results), but positive effects for elevated GATA-3 expression can already be observed when overexpressed in human $CD34^{+}CD1^{-}$ uncommitted T cell precursors [179], thus earlier compared to during mouse T cell development. As such, GATA-3 seems only capable of inducing its positive regulatory effects on T cell differentiation in both mouse and human when Notch activation has declined. Thus, although Notch activation is required for the early upregulation of GATA-3 [153], Notch may prevent premature GATA-3 protein activity. This is critical as elevated GATA-3 expression induces rapid differentiation, at the expense of proper TCR rearrangements [179]. Yet, GATA-3 is critical for inducing T-lineage differentiation and also seems essential as a survival factor during early T cell development [149;153]. Strikingly, inhibition of Notch signaling in human $CD34^{+}CD1^{-}$ late pro-T cells induces a similar effect as GATA-3 overexpression: inhibition of TCR rearrangements and accelerated differentiation towards the DP stage[101]. Thus, while both factors are essential, the activities of Notch and GATA-3 seem to be well-controlled during the early stages of T cell differentiation in order to obtain a perfect balance between the acquisition of essential T cell proteins (such as T cell receptors) and proliferation on the one hand (Notch mediated) and further developmental progression on the other hand (GATA-3 mediated).

Such opposing, but developmentally important sequential roles for Notch and GATA-3 are more apparent during early human T cell development. Positive differentiation effects for GATA-3 can already be observed when overexpressed in uncommitted $CD34^{+}CD1^{-}$ human thymocytes [179] (Figure 4). While not corresponding with mouse observations with respect to stage specificity, there is a clear correlation with respect to Notch signaling activity since, as in the mouse, the GATA-3 induced accelerated differentiation in human also correlates with the developmental stage where Notch activation starts to decline [114], as illustrated in Figure 4. In fact, the enhanced differentiation towards the DP stage of T cell development that is induced by enforced GATA-3 expression [179] strikingly resembles the accelerated and aberrant DP formation that is observed when Notch signaling is inhibited in human

postnatal CD34$^+$ thymocytes [21;101]. These observations suggest that Notch, although positively required to induce GATA-3 gene expression, may also be critical as a negative regulator of GATA-3 protein activity, or vice versa. In each case, GATA-3 seems to have a clearer and more straightforward role in the human T cell specification and commitment process as compared to in mouse. With respect to the other regulatory factors that are involved in early T-lineage specification and commitment, it is presently unclear how these factors collaborate with Notch signaling to promote T cell development. Based on studies that have shown collaborative activities between Notch signaling and the Wnt [168] and Runx1 [180] pathways in hematopoietic stem cells, such studies will be critical to delineate the full molecular network that induces T cell specification and commitment. Based on the current picture from just two of these factors, GATA-3 and Notch, one can predict a huge level of complexity within this process.

Initial intra T-Cell Lineage Decisions: TCR-αβ versus TCR-γδ Differentiation

Following T cell lineage commitment, the first lineage choice that T cell precursors need to take is the decision to develop into αβ- or γδ-lineage T cells. As recently reviewed more extensively [1;181-185], the outcome of the TCR rearrangements at the early pre-T cell stage will mainly determine the developmental outcome, but this is not the only factor that mediates this decision. There are a number of additional transcriptional regulators as well as environmental factors that mediate the further development of these early pre-T cells. Importantly, the developmental outcome of this lineage decision is quite distinct. Developing TCR-γδ T cells almost immediately reach their end stage of differentiation and mainly remain CD4$^-$CD8$^-$ DN cells that show very little proliferation. In contrast, progression towards the αβ-lineage results in β-selection that induces strong proliferation and differentiation towards the DP stage where many developmental options are still available to the cells, including the potential to differentiate into CD4$^+$ T helper cells, CD8$^+$ cytotoxic T cells, regulatory T cells and NKT cells as illustrated earlier in Figure 1. Thus, γδ T cells show very little apparent developmental complexity compared to αβ-lineage cells.

TCR-αβ T cells are central mediators of the adaptive immune response and recognize a huge variety of structures through the immense diversity in existing TCR complexes. They comprise the majority of circulating T cells in mice and human. In contrast, γδ T cells are mainly located in sites of the body that are exposed to the exterior, such as the skin and the gut, and behave as innate like lymphocytes by providing a first line of defense against pathogens that can enter at those sites. As a result, they recognize more conserved structures such as heat-shock proteins and lipid structures. Both lineages diverge from each other mainly at the early-pre T cell stage as a result of TCR recombination events [11;115]. In frame rearrangements at the TCR-γ and TCR-δ loci will result in the generation of a TCR-γδ complex and further differentiation along the γδ-lineage. Both IL-7 [186] and IL-15 [187] signal responses have been shown to enhance chromatine accessibility of the TCR-γ locus. Importantly, artificially prolonged IL-7 signals can even delay αβ-lineage differentiation

through inhibition of TCF7, LEF1 and RORγt activation [188], factors that are critical for the generation of DP thymocytes [146;189;190]. Thus, IL-7 enhances the likelihood of TCR-γδ development. Differentiation along the αβ-lineage depends on the successful rearrangement of the TCR-β chain that will partner with pTα to form the preTCR complex that drives β-selection and differentiation towards the DP stage. This rearrangement process is critically dependent on Notch-Delta-like interactions as discussed earlier [11;169;191;192]. In either case, the completion of a TCR-γδ or pTα-TCR-β complex seems to prevent further rearrangements at the other TCR loci that could support the generation of the alternative lineage pathway [24;193-197]. Although not absolute [198], it is clear that the outcome of the TCR rearrangements will dramatically influence the lineage outcome.

However, γδ and αβ T cells also require different regulatory factors for their differentiation. Consistent with the limited complexity of their further developmental program, γδ T cells do not seem to require many additional regulatory factors and mainly depend on genes whose expression is already activated at earlier stages of T cell development, such as Sox13 [10;199], Runx3 [11], Icer, Nor1, Nurr1 and Nurr77 [200]. In contrast, the further differentiation of αβ T cells requires the input from novel regulatory factors that are critical for the proliferation and survival as the cells pass through the β-selection checkpoint, including Ets1, RORγt, Akt, Notch-Delta signaling, the Wnt/TCF7/LEF1 pathway, HEB and Bcl11b (see [1] for detailed review). Also epigenetic phenomena such as chromatin remodeling and methylation patterns seem to be more critical during αβ-lineage differentiation as compared to during γδ T cell development as illustrated by the selective αβ-lineage requirement for proteins that mediate these events, such as Dnmt1 [201], Bmi1 [202] and Brg1 [203]. MicroRNA-mediated regulatory mechanisms are also selectively involved in TCR-αβ T cell development [204], and the observation that the ribosomal protein Rpl22 is only required for the development of the αβ T cell lineage [205] emphasises the requirement for additional levels of regulation during αβ T cell differentiation compared to during γδ T cell development. Since the thymus mainly generates T cells that belong to the TCR-αβ lineage, this further illustrates the complexity of the molecular network that controls the T cell differentiation program, as also illustrated by the specific changes that occur during this process with continuous alterations in factors that are gained and lost from one stage to the next. Importantly, despite this complexity, the outcome of commitment processes can be severely influenced by specific environmental signals, as illustrated by the activity of IL-7.

Conclusion

T cell development is a complex, multi-stage process in which hematopoietic precursor cells progressively lose alternative differentiation potentials while gradually obtaining a T-lineage restricted identity. T cell development depends on a large number of regulatory factors of which the composition continuously alters, depending on the developmental stage. As exemplified by just two of these factors, the interpretation of the interactions between all of these factors will be less than simple. The complexity of this developmental model may result from several different phenomena. First, the anatomical dissociation of T cell

development away from the differentiation of other hematopoietic lineages in the bone marrow has allowed thymopoiesis to generate a large variety of T-lineage cells, thereby generating an additional level of complexity as the early precursor cells need to maintain these developmental options. Second, the notion that the earliest T cell precursors have multi-lineage capacity results in a large variety of transcriptional regulators that are expressed in these T-lineage progenitor cells. This highly diverse network thus needs to be tightly controlled and this is achieved by a variety of environmental inputs that will initiate T cell differentiation and repress the alternative lineage potentials. While Notch signaling is a critical factor that mediates this process, many additional regulators are required to induce and support the T cell program. As such, the precise regulatory inputs that control this process and their modes of interaction are only at the verge of being elucidated.

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References

[1] Taghon,T. and Rothenberg,E.V., Molecular mechanisms that control mouse and human TCR-alphabeta and TCR-gammadelta T cell development. *Semin.Immunopathol.* 2008. 4: 383-398.
[2] Ceredig,R. and Rolink,T., A positive look at double-negative thymocytes. *Nat.Rev.Immunol.* 2002. 2: 888-897.
[3] MacDonald,H.R., Radtke,F., and Wilson,A., T cell fate specification and alphabeta/gammadelta lineage commitment. *Curr.Opin.Immunol.* 2001. 13: 219-224.
[4] Porritt,H.E., Rumfelt,L.L., Tabrizifard,S., Schmitt,T.M., Zuniga-Pflucker,J.C., and Petrie,H.T., Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. *Immunity.* 2004. 20: 735-745.
[5] Allman,D., Sambandam,A., Kim,S., Miller,J.P., Pagan,A., Well,D., Meraz,A., and Bhandoola,A., Thymopoiesis independent of common lymphoid progenitors. *Nat.Immunol.* 2003. 4: 168-174.
[6] Benz,C., Martins,V.C., Radtke,F., and Bleul,C.C., The stream of precursors that colonizes the thymus proceeds selectively through the early T lineage precursor stage of T cell development. *J.Exp.Med.* 2008. 205: 1187-1199.

[7] Izon,D.J., T-cell development: Thymus-settling progenitors: settled? *Immunol.Cell Biol.* 2008. 7: 552-553.

[8] Ceredig,R., Bosco,N., and Rolink,T., Problems defining DN2 thymocytes. *Immunol.Cell Biol.* 2008. 7: 545-547.

[9] Masuda,K., Kakugawa,K., Nakayama,T., Minato,N., Katsura,Y., and Kawamoto,H., T cell lineage determination precedes the initiation of TCR beta gene rearrangement. *J.Immunol.* 2007. 179: 3699-3706.

[10] Rothenberg,E.V., Moore,J.E., and Yui,M.A., Launching the T-cell-lineage developmental programme. *Nat.Rev.Immunol.* 2008. 8: 9-21.

[11] Taghon,T., Yui,M.A., Pant,R., Diamond,R.A., and Rothenberg,E.V., Developmental and molecular characterization of emerging beta- and gammadelta-selected pre-T cells in the adult mouse thymus. *Immunity.* 2006. 24: 53-64.

[12] Williams,J.A., Hathcock,K.S., Klug,D., Harada,Y., Choudhury,B., Allison,J.P., Abe,R., and Hodes,R.J., Regulated costimulation in the thymus is critical for T cell development: dysregulated CD28 costimulation can bypass the pre-TCR checkpoint. *J.Immunol.* 2005. 175: 4199-4207.

[13] Wilson,A., Capone,M., and MacDonald,H.R., Unexpectedly late expression of intracellular CD3epsilon and TCR gammadelta proteins during adult thymus development. *Int.Immunol.* 1999. 11: 1641-1650.

[14] Hao,Q.L., George,A.A., Zhu,J., Barsky,L., Zielinska,E., Wang,X., Price,M., Ge,S., and Crooks,G.M., Human intrathymic lineage commitment is marked by differential CD7 expression: identification of CD7- lympho-myeloid thymic progenitors. *Blood* 2008. 111: 1318-1326.

[15] Six,A., Rast,J.P., McCormack,W.T., Dunon,D., Courtois,D., Li,Y., Chen,C.H., and Cooper,M.D., Characterization of avian T-cell receptor gamma genes. *Proc.Natl.Acad.Sci.U.S.A* 1996. 93: 15329-15334.

[16] Weerkamp,F., Pike-Overzet,K., and Staal,F.J., T-sing progenitors to commit. *Trends Immunol.* 2006. 27: 125-131.

[17] Ratajczak,M.Z., Phenotypic and functional characterization of hematopoietic stem cells. *Curr.Opin.Hematol.* 2008. 15: 293-300.

[18] Dik,W.A., Pike-Overzet,K., Weerkamp,F., de,R.D., de Haas,E.F., Baert,M.R., van der Spek,P., Koster,E.E., Reinders,M.J., van Dongen,J.J., Langerak,A.W., and Staal,F.J., New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. *J.Exp.Med.* 2005. 201: 1715-1723.

[19] Blom,B. and Spits,H., Development of human lymphoid cells. *Annu.Rev.Immunol.* 2006. 24: 287-320.

[20] Joachims,M.L., Chain,J.L., Hooker,S.W., Knott-Craig,C.J., and Thompson,L.F., Human alpha beta and gamma delta thymocyte development: TCR gene rearrangements, intracellular TCR beta expression, and gamma delta developmental potential--differences between men and mice. *J.Immunol.* 2006. 176: 1543-1552.

[21] Taghon,T., Van de Walle,I., De Smet,G., De Smedt,M., Leclercq,G., Vandekerckhove,B., and Plum,J., Notch signaling is required for proliferation but not

for differentiation at a well-defined beta-selection checkpoint during human T-cell development. *Blood* 2009. 113: 3254-3263.

[22] Lewis,J.M., Girardi,M., Roberts,S.J., Barbee,S.D., Hayday,A.C., and Tigelaar,R.E., Selection of the cutaneous intraepithelial gammadelta+ T cell repertoire by a thymic stromal determinant. *Nat.Immunol.* 2006. 7: 843-850.

[23] Hoffman,E.S., Passoni,L., Crompton,T., Leu,T.M., Schatz,D.G., Koff,A., Owen,M.J., and Hayday,A.C., Productive T-cell receptor beta-chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. *Genes Dev.* 1996. 10: 948-962.

[24] Aifantis,I., Azogui,O., Feinberg,J., Saint-Ruf,C., Buer,J., and von Boehmer,H., On the role of the pre-T cell receptor in alphabeta versus gammadelta T lineage commitment. *Immunity.* 1998. 9: 649-655.

[25] Aifantis,I., Borowski,C., Gounari,F., Lacorazza,H.D., Nikolich-Zugich,J., and von Boehmer,H., A critical role for the cytoplasmic tail of pTalpha in T lymphocyte development. *Nat.Immunol.* 2002. 3: 483-488.

[26] Plum,J., De Smedt,M., Leclercq,G., Taghon,T., Kerre,T., and Vandekerckhove,B., Human Intrathymic Development: A Selective Approach. *Seminars In Immunopathology* 2008. 4: 411-423.

[27] Bhandoola,A., von Boehmer,H., Petrie,H.T., and Zuniga-Pflucker,J.C., Commitment and developmental potential of extrathymic and intrathymic T cell precursors: plenty to choose from. *Immunity.* 2007. 26: 678-689.

[28] Spangrude,G.J., Heimfeld,S., and Weissman,I.L., Purification and characterization of mouse hematopoietic stem cells. *Science* 1988. 241: 58-62.

[29] Adolfsson,J., Borge,O.J., Bryder,D., Theilgaard-Monch,K., Astrand-Grundstrom,I., Sitnicka,E., Sasaki,Y., and Jacobsen,S.E., Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. *Immunity.* 2001. 15: 659-669.

[30] Goldschneider,I., Cyclical mobilization and gated importation of thymocyte progenitors in the adult mouse: evidence for a thymus-bone marrow feedback loop. *Immunol.Rev.* 2006. 209: 58-75.

[31] Schwarz,B.A., Sambandam,A., Maillard,I., Harman,B.C., Love,P.E., and Bhandoola,A., Selective thymus settling regulated by cytokine and chemokine receptors. *J.Immunol.* 2007. 178: 2008-2017.

[32] Liu,C., Saito,F., Liu,Z., Lei,Y., Uehara,S., Love,P., Lipp,M., Kondo,S., Manley,N., and Takahama,Y., Coordination between CCR7- and CCR9-mediated chemokine signals in prevascular fetal thymus colonization. *Blood* 2006. 108: 2531-2539.

[33] Rossi,F.M., Corbel,S.Y., Merzaban,J.S., Carlow,D.A., Gossens,K., Duenas,J., So,L., Yi,L., and Ziltener,H.J., Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. *Nat.Immunol.* 2005. 6: 626-634.

[34] Lesley,J., Hyman,R., and Schulte,R., Evidence that the Pgp-1 glycoprotein is expressed on thymus-homing progenitor cells of the thymus. *Cell Immunol.* 1985. 91: 397-403.

[35] Goldschneider,I., Komschlies,K.L., and Greiner,D.L., Studies of thymocytopoiesis in rats and mice. I. Kinetics of appearance of thymocytes using a direct intrathymic adoptive transfer assay for thymocyte precursors. *J.Exp.Med.* 1986. 163: 1-17.

[36] Mori,S., Shortman,K., and Wu,L., Characterization of thymus-seeding precursor cells from mouse bone marrow. *Blood* 2001. 98: 696-704.

[37] Sambandam,A., Maillard,I., Zediak,V.P., Xu,L., Gerstein,R.M., Aster,J.C., Pear,W.S., and Bhandoola,A., Notch signaling controls the generation and differentiation of early T lineage progenitors. *Nat.Immunol.* 2005. 6: 663-670.

[38] Benz,C. and Bleul,C.C., A multipotent precursor in the thymus maps to the branching point of the T versus B lineage decision. *J.Exp.Med.* 2005. 202: 21-31.

[39] Heinzel,K., Benz,C., Martins,V.C., Haidl,I.D., and Bleul,C.C., Bone marrow-derived hemopoietic precursors commit to the T cell lineage only after arrival in the thymic microenvironment. *J.Immunol.* 2007. 178: 858-868.

[40] Balciunaite,G., Ceredig,R., and Rolink,A.G., The earliest subpopulation of mouse thymocytes contains potent T, significant macrophage, and natural killer cell but no B-lymphocyte potential. *Blood* 2005. 105: 1930-1936.

[41] Bell,J.J. and Bhandoola,A., The earliest thymic progenitors for T cells possess myeloid lineage potential. *Nature* 2008. 452: 764-767.

[42] Wada,H., Masuda,K., Satoh,R., Kakugawa,K., Ikawa,T., Katsura,Y., and Kawamoto,H., Adult T-cell progenitors retain myeloid potential. *Nature* 2008. 452: 768-772.

[43] Ceredig,R., Bosco,N., and Rolink,A.G., The B lineage potential of thymus settling progenitors is critically dependent on mouse age. *Eur.J.Immunol.* 2007. 37: 830-837.

[44] King,A.G., Kondo,M., Scherer,D.C., and Weissman,I.L., Lineage infidelity in myeloid cells with TCR gene rearrangement: a latent developmental potential of proT cells revealed by ectopic cytokine receptor signaling. *Proc.Natl.Acad.Sci.U.S.A* 2002. 99: 4508-4513.

[45] Petrie,H.T., Early commitment: T cell progenitors in the blood. *Immunity.* 2007. 26: 7-8.

[46] Adolfsson,J., Mansson,R., Buza-Vidas,N., Hultquist,A., Liuba,K., Jensen,C.T., Bryder,D., Yang,L., Borge,O.J., Thoren,L.A., Anderson,K., Sitnicka,E., Sasaki,Y., Sigvardsson,M., and Jacobsen,S.E., Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell* 2005. 121: 295-306.

[47] Igarashi,H., Gregory,S.C., Yokota,T., Sakaguchi,N., and Kincade,P.W., Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity.* 2002. 17: 117-130.

[48] Lai,A.Y. and Kondo,M., Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *J.Exp.Med.* 2006. 203: 1867-1873.

[49] Perry,S.S., Wang,H., Pierce,L.J., Yang,A.M., Tsai,S., and Spangrude,G.J., L-selectin defines a bone marrow analog to the thymic early T-lineage progenitor. *Blood* 2004. 103: 2990-2996.

[50] Balciunaite,G., Ceredig,R., Massa,S., and Rolink,A.G., A B220+ CD117+ CD19- hematopoietic progenitor with potent lymphoid and myeloid developmental potential. *Eur.J.Immunol.* 2005. 35: 2019-2030.

[51] Kondo,M., Weissman,I.L., and Akashi,K., Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 1997. 91: 661-672.

[52] Martin,C.H., Aifantis,I., Scimone,M.L., von Andrian,U.H., Reizis,B., von Boehmer,H., and Gounari,F., Efficient thymic immigration of B220+ lymphoid-restricted bone marrow cells with T precursor potential. *Nat.Immunol.* 2003. 4: 866-873.

[53] Perry,S.S., Welner,R.S., Kouro,T., Kincade,P.W., and Sun,X.H., Primitive lymphoid progenitors in bone marrow with T lineage reconstituting potential. *J.Immunol.* 2006. 177: 2880-2887.

[54] Schwarz,B.A. and Bhandoola,A., Circulating hematopoietic progenitors with T lineage potential. *Nat.Immunol.* 2004. 5: 953-960.

[55] Umland,O., Mwangi,W.N., Anderson,B.M., Walker,J.C., and Petrie,H.T., The blood contains multiple distinct progenitor populations with clonogenic B and T lineage potential. *J.Immunol.* 2007. 178: 4147-4152.

[56] Krueger,A. and von Boehmer,H., Identification of a T lineage-committed progenitor in adult blood. *Immunity.* 2007. 26: 105-116.

[57] Carlyle,J.R. and Zuniga-Pflucker,J.C., Requirement for the thymus in alphabeta T lymphocyte lineage commitment. *Immunity.* 1998. 9: 187-197.

[58] Rodewald,H.R., Kretzschmar,K., Takeda,S., Hohl,C., and Dessing,M., Identification of pro-thymocytes in murine fetal blood: T lineage commitment can precede thymus colonization. *EMBO J.* 1994. 13: 4229-4240.

[59] Goodell,M.A., Rosenzweig,M., Kim,H., Marks,D.F., DeMaria,M., Paradis,G., Grupp,S.A., Sieff,C.A., Mulligan,R.C., and Johnson,R.P., Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat.Med.* 1997. 3: 1337-1345.

[60] Kawashima,I., Zanjani,E.D., maida-Porada,G., Flake,A.W., Zeng,H., and Ogawa,M., CD34+ human marrow cells that express low levels of Kit protein are enriched for long-term marrow-engrafting cells. *Blood* 1996. 87: 4136-4142.

[61] Sitnicka,E., Buza-Vidas,N., Larsson,S., Nygren,J.M., Liuba,K., and Jacobsen,S.E., Human CD34+ hematopoietic stem cells capable of multilineage engrafting NOD/SCID mice express flt3: distinct flt3 and c-kit expression and response patterns on mouse and candidate human hematopoietic stem cells. *Blood* 2003. 102: 881-886.

[62] Offner,F., Kerre,T., De Smedt,M., and Plum,J., Bone marrow CD34 cells generate fewer T cells in vitro with increasing age and following chemotherapy. *Br.J.Haematol.* 1999. 104: 801-808.

[63] Zuniga-Pflucker,J.C., T-cell development made simple. *Nat.Rev.Immunol.* 2004. 4: 67-72.

[64] Gimeno,R., Weijer,K., Voordouw,A., Uittenbogaart,C.H., Legrand,N., Alves,N.L., Wijnands,E., Blom,B., and Spits,H., Monitoring the effect of gene silencing by RNA interference in human CD34+ cells injected into newborn RAG2-/- gammac-/- mice: functional inactivation of p53 in developing T cells. *Blood* 2004. 104: 3886-3893.

[65] De Smedt,M., Hoebeke,I., and Plum,J., Human bone marrow CD34+ progenitor cells mature to T cells on OP9-DL1 stromal cell line without thymus microenvironment. *Blood Cells Mol.Dis.* 2004. 33: 227-232.

[66] Robin,C., Bennaceur-Griscelli,A., Louache,F., Vainchenker,W., and Coulombel,L., Identification of human T-lymphoid progenitor cells in CD34+ CD38low and CD34+

CD38+ subsets of human cord blood and bone marrow cells using NOD-SCID fetal thymus organ cultures. *Br.J.Haematol.* 1999. 104: 809-819.

[67] Weekx,S.F., Snoeck,H.W., Offner,F., De Smedt,M., Van Bockstaele,D.R., Nijs,G., Lenjou,M., Moulijn,A., Rodrigus,I., Berneman,Z.N., and Plum,J.**,** Generation of T cells from adult human hematopoietic stem cells and progenitors in a fetal thymic organ culture system: stimulation by tumor necrosis factor-alpha. *Blood* 2000. 95: 2806-2812.

[68] Galy,A., Travis,M., Cen,D., and Chen,B.**,** Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity.* 1995. 3: 459-473.

[69] Six,E.M., Bonhomme,D., Monteiro,M., Beldjord,K., Jurkowska,M., Cordier-Garcia,C., Garrigue,A., Dal,C.L., Rocha,B., Fischer,A., Cavazzana-Calvo,M., and Andre-Schmutz,I.**,** A human postnatal lymphoid progenitor capable of circulating and seeding the thymus. *J.Exp.Med.* 2007. 204: 3085-3093.

[70] Kerre,T.C., De Smet,G., De Smedt,M., Zippelius,A., Pittet,M.J., Langerak,A.W., De Bosscher,J., Offner,F., Vandekerckhove,B., and Plum,J.**,** Adapted NOD/SCID model supports development of phenotypically and functionally mature T cells from human umbilical cord blood CD34(+) cells. *Blood* 2002. 99: 1620-1626.

[71] Hao,Q.L., Zhu,J., Price,M.A., Payne,K.J., Barsky,L.W., and Crooks,G.M.**,** Identification of a novel, human multilymphoid progenitor in cord blood. *Blood* 2001. 97: 3683-3690.

[72] Haddad,R., Guardiola,P., Izac,B., Thibault,C., Radich,J., Delezoide,A.L., Baillou,C., Lemoine,F.M., Gluckman,J.C., Pflumio,F., and Canque,B.**,** Molecular characterization of early human T/NK and B-lymphoid progenitor cells in umbilical cord blood. *Blood* 2004. 104: 3918-3926.

[73] Hoebeke,I., De Smedt,M., Stolz,F., Pike-Overzet,K., Staal,F.J., Plum,J., and Leclercq,G.**,** T-, B- and NK-lymphoid, but not myeloid cells arise from human CD34(+)CD38(-)CD7(+) common lymphoid progenitors expressing lymphoid-specific genes. *Leukemia* 2007. 21: 311-319.

[74] Haddad,R., Guimiot,F., Six,E., Jourquin,F., Setterblad,N., Kahn,E., Yagello,M., Schiffer,C., Andre-Schmutz,I., Cavazzana-Calvo,M., Gluckman,J.C., Delezoide,A.L., Pflumio,F., and Canque,B.**,** Dynamics of thymus-colonizing cells during human development. *Immunity.* 2006. 24: 217-230.

[75] Ramiro,A.R., Trigueros,C., Marquez,C., San Millan,J.L., and Toribio,M.L.**,** Regulation of pre-T cell receptor (pT alpha-TCR beta) gene expression during human thymic development. *J.Exp.Med.* 1996. 184: 519-530.

[76] Klein,F., Feldhahn,N., Lee,S., Wang,H., Ciuffi,F., von,E.M., Toribio,M.L., Sauer,H., Wartenberg,M., Barath,V.S., Kronke,M., Wernet,P., Rowley,J.D., and Muschen,M.**,** T lymphoid differentiation in human bone marrow. *Proc.Natl.Acad.Sci.U.S.A* 2003. 100: 6747-6752.

[77] Ktorza,S., Blanc,C., Laurent,C., Sarun,S., Verpilleux,M.P., Debre,P., and Schmitt,C.**,** Complete TCR-delta rearrangements and partial (D-J) recombination of the TCR-beta locus in CD34+7+ precursors from human cord blood. *J.Immunol.* 1996. 156: 4120-4127.

[78] Blom,B., Res,P., Noteboom,E., Weijer,K., and Spits,H., Prethymic CD34+ progenitors capable of developing into T cells are not committed to the T cell lineage. *J.Immunol.* 1997. 158: 3571-3577.
[79] Kurtzberg,J., Denning,S.M., Nycum,L.M., Singer,K.H., and Haynes,B.F., Immature human thymocytes can be driven to differentiate into nonlymphoid lineages by cytokines from thymic epithelial cells. *Proc.Natl.Acad.Sci.U.S.A* 1989. 86: 7575-7579.
[80] Schmitt,C., Ktorza,S., Sarun,S., Blanc,C., De Jong,R., and Debre,P., CD34-expressing human thymocyte precursors proliferate in response to interleukin-7 but have lost myeloid differentiation potential. *Blood* 1993. 82: 3675-3685.
[81] Res,P., Martinez-Caceres,E., Cristina,J.A., Staal,F., Noteboom,E., Weijer,K., and Spits,H., CD34+CD38dim cells in the human thymus can differentiate into T, natural killer, and dendritic cells but are distinct from pluripotent stem cells. *Blood* 1996. 87: 5196-5206.
[82] Sanchez,M.J., Muench,M.O., Roncarolo,M.G., Lanier,L.L., and Phillips,J.H., Identification of a common T/natural killer cell progenitor in human fetal thymus. *J.Exp.Med.* 1994. 180: 569-576.
[83] Marquez,C., Trigueros,C., Fernandez,E., and Toribio,M.L., The development of T and non-T cell lineages from CD34+ human thymic precursors can be traced by the differential expression of CD44. *J.Exp.Med.* 1995. 181: 475-483.
[84] de Yebenes,V., Carrasco,Y.R., Ramiro,A.R., and Toribio,M.L., Identification of a myeloid intrathymic pathway of dendritic cell development marked by expression of the granulocyte macrophage-colony-stimulating factor receptor. *Blood* 2002. 99: 2948-2956.
[85] Weerkamp,F., Baert,M.R., Brugman,M.H., Dik,W.A., de Haas,E.F., Visser,T.P., de Groot,C.J., Wagemaker,G., van Dongen,J.J., and Staal,F.J., Human thymus contains multipotent progenitors with T/B lymphoid, myeloid, and erythroid lineage potential. *Blood* 2006. 107: 3131-3137.
[86] Kerre,T.C., De Smet,G., De Smedt,M., Offner,F., De Bosscher,J., Plum,J., and Vandekerckhove,B., Both CD34+38+ and CD34+38- cells home specifically to the bone marrow of NOD/LtSZ scid/scid mice but show different kinetics in expansion. *J.Immunol.* 2001. 167: 3692-3698.
[87] Artavanis-Tsakonas,S., Rand,M.D., and Lake,R.J., Notch signaling: cell fate control and signal integration in development. *Science* 1999. 284: 770-776.
[88] Maillard,I., Fang,T., and Pear,W.S., Regulation of lymphoid development, differentiation, and function by the Notch pathway. *Annu.Rev.Immunol.* 2005. 23: 945-974.
[89] Radtke,F., Wilson,A., Mancini,S.J., and MacDonald,H.R., Notch regulation of lymphocyte development and function. *Nat.Immunol.* 2004. 5: 247-253.
[90] Jouve,C., Palmeirim,I., Henrique,D., Beckers,J., Gossler,A., Ish-Horowicz,D., and Pourquie,O., Notch signalling is required for cyclic expression of the hairy-like gene HES1 in the presomitic mesoderm. *Development* 2000. 127: 1421-1429.
[91] Lee,J.S., Ishimoto,A., Honjo,T., and Yanagawa,S., Murine leukemia provirus-mediated activation of the Notch1 gene leads to induction of HES-1 in a mouse T lymphoma cell line, DL-3. *FEBS Lett.* 1999. 455: 276-280.

[92] Krebs,L.T., Deftos,M.L., Bevan,M.J., and Gridley,T., The Nrarp gene encodes an ankyrin-repeat protein that is transcriptionally regulated by the notch signaling pathway. *Dev.Biol.* 2001. 238: 110-119.

[93] Lamar,E., Deblandre,G., Wettstein,D., Gawantka,V., Pollet,N., Niehrs,C., and Kintner,C., Nrarp is a novel intracellular component of the Notch signaling pathway. *Genes Dev.* 2001. 15: 1885-1899.

[94] Pirot,P., van Grunsven,L.A., Marine,J.C., Huylebroeck,D., and Bellefroid,E.J., Direct regulation of the Nrarp gene promoter by the Notch signaling pathway. *Biochem.Biophys.Res.Commun.* 2004. 322: 526-534.

[95] Kishi,N., Tang,Z., Maeda,Y., Hirai,A., Mo,R., Ito,M., Suzuki,S., Nakao,K., Kinoshita,T., Kadesch,T., Hui,C., Artavanis-Tsakonas,S., Okano,H., and Matsuno,K., Murine homologs of deltex define a novel gene family involved in vertebrate Notch signaling and neurogenesis. *Int.J.Dev.Neurosci.* 2001. 19: 21-35.

[96] Yamamoto,N., Yamamoto,S., Inagaki,F., Kawaichi,M., Fukamizu,A., Kishi,N., Matsuno,K., Nakamura,K., Weinmaster,G., Okano,H., and Nakafuku,M., Role of Deltex-1 as a transcriptional regulator downstream of the Notch receptor. *J.Biol.Chem.* 2001. 276: 45031-45040.

[97] Reizis,B. and Leder,P., Direct induction of T lymphocyte-specific gene expression by the mammalian Notch signaling pathway. *Genes Dev.* 2002. 16: 295-300.

[98] Palomero,T., Lim,W.K., Odom,D.T., Sulis,M.L., Real,P.J., Margolin,A., Barnes,K.C., O'Neil,J., Neuberg,D., Weng,A.P., Aster,J.C., Sigaux,F., Soulier,J., Look,A.T., Young,R.A., Califano,A., and Ferrando,A.A., NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc.Natl.Acad.Sci.U.S.A* 2006. 103: 18261-18266.

[99] Weng,A.P., Millholland,J.M., Yashiro-Ohtani,Y., Arcangeli,M.L., Lau,A., Wai,C., Del,B.C., Rodriguez,C.G., Sai,H., Tobias,J., Li,Y., Wolfe,M.S., Shachaf,C., Felsher,D., Blacklow,S.C., Pear,W.S., and Aster,J.C., c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev.* 2006. 20: 2096-2109.

[100] Besseyrias,V., Fiorini,E., Strobl,L.J., Zimber-Strobl,U., Dumortier,A., Koch,U., Arcangeli,M.L., Ezine,S., MacDonald,H.R., and Radtke,F., Hierarchy of Notch-Delta interactions promoting T cell lineage commitment and maturation. *J.Exp.Med.* 2007. 204: 331-343.

[101] De Smedt,M., Hoebeke,I., Reynvoet,K., Leclercq,G., and Plum,J., Different thresholds of Notch signaling bias human precursor cells toward B-, NK-, monocytic/dendritic-, or T-cell lineage in thymus microenvironment. *Blood* 2005. 106: 3498-3506.

[102] Schmitt,T.M., Ciofani,M., Petrie,H.T., and Zuniga-Pflucker,J.C., Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J.Exp.Med.* 2004. 200: 469-479.

[103] Radtke,F., Wilson,A., Stark,G., Bauer,M., van Meerwijk,J., MacDonald,H.R., and Aguet,M., Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity.* 1999. 10: 547-558.

[104] Wilson,A., MacDonald,H.R., and Radtke,F., Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *J.Exp.Med.* 2001. 194: 1003-1012.

[105] De Smedt,M., Reynvoet,K., Kerre,T., Taghon,T., Verhasselt,B., Vandekerckhove,B., Leclercq,G., and Plum,J., Active form of Notch imposes T cell fate in human progenitor cells. *J.Immunol.* 2002. 169: 3021-3029.

[106] Schmitt,T.M. and Zuniga-Pflucker,J.C., Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity.* 2002. 17: 749-756.

[107] Mohtashami,M. and Zuniga-Pflucker,J.C., Three-dimensional architecture of the thymus is required to maintain delta-like expression necessary for inducing T cell development. *J.Immunol.* 2006. 176: 730-734.

[108] La Motte-Mohs,R.N., Herer,E., and Zuniga-Pflucker,J.C., Induction of T-cell development from human cord blood hematopoietic stem cells by Delta-like 1 in vitro. *Blood* 2005. 105: 1431-1439.

[109] Schmitt,T.M., de Pooter,R.F., Gronski,M.A., Cho,S.K., Ohashi,P.S., and Zuniga-Pflucker,J.C., Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. *Nat.Immunol.* 2004. 5: 410-417.

[110] Timmermans,F., Velghe,I., Vanwalleghem,L., De Smedt,M., Van Coppernolle,S., Taghon,T., Moore,H.D., Leclercq,G., Langerak,A.W., Kerre,T., Plum,J., and Vandekerckhove,B., Generation of T cells from human embryonic stem cell-derived hematopoietic zones. *J.Immunol.* 2009. 182: 6879-6888.

[111] Plum,J., De Smedt,M., Verhasselt,B., Offner,F., Kerre,T., Vanhecke,D., Leclercq,G., and Vandekerckhove,B., In vitro intrathymic differentiation kinetics of human fetal liver CD34+. *J.Immunol.* 1999. 162: 60-68.

[112] Delaney,C., Varnum-Finney,B., Aoyama,K., Brashem-Stein,C., and Bernstein,I.D., Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. *Blood* 2005. 106: 2693-2699.

[113] Lefort,N., Benne,C., Lelievre,J.D., Dorival,C., Balbo,M., Sakano,S., Coulombel,L., and Levy,Y., Short exposure to Notch ligand Delta-4 is sufficient to induce T-cell differentiation program and to increase the T cell potential of primary human CD34+ cells. *Exp.Hematol.* 2006. 34: 1720-1729.

[114] Van de Walle,I., De Smet,G., De Smedt,M., Vandekerckhove,B., Leclercq,G., Plum,J., and Taghon,T., An early decrease in Notch activation is required for human TCR-alphabeta lineage differentiation at the expense of TCR-gammadelta T cells. *Blood* 2009. 113: 2988-2998.

[115] Prinz,I., Sansoni,A., Kissenpfennig,A., Ardouin,L., Malissen,M., and Malissen,B., Visualization of the earliest steps of gammadelta T cell development in the adult thymus. *Nat.Immunol.* 2006. 7: 995-1003.

[116] Tydell,C.C., David-Fung,E.S., Moore,J.E., Rowen,L., Taghon,T., and Rothenberg,E.V., Molecular dissection of prethymic progenitor entry into the T lymphocyte developmental pathway. *J.Immunol.* 2007. 179: 421-438.

[117] Tsai,F.Y., Keller,G., Kuo,F.C., Weiss,M., Chen,J., Rosenblatt,M., Alt,F.W., and Orkin,S.H., An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* 1994. 371: 221-226.

[118] Zhang,P., Iwasaki-Arai,J., Iwasaki,H., Fenyus,M.L., Dayaram,T., Owens,B.M., Shigematsu,H., Levantini,E., Huettner,C.S., Lekstrom-Himes,J.A., Akashi,K., and Tenen,D.G., Enhancement of hematopoietic stem cell repopulating capacity and self-

renewal in the absence of the transcription factor C/EBP alpha. *Immunity*. 2004. 21: 853-863.
[119] Shivdasani,R.A., Mayer,E.L., and Orkin,S.H.**,** Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature* 1995. 373: 432-434.
[120] Laiosa,C.V., Stadtfeld,M., Xie,H., de Andres-Aguayo,L., and Graf,T.**,** Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBP alpha and PU.1 transcription factors. *Immunity*. 2006. 25: 731-744.
[121] Anderson,M.K., Weiss,A.H., Hernandez-Hoyos,G., Dionne,C.J., and Rothenberg,E.V.**,** Constitutive expression of PU.1 in fetal hematopoietic progenitors blocks T cell development at the pro-T cell stage. *Immunity*. 2002. 16: 285-296.
[122] Franco,C.B., Scripture-Adams,D.D., Proekt,I., Taghon,T., Weiss,A.H., Yui,M.A., Adams,S.L., Diamond,R.A., and Rothenberg,E.V.**,** Notch/Delta signaling constrains reengineering of pro-T cells by PU.1. *Proc.Natl.Acad.Sci.U.S.A* 2006. 103: 11993-11998.
[123] Egawa,T., Tillman,R.E., Naoe,Y., Taniuchi,I., and Littman,D.R.**,** The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. *J.Exp.Med.* 2007. 204: 1945-1957.
[124] Growney,J.D., Shigematsu,H., Li,Z., Lee,B.H., Adelsperger,J., Rowan,R., Curley,D.P., Kutok,J.L., Akashi,K., Williams,I.R., Speck,N.A., and Gilliland,D.G.**,** Loss of Runx1 perturbs adult hematopoiesis and is associated with a myeloproliferative phenotype. *Blood* 2005. 106: 494-504.
[125] Guo,Y., Maillard,I., Chakraborti,S., Rothenberg,E.V., and Speck,N.A.**,** Core binding factors are necessary for natural killer cell development and cooperate with Notch signaling during T-cell specification. *Blood* 2008. 112: 480-492.
[126] Lorsbach,R.B., Moore,J., Ang,S.O., Sun,W., Lenny,N., and Downing,J.R.**,** Role of RUNX1 in adult hematopoiesis: analysis of RUNX1-IRES-GFP knock-in mice reveals differential lineage expression. *Blood* 2004. 103: 2522-2529.
[127] Talebian,L., Li,Z., Guo,Y., Gaudet,J., Speck,M.E., Sugiyama,D., Kaur,P., Pear,W.S., Maillard,I., and Speck,N.A.**,** T-lymphoid, megakaryocyte, and granulocyte development are sensitive to decreases in CBFbeta dosage. *Blood* 2007. 109: 11-21.
[128] Taniuchi,I., Osato,M., Egawa,T., Sunshine,M.J., Bae,S.C., Komori,T., Ito,Y., and Littman,D.R.**,** Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell* 2002. 111: 621-633.
[129] Hahm,K., Cobb,B.S., McCarty,A.S., Brown,K.E., Klug,C.A., Lee,R., Akashi,K., Weissman,I.L., Fisher,A.G., and Smale,S.T.**,** Helios, a T cell-restricted Ikaros family member that quantitatively associates with Ikaros at centromeric heterochromatin. *Genes Dev*. 1998. 12: 782-796.
[130] Rothenberg,E.V. and Taghon,T.**,** Molecular genetics of T cell development. *Annu.Rev.Immunol.* 2005. 23: 601-649.
[131] Taghon,T.N., David,E.S., Zuniga-Pflucker,J.C., and Rothenberg,E.V.**,** Delayed, asynchronous, and reversible T-lineage specification induced by Notch/Delta signaling. *Genes Dev*. 2005. 19: 965-978.

[132] Winandy,S., Wu,L., Wang,J.H., and Georgopoulos,K., Pre-T cell receptor (TCR) and TCR-controlled checkpoints in T cell differentiation are set by Ikaros. *J.Exp.Med.* 1999. 190: 1039-1048.

[133] Bender,T.P., Kremer,C.S., Kraus,M., Buch,T., and Rajewsky,K., Critical functions for c-Myb at three checkpoints during thymocyte development. *Nat.Immunol.* 2004. 5: 721-729.

[134] Greig,K.T., Carotta,S., and Nutt,S.L., Critical roles for c-Myb in hematopoietic progenitor cells. *Semin.Immunol.* 2008. 20: 247-256.

[135] Lieu,Y.K., Kumar,A., Pajerowski,A.G., Rogers,T.J., and Reddy,E.P., Requirement of c-myb in T cell development and in mature T cell function. *Proc.Natl.Acad.Sci.U.S.A* 2004. 101: 14853-14858.

[136] Hock,H., Hamblen,M.J., Rooke,H.M., Schindler,J.W., Saleque,S., Fujiwara,Y., and Orkin,S.H., Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. *Nature* 2004. 431: 1002-1007.

[137] Pargmann,D., Yucel,R., Kosan,C., Saba,I., Klein-Hitpass,L., Schimmer,S., Heyd,F., Dittmer,U., and Moroy,T., Differential impact of the transcriptional repressor Gfi1 on mature CD4+ and CD8+ T lymphocyte function. *Eur.J.Immunol.* 2007. 37: 3551-3563.

[138] Schmidt,T., Karsunky,H., Rodel,B., Zevnik,B., Elsasser,H.P., and Moroy,T., Evidence implicating Gfi-1 and Pim-1 in pre-T-cell differentiation steps associated with beta-selection. *EMBO J.* 1998. 17: 5349-5359.

[139] Yucel,R., Karsunky,H., Klein-Hitpass,L., and Moroy,T., The transcriptional repressor Gfi1 affects development of early, uncommitted c-Kit+ T cell progenitors and CD4/CD8 lineage decision in the thymus. *J.Exp.Med.* 2003. 197: 831-844.

[140] Wang,H., Pierce,L.J., and Spangrude,G.J., Distinct roles of IL-7 and stem cell factor in the OP9-DL1 T-cell differentiation culture system. *Exp.Hematol.* 2006. 34: 1730-1740.

[141] Wakabayashi,Y., Watanabe,H., Inoue,J., Takeda,N., Sakata,J., Mishima,Y., Hitomi,J., Yamamoto,T., Utsuyama,M., Niwa,O., Aizawa,S., and Kominami,R., Bcl11b is required for differentiation and survival of alphabeta T lymphocytes. *Nat.Immunol.* 2003. 4: 533-539.

[142] El Andaloussi A., Graves,S., Meng,F., Mandal,M., Mashayekhi,M., and Aifantis,I., Hedgehog signaling controls thymocyte progenitor homeostasis and differentiation in the thymus. *Nat.Immunol.* 2006. 7: 418-426.

[143] Ohteki,T., Wilson,A., Verbeek,S., MacDonald,H.R., and Clevers,H., Selectively impaired development of intestinal T cell receptor gamma delta+ cells and liver CD4+ NK1+ T cell receptor alpha beta+ cells in T cell factor-1-deficient mice. *Eur.J.Immunol.* 1996. 26: 351-355.

[144] Schilham,M.W., Wilson,A., Moerer,P., Benaissa-Trouw,B.J., Cumano,A., and Clevers,H.C., Critical involvement of Tcf-1 in expansion of thymocytes. *J.Immunol.* 1998. 161: 3984-3991.

[145] Verbeek,S., Izon,D., Hofhuis,F., Robanus-Maandag,E., te Riele,H., van de Watering,M., Oosterwegel,M., Wilson,A., MacDonald,H.R., and Clevers,H., An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature* 1995. 374: 70-74.

[146] Okamura,R.M., Sigvardsson,M., Galceran,J., Verbeek,S., Clevers,H., and Grosschedl,R., Redundant regulation of T cell differentiation and TCRalpha gene expression by the transcription factors LEF-1 and TCF-1. *Immunity.* 1998. 8: 11-20.

[147] David-Fung,E.S., Yui,M.A., Morales,M., Wang,H., Taghon,T., Diamond,R.A., and Rothenberg,E.V., Progression of regulatory gene expression states in fetal and adult pro-T-cell development. *Immunol.Rev.* 2006. 209: 212-236.

[148] Eyquem,S., Chemin,K., Fasseu,M., and Bories,J.C., The Ets-1 transcription factor is required for complete pre-T cell receptor function and allelic exclusion at the T cell receptor beta locus. *Proc.Natl.Acad.Sci.U.S.A* 2004. 101: 15712-15717.

[149] Hendriks,R.W., Nawijn,M.C., Engel,J.D., van Doorninck,H., Grosveld,F., and Karis,A., Expression of the transcription factor GATA-3 is required for the development of the earliest T cell progenitors and correlates with stages of cellular proliferation in the thymus. *Eur.J.Immunol.* 1999. 29: 1912-1918.

[150] Ting,C.N., Olson,M.C., Barton,K.P., and Leiden,J.M., Transcription factor GATA-3 is required for development of the T-cell lineage. *Nature* 1996. 384: 474-478.

[151] Pandolfi,P.P., Roth,M.E., Karis,A., Leonard,M.W., Dzierzak,E., Grosveld,F.G., Engel,J.D., and Lindenbaum,M.H., Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. *Nat.Genet.* 1995. 11: 40-44.

[152] Samson,S.I., Richard,O., Tavian,M., Ranson,T., Vosshenrich,C.A., Colucci,F., Buer,J., Grosveld,F., Godin,I., and Di Santo,J.P., GATA-3 promotes maturation, IFN-gamma production, and liver-specific homing of NK cells. *Immunity.* 2003. 19: 701-711.

[153] Hozumi,K., Negishi,N., Tsuchiya,I., Abe,N., Hirano,K., Suzuki,D., Yamamoto,M., Engel,J.D., and Habu,S., Notch signaling is necessary for GATA3 function in the initiation of T cell development. *Eur.J.Immunol.* 2008. 38: 977-985.

[154] Weerkamp,F., Luis,T.C., Naber,B.A., Koster,E.E., Jeannotte,L., van Dongen,J.J., and Staal,F.J., Identification of Notch target genes in uncommitted T-cell progenitors: No direct induction of a T-cell specific gene program. *Leukemia* 2006. 20: 1967-1977.

[155] Staal,F.J., Luis,T.C., and Tiemessen,M.M., WNT signalling in the immune system: WNT is spreading its wings. *Nat.Rev.Immunol.* 2008. 8: 581-593.

[156] Nutt,S.L., Heavey,B., Rolink,A.G., and Busslinger,M., Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature* 1999. 401: 556-562.

[157] Rolink,A.G., Nutt,S.L., Melchers,F., and Busslinger,M., Long-term in vivo reconstitution of T-cell development by Pax5-deficient B-cell progenitors. *Nature* 1999. 401: 603-606.

[158] Pongubala,J.M., Northrup,D.L., Lancki,D.W., Medina,K.L., Treiber,T., Bertolino,E., Thomas,M., Grosschedl,R., Allman,D., and Singh,H., Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5. *Nat.Immunol.* 2008. 9: 203-215.

[159] Pevny,L., Simon,M.C., Robertson,E., Klein,W.H., Tsai,S.F., D'Agati,V., Orkin,S.H., and Costantini,F., Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* 1991. 349: 257-260.

[160] Orkin,S.H., Shivdasani,R.A., Fujiwara,Y., and McDevitt,M.A., Transcription factor GATA-1 in megakaryocyte development. *Stem Cells* 1998. 16 Suppl 2: 79-83.

[161] Maeda,T., Merghoub,T., Hobbs,R.M., Dong,L., Maeda,M., Zakrzewski,J., van den Brink,M.R., Zelent,A., Shigematsu,H., Akashi,K., Teruya-Feldstein,J., Cattoretti,G., and Pandolfi,P.P., Regulation of B versus T lymphoid lineage fate decision by the proto-oncogene LRF. *Science* 2007. 316: 860-866.

[162] Lehar,S.M. and Bevan,M.J., T cells develop normally in the absence of both Deltex1 and Deltex2. *Mol.Cell Biol.* 2006. 26: 7358-7371.

[163] Izon,D.J., Aster,J.C., He,Y., Weng,A., Karnell,F.G., Patriub,V., Xu,L., Bakkour,S., Rodriguez,C., Allman,D., and Pear,W.S., Deltex1 redirects lymphoid progenitors to the B cell lineage by antagonizing Notch1. *Immunity.* 2002. 16: 231-243.

[164] Yun,T.J. and Bevan,M.J., Notch-regulated ankyrin-repeat protein inhibits Notch1 signaling: multiple Notch1 signaling pathways involved in T cell development. *J.Immunol.* 2003. 170: 5834-5841.

[165] Tsuji,M., Shinkura,R., Kuroda,K., Yabe,D., and Honjo,T., Msx2-interacting nuclear target protein (Mint) deficiency reveals negative regulation of early thymocyte differentiation by Notch/RBP-J signaling. *Proc.Natl.Acad.Sci.U.S.A* 2007. 104: 1610-1615.

[166] Rolink,A.G., Balciunaite,G., Demoliere,C., and Ceredig,R., The potential involvement of Notch signaling in NK cell development. *Immunol.Lett.* 2006. 107: 50-57.

[167] Taghon,T., Yui,M.A., and Rothenberg,E.V., Mast cell lineage diversion of T lineage precursors by the essential T cell transcription factor GATA-3. *Nat.Immunol.* 2007. 8: 845-855.

[168] Duncan,A.W., Rattis,F.M., DiMascio,L.N., Congdon,K.L., Pazianos,G., Zhao,C., Yoon,K., Cook,J.M., Willert,K., Gaiano,N., and Reya,T., Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat.Immunol.* 2005. 6: 314-322.

[169] Wolfer,A., Wilson,A., Nemir,M., MacDonald,H.R., and Radtke,F., Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes. *Immunity.* 2002. 16: 869-879.

[170] Ciofani,M., Schmitt,T.M., Ciofani,A., Michie,A.M., Cuburu,N., Aublin,A., Maryanski,J.L., and Zuniga-Pflucker,J.C., Obligatory role for cooperative signaling by pre-TCR and Notch during thymocyte differentiation. *J.Immunol.* 2004. 172: 5230-5239.

[171] Ciofani,M. and Zuniga-Pflucker,J.C., Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. *Nat.Immunol.* 2005. 6: 881-888.

[172] Maillard,I., Tu,L., Sambandam,A., Yashiro-Ohtani,Y., Millholland,J., Keeshan,K., Shestova,O., Xu,L., Bhandoola,A., and Pear,W.S., The requirement for Notch signaling at the beta-selection checkpoint in vivo is absolute and independent of the pre-T cell receptor. *J.Exp.Med.* 2006. 203: 2239-2245.

[173] De Smedt,M., Taghon,T., Van de Walle,I., De Smet,G., Leclercq,G., and Plum,J., Notch signaling induces cytoplasmic CD3 epsilon expression in human differentiating NK cells. *Blood* 2007. 110: 2696-2703.

[174] Anderson,M.K., Hernandez-Hoyos,G., Dionne,C.J., Arias,A.M., Chen,D., and Rothenberg,E.V., Definition of regulatory network elements for T cell development by perturbation analysis with PU.1 and GATA-3. *Dev.Biol.* 2002. 246: 103-121.

[175] Pai,S.Y., Truitt,M.L., Ting,C.N., Leiden,J.M., Glimcher,L.H., and Ho,I.C., Critical roles for transcription factor GATA-3 in thymocyte development. *Immunity.* 2003. 19: 863-875.

[176] Hernandez-Hoyos,G., Anderson,M.K., Wang,C., Rothenberg,E.V., and berola-Ila,J., GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. *Immunity.* 2003. 19: 83-94.

[177] Nawijn,M.C., Ferreira,R., Dingjan,G.M., Kahre,O., Drabek,D., Karis,A., Grosveld,F., and Hendriks,R.W., Enforced expression of GATA-3 during T cell development inhibits maturation of CD8 single-positive cells and induces thymic lymphoma in transgenic mice. *J.Immunol.* 2001. 167: 715-723.

[178] Chen,D. and Zhang,G., Enforced expression of the GATA-3 transcription factor affects cell fate decisions in hematopoiesis. *Exp.Hematol.* 2001. 29: 971-980.

[179] Taghon,T., De Smedt,M., Stolz,F., Cnockaert,M., Plum,J., and Leclercq,G., Enforced expression of GATA-3 severely reduces human thymic cellularity. *J.Immunol.* 2001. 167: 4468-4475.

[180] Burns,C.E., Traver,D., Mayhall,E., Shepard,J.L., and Zon,L.I., Hematopoietic stem cell fate is established by the Notch-Runx pathway. *Genes Dev.* 2005. 19: 2331-2342.

[181] Garbe,A.I. and von Boehmer,H., TCR and Notch synergize in alphabeta versus gammadelta lineage choice. *Trends Immunol.* 2007. 28: 124-131.

[182] Hayes,S.M. and Love,P.E., Strength of signal: a fundamental mechanism for cell fate specification. *Immunol.Rev.* 2006. 209: 170-175.

[183] Hayes,S.M. and Love,P.E., A retrospective on the requirements for gammadelta T-cell development. *Immunol.Rev.* 2007. 215: 8-14.

[184] Lauritsen,J.P., Haks,M.C., Lefebvre,J.M., Kappes,D.J., and Wiest,D.L., Recent insights into the signals that control alphabeta/gammadelta-lineage fate. *Immunol.Rev.* 2006. 209: 176-190.

[185] Melichar,H. and Kang,J., Integrated morphogen signal inputs in gammadelta versus alphabeta T-cell differentiation. *Immunol.Rev.* 2007. 215: 32-45.

[186] Ye,S.K., Agata,Y., Lee,H.C., Kurooka,H., Kitamura,T., Shimizu,A., Honjo,T., and Ikuta,K., The IL-7 receptor controls the accessibility of the TCRgamma locus by Stat5 and histone acetylation. *Immunity.* 2001. 15: 813-823.

[187] Zhao,H., Nguyen,H., and Kang,J., Interleukin 15 controls the generation of the restricted T cell receptor repertoire of gamma delta intestinal intraepithelial lymphocytes. *Nat.Immunol.* 2005. 6: 1263-1271.

[188] Yu,Q., Erman,B., Park,J.H., Feigenbaum,L., and Singer,A., IL-7 receptor signals inhibit expression of transcription factors TCF-1, LEF-1, and RORgammat: impact on thymocyte development. *J.Exp.Med.* 2004. 200: 797-803.

[189] Goux,D., Coudert,J.D., Maurice,D., Scarpellino,L., Jeannet,G., Piccolo,S., Weston,K., Huelsken,J., and Held,W., Cooperating pre-T-cell receptor and TCF-1-dependent signals ensure thymocyte survival. *Blood* 2005. 106: 1726-1733.

[190] Sun,Z., Unutmaz,D., Zou,Y.R., Sunshine,M.J., Pierani,A., Brenner-Morton,S., Mebius,R.E., and Littman,D.R., Requirement for RORgamma in thymocyte survival and lymphoid organ development. *Science* 2000. 288: 2369-2373.

[191] Ciofani,M., Knowles,G.C., Wiest,D.L., von Boehmer,H., and Zuniga-Pflucker,J.C., Stage-specific and differential notch dependency at the alphabeta and gammadelta T lineage bifurcation. *Immunity.* 2006. 25: 105-116.

[192] Garbe,A.I., Krueger,A., Gounari,F., Zuniga-Pflucker,J.C., and von Boehmer,H., Differential synergy of Notch and T cell receptor signaling determines alphabeta versus gammadelta lineage fate. *J.Exp.Med.* 2006. 203: 1579-1590.

[193] Bonneville,M., Ishida,I., Mombaerts,P., Katsuki,M., Verbeek,S., Berns,A., and Tonegawa,S., Blockage of alpha beta T-cell development by TCR gamma delta transgenes. *Nature* 1989. 342: 931-934.

[194] Chien,Y.H., Iwashima,M., Kaplan,K.B., Elliott,J.F., and Davis,M.M., A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. *Nature* 1987. 327: 677-682.

[195] Ferrero,I., Mancini,S.J., Grosjean,F., Wilson,A., Otten,L., and MacDonald,H.R., TCRgamma silencing during alphabeta T cell development depends upon pre-TCR-induced proliferation. *J.Immunol.* 2006. 177: 6038-6043.

[196] Ishida,I., Verbeek,S., Bonneville,M., Itohara,S., Berns,A., and Tonegawa,S., T-cell receptor gamma delta and gamma transgenic mice suggest a role of a gamma gene silencer in the generation of alpha beta T cells. *Proc.Natl.Acad.Sci.U.S.A* 1990. 87: 3067-3071.

[197] von Boehmer,H., Bonneville,M., Ishida,I., Ryser,S., Lincoln,G., Smith,R.T., Kishi,H., Scott,B., Kisielow,P., and Tonegawa,S., Early expression of a T-cell receptor beta-chain transgene suppresses rearrangement of the V gamma 4 gene segment. *Proc.Natl.Acad.Sci.U.S.A* 1988. 85: 9729-9732.

[198] Wilson,A. and MacDonald,H.R., A limited role for beta-selection during gamma delta T cell development. *J.Immunol.* 1998. 161: 5851-5854.

[199] Melichar,H.J., Narayan,K., Der,S.D., Hiraoka,Y., Gardiol,N., Jeannet,G., Held,W., Chambers,C.A., and Kang,J., Regulation of gammadelta versus alphabeta T lymphocyte differentiation by the transcription factor SOX13. *Science* 2007. 315: 230-233.

[200] Silva-Santos,B., Pennington,D.J., and Hayday,A.C., Lymphotoxin-mediated regulation of gammadelta cell differentiation by alphabeta T cell progenitors. *Science* 2005. 307: 925-928.

[201] Lee,P.P., Fitzpatrick,D.R., Beard,C., Jessup,H.K., Lehar,S., Makar,K.W., Perez-Melgosa,M., Sweetser,M.T., Schlissel,M.S., Nguyen,S., Cherry,S.R., Tsai,J.H., Tucker,S.M., Weaver,W.M., Kelso,A., Jaenisch,R., and Wilson,C.B., A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity.* 2001. 15: 763-774.

[202] Miyazaki,M., Miyazaki,K., Itoi,M., Katoh,Y., Guo,Y., Kanno,R., Katoh-Fukui,Y., Honda,H., Amagai,T., van Lohuizen,M., Kawamoto,H., and Kanno,M., Thymocyte proliferation induced by pre-T cell receptor signaling is maintained through polycomb gene product Bmi-1-mediated Cdkn2a repression. *Immunity.* 2008. 28: 231-245.

[203] Gebuhr,T.C., Kovalev,G.I., Bultman,S., Godfrey,V., Su,L., and Magnuson,T., The role of Brg1, a catalytic subunit of mammalian chromatin-remodeling complexes, in T cell development. *J.Exp.Med.* 2003. 198: 1937-1949.

[204] Cobb,B.S., Nesterova,T.B., Thompson,E., Hertweck,A., O'Connor,E., Godwin,J., Wilson,C.B., Brockdorff,N., Fisher,A.G., Smale,S.T., and Merkenschlager,M.**,** T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. *J.Exp.Med.* 2005. 201: 1367-1373.

[205] Anderson,S.J., Lauritsen,J.P., Hartman,M.G., Foushee,A.M., Lefebvre,J.M., Shinton,S.A., Gerhardt,B., Hardy,R.R., Oravecz,T., and Wiest,D.L.**,** Ablation of ribosomal protein L22 selectively impairs alphabeta T cell development by activation of a p53-dependent checkpoint. *Immunity.* 2007. 26: 759-772.

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Chapter 7

Development of Natural Killer Cell Diversity

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Abstract

Natural killer (NK) cells were discovered some 30 years ago due to their capacity to spontaneously lyse tumour target cells *in vitro*. Although initially received with scepticism, the 'natural' cytotoxicity exhibited by NK cells has been molecularly dissected and is now generally accepted as an alternative cellular mechanism to detect infected, stressed or transformed cells. Subsequent studies showed that during viral or bacterial infections, NK cells are also prodigious cytokine producers, and by their ability to rapidly amplify and recruit inflammatory cells they play an important role in innate immunity. Thus, in a short period of time, NK cells have evolved from a 'tissue culture artefact' to an essential player in the innate immune defence system. Most immunologists view NK cells as rapidly-reacting innate lymphocytes that perform stereotyped roles including target cell lysis and IFN-γ secretion to promote Th1 responses. Nevertheless, several reports suggest that NK cells exhibit functional diversity that may be subserved through distinct NK cell subsets. In addition to the bone marrow, multiple tissue sites (including the thymus, lymph node and intestine) can generate cells bearing NK-specific markers that show distinct functional properties. In some cases (for example, at mucosal surfaces), NKp46^{+} cells play an important role in tissue homeostasis through a cross-talk with epithelial cells. In this situation, these 'NK' cells are clearly distinguished from classical NK cells that are involved in immune defence. These recent observations suggest that NK cells should be considered not only in 'reactive' innate immunity both also in 'pre-emptive' defence that operates by improving barrier functions at epithelial surfaces.

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Introduction

Natural Killer (NK) cells owe their name to their ability to spontaneously lyse tumour cells without prior activation. It was this characteristic that initially attracted the interest of several research groups in the 1970's and led to their discovery [1-4]. Much of the early effort was focused on understanding the mechanisms controlling the spontaneous cytotoxic activity of NK cells. An equally important issue was why and how 'natural killers' did not attack self-tissues (NK cell 'tolerance'). It was later learned that NK cells belong to the lymphoid lineage, and like B and T lymphocytes, have the ability to distinguish self from non-self [5]. Nevertheless, the tolerance mechanisms used by NK cells and their adaptive counterparts differ greatly. Unlike B and T lymphocytes that use rearranged antigen receptors that are restricted by self-encoded Major Compatibility Complex (MHC) molecules and recognize foreign peptides complexed to MHC, NK cells use germ line-encoded inhibitory NK cell receptors that distinguish self-MHC from 'altered', 'stressed' or non-self-MHC molecules expressed at the cell surface [6-8]. Thus, NK cells and adaptive lymphocytes (B, T) use fundamentally different strategies to detect invading pathogens, with NK cells surveying self MHC for alterations (stress, transformations), as opposed to B and T lymphocytes that detect foreign pathogens or pathogen-derived peptides, respectively.

Infection and cellular transformation often lead to changes in the expression of cell surface molecules. This indirect 'signature' can be 'read' by lymphocytes that patrol tissues. For example, viruses generally down-modulate MHC class I expression on the cells they infect. As such, these cells become 'invisible' to CD8 T lymphocyte recognition and destruction. Nevertheless, virally infected cells lacking MHC do not escape immune detection since they become targets for NK cells that survey self-MHC molecules at the cell surface [9]. However, some viruses are experts at this game of 'hide and seek' and have evolved an amazing repertoire of molecules and mechanisms that allow their potential escape from immune recognition [10]. These include virus encoded proteins that complex and inhibit expression of ligands for NK cell activating receptors, as well as proteins that interfere with soluble factors required for the inflammatory response.

In contrast, cellular transformation does not necessarily lead to the reduced expression of MHC class I molecules but is more generally associated with the inducible cell surface expression of a family of 'stress' proteins. Ligands for NK cell activating receptors (including NKG2D or natural-killer group 2, member D expressed by all NK cells) represent another "pathogenic alteration" system that operates independently of the inhibitory MHC-specific NK cell receptors [7].

Despite their designation, we now know that only a fraction of peripheral NK cells (in mice or man) truly act as 'natural killers'. In addition, NK cells possess a variety of functional effector molecules that allow them to modify cells that are within their microenvironment. These include soluble factors that can amplify immune recognition via up-regulation of MHC molecules on target cells (IFN-γ), while others activate neutrophils (TNF-α), recruit hematopoietic cells (GM-CSF, chemokines) and potentially suppress the inflammatory response (IL-10) [11]. Thus, NK cells are involved in different steps of the immune response (detection, first-line defence, and subsequent amplification), they interface directly with cells involved in the adaptive immune responses (DC cross-talk), and they are

potentially involved in the regulation of autoimmunity [12]. This diversity of NK cell functions is an important concept, since NK cell functions have long been considered as stereotyped (mainly involving target cell destruction). NK cell functions can be modulated and in some cases evolve to provide new biological effectors depending on environmental signals [13].

Considering their roles as multi-tasking, rapidly-acting innate immune effectors, we have proposed that NK cells might perform unique functions following diversification into specialized subsets [14, 15]. Still, we are just beginning to accumulate detailed knowledge about the developmental pathways that lead to NK cell diversity. We realize that the tissue microenvironment impacts strongly on the development of NK cell effector functions. Whether this process acts on common hematopoietic progenitors that seed distinct tissue sites or involves an entirely local developmental scheme remains unclear. Whatever the mechanism, NK cell diversity might reflect unanticipated functional specialization of tissue-bound/location-bound NK populations that could provide the respective tissues/sites with effector cells that are adapted to fit optimally to tissue/site-specific immune requirements.

This review will consider the developmental origins and biological implications of NK cell diversity. As almost all processes of NK cell differentiation appear to occur in the BM, an in-depth description of BM NK cell development provides details about the basic processes that are likely to occur for NK cell generation at other tissue site. Following the description of BM NK cell development, we will discuss variations on this developmental scheme that are exemplified at other sites of the body. We will conclude by commenting on the possible inter-relatedness of NK cell development at these different tissue sites and how this knowledge indicates potential new biological roles for NK cells.

NK Cell Development: General Comments

The developmental process in the immune system starts with the commitment of hematopoietic precursors to the lymphoid lineage and cumulates with the generation of fully functional mature cells. Along the way, intermediate stages, where progenitor cells acquire the expression of lineage specific proteins and lineage-specific functional attributes, can be identified. The cells undergoing this developmental process also undergo ordered changes in their cell surface phenotype that allows discrete developmental events to be associated with cell surface marker expression. Complementing this analysis is the identification of precursor-product relationships, whereby developmental intermediates may be linked following *in vitro* culture or *in vivo* adoptive transfer. Together, these approaches have led to models of NK cell development with the definition of several developmental stages.

The best-known pathway of NK cell development takes place in the bone marrow (BM). It is generally assumed that the BM represents the primary site for NK development since impairment of this environment has profound deleterious consequences for the homeostasis of the peripheral NK cell compartment. The earliest stage of BM NK development is characterized by the NK-cell precursor (NKP), which by definition has committed to the NK cell lineage and thus lost the potential to develop into any other haematopoietic lineage. NKPs give rise to immature NK cells (iNK cells), which express some (but not all) NK-cell-

specific markers. At the iNK stage, developing NK cells begin to express NK-cell receptors specific for MHC class I molecules. Only if a developing NK cell expresses a MHC class I receptor specific for self-MHC class I molecules can this NK cell achieve full functional competence. This process has been termed "education". Education represents an important checkpoint in NK cell development as expressing a self-specific MHC class I receptor equates with the capacity to distinguish self-MHC from altered/non-self MHC. It is important to note, however, that 'un-educated' NK cell precursors are not deleted and have been identified (albeit as a small population) in the periphery of normal humans and mice [16-18]. Although MHC class I receptor-mediated immune activation is impaired in 'un-educated' NK cells, they appear indistinguishable from educated NK cells following MHC I-independent stimulation. Whether iNK cells have functional roles on their own other than being precursors for more mature NK cells is likewise not clear. NK cells that complete the education process have a mature phenotype and represent the majority population of NK cells in the peripheral lymphoid tissues.

Bone Marrow NK Cell Development

Natural Killer Cell Precursors (NKP)

NKP are hematopoietic cells that have a restricted lineage potential and give rise only to mature NK cells. As such, NKP differ from other lymphoid lineage precursors that additionally have B and/or T lymphocyte potential (common lymphoid precursors, early lymphoid progenitors, etc). The only cell population in the adult mouse bone marrow that fulfils the NKP definition lacks expression of other hematopoietic lineage markers (Lin^-) including the NK markers NK1.1 and CD49b (α_2-integrin, which is recognized by the monoclonal antibody DX5) yet expresses CD122 (IL-2Rβ, a shared cytokine receptor for IL-2 and IL-15). This $Lin^-CD122^+NK1.1^-CD49b^-$ population has been shown to generate mature NK cells *in vitro* or in foetal thymic organ cultures [19]. Still, this population is not homogenous, neither phenotypically (only a fraction of NKP express activating receptors NKG2D, the CD117 growth factor receptor for stem cell factor or the CD127 cytokine receptor for IL-7), nor functionally (only about 1 in 10 of such defined NKP gives rise to a NK cell *in vitro*). It remains to be shown whether the NK potential of NKP segregates with the expression of these different cytokine and growth factor receptors [20]. Finally, NKP provide a means to identify other markers that may more accurately define NK cell precursors.

Immature NK Cells (iNK)

NKP develop to mature NK cells via several phenotypically-defined intermediates (see Figure 1). As a group, these are denoted as immature NK cells or iNK cells. Mice that express the allelic variant of CD161c or NKR-P1C, detected by the PK136 antibody (NK1.1, expressed by C57BL/6 and C57BL/10 mouse strains) are useful for defining iNK cells as

CD161c is one of the first NK cell markers that is acquired by NKP as they mature. It should be emphasized here that NK1.1 is not a 'NK cell-specific' marker since it is also expressed by subsets of T cells. Nevertheless, when $CD3^+$ T lymphocytes are excluded, expression of this marker is restricted to NK cells and their precursors. Immature NK cells also express the natural cytotoxicity receptor (NCR) NKp46. NCRs are a family of activating receptors (including NKp30, NKp44 and NKp46 in humans; NKp46 is the sole NCR expressed in mice) that have a highly selective expression in NK cells [21, 22]. Unlike mature NK cells, iNK do not express CD49b (DX5), CD94 (a C-type lectin forming heterodimers with members of the NKG2 family) or the Ly49 family of MHC class I-specific inhibitory receptors. Of the other phenotypic markers used to characterize NK cells, CD27 is well expressed but neither CD43 nor KLRG-1 and only low levels of CD11b (a_M-integrin) and CD11c (α_X-integrin) are found on iNK. Functionally, iNK cells are not cytotoxic for YAC-1 tumour cells *in vitro* and fail to secrete IFN-γ when stimulated by cell surface receptor triggering or by soluble factors [19]. As such, iNK cells have an appropriate designation with respect to classical NK cell effector functions. Nevertheless, it remains possible that iNK cells have biological roles beyond those defined by cytotoxicity and IFN-γ production.

NK Cell Education towards Self-MHC

The process of education likely begins as developing NK cells start to express MHC class I-specific inhibitory receptors. In the mouse, NK cells express two different classes of MHC class I-specific inhibitory receptors, CD94/NKG2 heterodimers and Ly49 molecules [23, 24]. While most NK cells express one or more of these receptors, no single CD94/NKG2 heterodimer or Ly49 molecule is expressed by all NK cells. Instead, CD94/NKG2 heterodimers or Ly49 molecules (or combinations thereof) are expressed at relatively constant frequencies by NK cells in a given inbred mouse strain. NK cells usually express more than one Ly49 receptor and can co-express both CD94/NKG2 and Ly49 family members at the same time. In general terms, CD94/NKG2 heterodimers are expressed earlier during NK cell development and allow for a more global recognition of MHC class I expression by surrounding cells as they recognize MHC class I-derived leader peptides presented via non-classical MHC class I Qa-1b molecules [25]. In contrast, Ly49 molecules have a more restricted capacity to interact with particular MHC class I alleles. Both classes of molecules are C-type lectins and contain canonical immunotyrosine-based inhibitory motifs (ITIM) that account for their inhibitory function [26]. In addition, there are activating members in both families whose expression appears somewhat delayed during NK cell maturation. That iNK cells first express CD94/NKG2 heterodimers (at quite high frequency) might indicate a so far undisclosed function. Ly49 expression follows that of CD94/NKG2 heterodimers. As the frequencies of NK cells expressing a specific Ly49 molecule increases during development, the frequency of CD94/NKG2 expressing NK cells decreases to around 50% which remains stable on mature NK cells. Whether this dynamic equilibration of NK cell inhibitory receptors is simply due to differential expression of the genes involved or could indicate an ongoing selection process is not known. Interestingly, most iNK express

	NKP	iNK		mNK		
Stage	A	B	C	D	E	F
CD122	+	+	+	+	+	+
NKG2D	+	+	+	+	+	+
NK1.1	-	+	+	+	+	+
CD94	-	+	+	+	+	+
TRAIL	-	+	+	-	-	+
CD51	-	+	+	-	-	-
IL-21R	-	+	+	-	-	-
Ly49	-	-	+	+	+	+
DX5	-	-	-	+	+	+
CD11b	-	-	lo	lo	hi	hi
CD43	-	-	lo	lo	lo	hi
CD27	-	+	+	+	+	-
KLRG-1	-	-	-	-	-	+
lytic	-	-	-	+*	+	+
IFN-γ	-	-	?	+*	+	+

Figure 1. Model for bone marrow NK cell development
Stages (A-F) of developing NK cells in lineage (CD3, CD19, Gr-1, Ter-119)-negative mouse bone marrow can be defined using the cell surface markers shown on the left. NKP, NK cell precursor; iNK, immature NK cell; mNK, mature NK cell.

CD69, which might indicate receptor engagement during the NK cell education process in an analogous fashion to that observed during thymocyte selection. Alternatively, CD69 expression might relate to lymphocyte retention through inhibition of S1P activity as demonstrated for T lymphocytes [27].

The process of education generates two broad types of NK cells that show self-tolerance. The first type includes the vast majority of NK cells that expresses at least one inhibitory NK cell receptor that recognizes self-MHC class I molecules, and as such, these cells have the capacity to sense variations in MHC class I expression levels on target cells caused by pathogenic alterations. A second type of NK cell (estimated to be about 10-20% of the peripheral NK cell pool) does not express self-specific MHC class I receptors, and as such, cannot sense self-MHC class I molecules. The existence of this second type of NK cells poses a problem, as these cells should be self-reactive. However, multiple reports have documented the existence of NK cells lacking self-MHC reactive inhibitory receptors and have shown that they are, in fact, tolerant due to functional hypo-responsiveness [16-18]. Several mechanisms

have been proposed that could allow ‘hypo-responsive’ NK cells to develop. As already indicated above, it has been shown that engagement of inhibitory MHC class I receptors on iNK cells is required for the acquisition of full functional competence by these cells. According to the “receptor calibration model” [28] signals derived from inhibitory receptors would balance signals from activating receptors. NK cells expressing inhibitory receptors that can fully balance these activating signals create a strong activating potential. In contrast, NK cells whose inhibitory receptors cannot fully balance the activating signals receive a more or less constant activating signal that would then render those cells ‘hypo-responsive’. The degree of NK cell responsiveness would be directly correlated to the extent of inhibitory receptor signalling during development. Still, the identification of the activating signal(s) (and the activating receptor(s) that is (are) triggered) involved in this process remains undefined. Moreover, this process could require multiple signals that might act in an independent or cumulative fashion.

Developing NK cells that express high-affinity self-MHC inhibitory receptors or higher numbers of self-MHC inhibitory receptors complete their differentiation with a higher activation potential than NK cells expressing low-affinity or reduced amounts of self-MHC inhibitory receptors. An elegant report from the Hoglund group recently provided data to support this model by using a group of mice carrying single MHC I alleles that were bound by the Ly49 with different affinities for the self-MHC [29]. The functional activities of the NK cells from these ‘single MHC’ mice correlated with the affinity of their Ly49 molecules for the self-MHC allele that was present. Furthermore, the presence of a second or third self-MHC recognized by the Ly49 repertoire expressed by the NK cells revealed an additive effect on the NK cell activity. Thus, the quality and quantity of self-MHC recognition by Ly49 inhibitory molecules influence the activating potential of developing NK cells. This can be explained by the fact that an MHC-I allele is often recognized by multiple Ly49s (albeit with different affinities) and that one Ly49 molecule displays promiscuity in binding (with different affinities) to more than one MHC-I allele. Taking into account that one NK cell expresses usually more than one (and up to six different) Ly49 molecule, the possibility that NK cells expressing more than one self-MHC-I Ly49 receptor provides a large and heterogeneous pool that can be ‘tuned’ against the activating signals present during NK cell development in the BM.

However, NK cells do not express unlimited numbers of self-MHC-I receptors. Transgenic expression of Ly49A by all NK cells in mice expressing the respective MHC-I molecule (H-2^{d}) reduces the frequency of expression of other H-2^{d}-specific Ly49 molecules by NK cells [30]. Furthermore, it was shown that NK cells from mice lacking MHC-I molecules express more Ly49 molecules specific for a given MHC-I allele on a per cell basis. Thus, expression of one self-MHC receptor appears to impact on the expression of other self-MHC I receptors on the same NK cell, which is consistent with the idea that developing NK cells sequentially express Ly49 molecule until engagement of these by self-MHC-I molecules induces the cessation of expression of further different Ly49 molecules. Moreover, Ly49 repertoire formation is dynamic and dependent on environmental cues. In the presence of the respective MHC-I molecule, Ly49 expression can be down-modulated (although it is not clear whether the MHC-I/Ly49 interactions occur in ‘*cis*’ or ‘*trans*’; [31]. Nevertheless, this reduction in Ly49 expression equips the cell with the capacity to respond to modulation of

MHC-I expression by surrounding cells. Although it is not known whether a qualitative threshold exists, it is conceivable that NK cell expressing many self-MHC receptors could more easily modulate its Ly49 receptors compared to a NK cell expressing fewer receptors. The chance of encountering sufficient interactions of Ly49 molecules with their respective MHC I ligand is higher when many of the Ly49 molecules are present – even when the target cell has down modulated its expression of MHC I molecules. On the other hand, when a NK cells with few self-MHC specific Ly49 inhibitory molecules interacts with a target cell, the chance of encountering not enough inhibitory signals increases if the target cells has down modulated its expression of MHC I molecules.

Since NK cells expressing no self-MHC receptors are not deleted, it can be assumed that they have a biological function. What is clear from published data is that these cells are not inert [16]. While they are incompetent in sensing variations in self-MHC class I expression levels, they seem to be indistinguishable from "educated" NK cells in their capacity to secrete IFN-γ in the context of infections *in vivo*. As such, NK cells expressing no self-MHC receptors might be specifically involved in MHC class I-independent immune responses or become activated as bystander cells during MHC class I-dependent responses where they could function as 'response amplifying units'. Another possibility is that these NK cells might also become specialized in performing regulatory functions. Along these lines, it is not known whether the outcome of a NK-DC interaction involving a 'competent' (self-MHC receptor expressing) NK cell is qualitatively different from one involving an 'incompetent' (no self-MHC receptor) NK cell. Alternatively, other MHC class I-independent ligand-receptor pairs might also contribute to the education of NK cells as it was shown for C-type lectins and NKP-R1 receptors [32]. This would imply the involvement of multiple educational processes in the development of NK cells generating a pool of cells with varying levels of competence and specialization. Clearly we have still much to learn about the complex interplay between different NK cell receptors during NK cell education and functional maturation and about the molecular mechanisms involved in this process.

Role of Activating Receptors in NK Cell Development

In contrast to inhibitory receptors, the precise role for activating receptors during NK cell development remains to be elucidated. Several NK cell activating receptors have been studied in the context of NK cell development, and we will summarize data from three of the main classes involved: the ITAM-bearing receptors, NKG2D and SLAM-family receptors (comprising SLAM, 2B4, Ly9 and others).

ITAM-Bearing Receptors

This is the largest group of activating receptors that includes activating Ly49 receptors (Ly49D,H,P) the CD94/NKG2C heterodimer, the CD16 FcR, the C-lectin NKRP1 receptor family (including NKRP1C recognized by NK1.1), and the natural cytotoxicity receptors (NKp46 is the only member of this family that is expressed in mice). All of these proteins have relatively short cytoplasmic tails and lack intrinsic signalling capacity. These receptors associate, via a charged amino acid in their transmembrane region, to immunotyrosine-based

activation motif (ITAM)-bearing signalling molecules, including FcεRIγ, CD3ζ, and DAP12, to form homo- or heterodimeric complexes [33]. After receptor engagement, Src-family kinase-mediated phosphorylation of the ITAM-tyrosines allows subsequent recruitment of Syk and ZAP-70 kinases that initiate a proximal signalling cascade. As all known ITAM signalling events are Syk-ZAP-70-dependent, Syk/ZAP-70 double-deficient mice provide a means of assessing the roles for ITAM-bearing receptors in NK development. Remarkably, BM and splenic NK cells develop normally in the absence of Syk/ZAP-70 and mature peripheral NK cells display normal cytotoxic activity and cytokine secretion when triggered by non-ITAM dependent receptors. Thus, Syk/ZAP-70-coupled ITAM-bearing receptors appear largely redundant for normal NK cell development and differentiation and for homeostasis of the peripheral NK cell pool [34].

Recently, two groups reported transgenic mice that constitutively express a ligand (the mouse cytomegalovirus encoded m157 protein) for the ITAM-dependent Ly49H molecule [35, 36]. Interestingly, $Ly49H^+$ NK cells in m157 transgenic mice were reduced and appeared phenotypically more immature than $Ly49H^-$ NK cells. Functionally, $Ly49H^-$ NK cells were normal while $Ly49H^+$ NK cells showed only reduced cytolytic and cytokine-producing capacity when stimulated in a Ly49H-dependent (but not Ly49H-independent) fashion. These data suggest that constitutive Ly49H signalling may 'desensitize' this activating pathway but does not globally impede NK cell development. Further studies will be required to determine the effects of Ly49H desensitization on other activating receptor pathways during specific immune responses.

NKG2D

This activating receptor is one of the first receptors expressed in the NK cell lineage starting at the NKP stage [20]. Whether NKG2D expression is a marker of NKP or whether its acquisition is the first step in NK cells developing from NKG2D- NKP is not yet known. However, since NKG2D is expressed before any known inhibitory Ly49 receptor, it could represent a key activating receptor that is tested during early NK cell development. Still, we have little data on the expression of NKG2D ligands in the BM. Mice lacking NKG2D do not show any obvious defects in NK cell development, and NKG2D-deficient NK cells display a relatively normal phenotype with functional defects restricted to NKG2D-dependent processes [37].

NKG2D ligands are MHC-I related molecules including H60 and the retinoic-acid inducible gene products Rae-1α, -1β, -1γ, -1δ,̃ ιε in the mouse [7] that are stress-induced proteins typically expressed by transformed or infected cells. The importance of these ligands in tumour immunosurveillance by NKG2D-expressing immune cells could explain the defective tumour surveillance seen in NKG2D-deficient mice [37].

Like ITAM-bearing receptors, the NKG2D molecule appears to be signalling incompetent and lacks any known signalling motif. NKG2D primarily interacts with the transmembrane signalling adaptor molecule DAP10. Upon DAP10 phosphorylation, the p85 subunit of PI3K and Grb2 are recruited to the NK2GD/DAP10 complex [38]. While DAP10-deficient NK cells showed reduced cytolytic capacity against targets expressing NKG2D ligands [39], engagement of NKG2D in $DAP10^{-/-}$ mice triggered cytokine production and

cytotoxicity probably due to the promiscuous association of DAP12 to an alternatively-spliced form of NKG2D that can be detected in mouse (but not human) NK cells [39, 40].

Constitutive expression of a NKG2D ligand (Rae-1ε), locally or systemically, led to a phenotype of reduced NKG2D expression. Constant engagement of the NKG2D receptor not only modified its expression, but also resulted in reduced responses following NKG2D stimulation [41]. Moreover, NK cells from the Rae-1 transgenic mice showed a more generalized defect in their ability to kill MHC-I deficient targets (from $\beta 2m^{-/-}$ mice) or RMA-S tumour cells (that lack MHC-I expression). Still, neither the absence nor the constitutive stimulation of the NKG2D receptor prevented normal NK cell development, suggesting that this receptor system was redundant for differentiation of most peripheral NK cells.

SLAM-Family Receptors

Several receptors form a family based on homology to signalling lymphocytic activation molecule (SLAM). These receptors belong to the immunoglobulin (Ig)-like super-family and include CD150 (SLAM), CD2, CD244 (2B4) and also CD48, CD84, CD229 (Ly9), NTBA, CD319 (CRACC). In the NK cell lineage, the best studied SLAM receptor is CD244 that is expressed by all NK cells and by subsets of γδ T cells, αβ T cells and other hematopoietic cells. The major ligand for CD244 is the Ig-family molecule CD48 that is expressed by all hematopoietic cells, including NK cells. In mice, two CD244 isoforms are expressed due to alternative splicing [42]. The long CD244 isoform is proposed to have inhibitory functions, whereas the short isoform might be activating [43], although definitive evidence for this dichotomy has yet to be provided. CD244 signals via the SH2-containing adaptor molecules SLAM-associated protein (SAP), EAT-2, or ERT [44]. SAP has been associated with activating signals. NK cells from SAP-deficient mice show a decreased capacity to kill target cells expressing the CD244-ligand CD48. In humans, lack of SAP causes the X-linked lymphoproliferative disease (XLP) resulting in either the reduction or inhibition of NK cell responses to CD244 cross-linking [45].

NK cells in CD244-deficient mice have a normal phenotype but display defective IFN-γ production and cytotoxicity towards CD48-bearing targets [46]. While this latter has initially been attributed to the lack of CD244-mediated activation of NK cells, it later became clear that CD244/CD48 interactions between NK cells are necessary to prevent perforin-dependent fratricide [47]. Consequently, in CD244-deficeint mice activated NK cells can eliminate each other leading to the observed reduction in NK cell responses upon stimulation. It was further shown that blocking CD244/CD48 interactions does not impact on the ability of NK cells to secrete granzymes upon stimulation.

As opposed to adaptive lymphocytes, NK cells express multiple classes of activating receptors that allow them to ‘cover’ a diversity of target cell ligands. These receptors operate independently of each other and the inactivation of any one activating receptor class does not impede the function of the others. This provides redundancy in the activation process that could explain why inhibition of a single activating pathway does not block the developmental program. Such a system might also provide a safeguard against pathogens that would selectively target one type of activating receptor.

Mature NK Cells in the Peripheral Tissues are Heterogeneous

NK cells that have completed their education (irrespective of whether or not they attained full functional competence) can be detected in the 'periphery'. What constitutes the 'periphery' is not fully defined, but includes those tissues sites that are distinct from the generative sites. This often includes the spleen, lymph nodes, lung, and the liver, although it potentially can include any tissue as mature NK cells are found in the circulation (blood, lymph). Considering their roles in innate immune defence, one would certainly expect NK cells to also be cellular actors at mucosal sites (gastrointestinal tract, skin, urogenital organs). The localization of NK cells in these different tissues has been documented in the spleen, LN, liver and uterus during gestation. However, it is not known whether different NK cell subsets (as defined by cell surface markers, or by their development - competent versus incompetent, bone marrow versus other sites, etc.) occupy the same niche.

Mature NK cells have been classically defined as: *(i)* $CD3^-NK1.1^+$ cells (in suitable mouse strains such as C57BL/6) or $CD3^-NKp46^+$ cells (in all strains) that express a repertoire of Ly49 receptors, *(ii)* cytolytic against YAC-1 tumor target cells, and *(iii)* they secrete IFN-γ after stimulation with target cells or appropriate cytokines. Although this basic description (for the most part) still holds true, recent data has allowed us to identify some additional features that suggest that NK cells are not homogeneous. A series of markers (including CD11b, CD27 and KLRG-1) can subdivide the peripheral NK cell pool into various subsets [48-50] that have distinct phenotypic and functional properties. Using CD11b and CD27 (see Figure 2), Smyth and colleagues define three NK cell subsets that are linked by a linear developmental program [49]. $CD27^+CD11b^{lo}$ cells appear to represent the most undifferentiated mature NK cells followed by cells with the $CD27^+CD11b^{hi}$ phenotype that ultimately give rise to CD27-$CD11b^{hi}$ cells. In additional to their functional differences, CD11b/CD27 subsets show an unequal distribution in different organs and tissues. For example, $CD27^+CD11b^{lo}$ cells readily produce cytokines after stimulation but are less cytotoxic than $CD27^+CD11b^{hi}$ cells. The former are found predominantly in lymph nodes and bone marrow, whereas $CD27^-CD11b^{hi}$ cells dominate in spleen, blood, and lung [51]. $CD27^+CD11b^{hi}$ cells are more homogenously distributed among the different tissues. Functionally, $CD27^-CD11b^{hi}$ cells seem to be less functional than the other subsets and may represent "exhausted" cells, as they are the only subset to express KLRG-1 (a marker of stimulation and proliferation). Based on tissue distribution and functional capacities [15], one can propose that $CD27^+CD11b^{lo}$ cells as resting (antigen-inexperienced) and $CD27^+CD11b^{hi}$ cells as primed (antigen-experienced) cells.

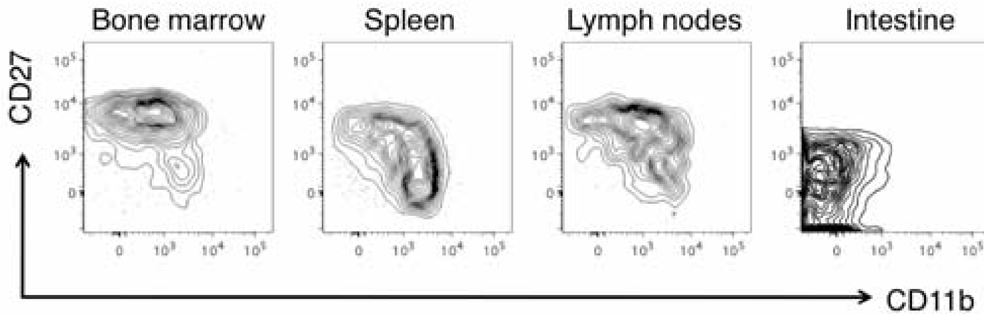

Figure 2. CD11b and CD27 define NK cell Subsets. CD11b versus CD27 expression on CD3$^-$CD19$^-$NKp46$^+$ cells from the indicated tissues is shown.

Although useful, CD11b and CD27 are not the only markers that define murine NK subsets. For example, expression of CD127 (IL-7 receptor alpha-chain) has been shown to mark a novel subset of NK cells distinct from bone-marrow NK cells. This subset is thymus-dependent, and requires GATA-3 and IL-7 for its development (see below). More recently, a novel subset of NKp46$^+$ cells (distinct from bone-marrow and thymus-derived NK cells) has been identified in the gut. These cells are also characterized by the expression of CD127 but appear unrelated to thymic CD127$^+$ cells (see below). It is likely that other markers (both cell surface protein as well as intracellular molecules) will be identified that segregate functionally distinct NK cell subsets in mice and man.

One should emphasize that the relative functional differences between these phenotypically defined subsets is still relatively modest (in the range of 2-to-3 fold). Thus, these subsets share overlapping functional capacities, at least as defined by common standard laboratory *in vitro* assays. One might argue that such assays are obsolete and inappropriate for trying to identify and characterize NK cell functional diversity. One major challenge in the NK field is the development of *in vitro* assays that will be useful in identifying functional NK cell subsets that have physiological relevance. It remains to be demonstrated that the aforementioned phenotypically defined NK cell subsets have any particular biological role *in vivo*.

Several activation states of mature NK cells can be proposed, including resting, primed, and exhausted (the latter may be considered as post-activation or chronically stimulated) (see Figure 3). Some of these states correlate with cell surface markers (*i.e*. the lymphocyte early-activation marker CD69 is often used to describe activated cells). We and others have found that NK1.1$^+$NKp46$^+$ NK cells express higher levels of CD11c and B220 and in the lymph nodes also MHC II upon activation. While some have proposed that these activated NK cells represent a new DC subset [52, 53], it appears that these cells are not involved in antigen presentation [54-56]. Finally, NK cell phenotypes can change with age: CD11b^{hi} NK cells become the most abundant subset in the aged mouse spleen. Whether this reflects differential migration or NK cell subsets within the organism or an accumulation of more ‘mature’ NK cells remains to be determined. The collective data suggests that NK cell phenotypes and functions are not fixed, but can evolve under normal circumstances, during both the

differentiation process and following their seeding of peripheral lymphoid and non-lymphoid tissues.

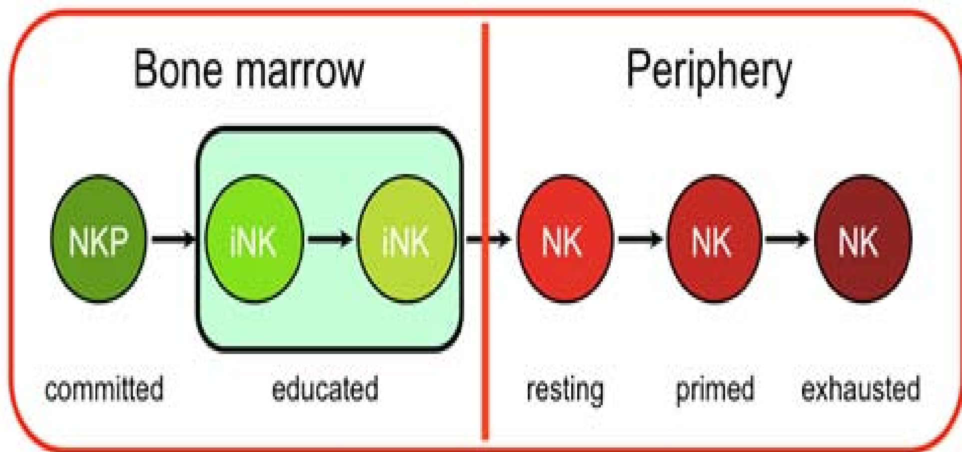

Figure 3. Activation states of developing and peripheral NK cells. NKP, NK cell precursor; iNK, immature NK cell; mNK, mature NK cell. For more details, see text.

Factors that Influence NK Cell Development and Homeostasis

Both intrinsic and extrinsic factors regulate the generation and maintenance of the peripheral NK cell pool. The major intrinsic mechanism involves transcription factors (TF), while a key extrinsic role is provided by soluble growth factors and cytokines elaborated by the tissue microenvironment. Interestingly, TFs are often involved in the regulation of cytokine receptor expression, so that the extrinsically and intrinsically regulated signals are likely coordinated and integrated in developing NK cells.

Transcription Factors

Members of several transcription factor (TF) families are involved at different stages in the regulation of the development of NK cells [57]. In some cases, several members of the same TF family are expressed by developing NK cells making it more difficult to define a clear function to a single factor within a family, since compensatory mechanisms might be operative.

At least two members of the Ets family of TFs are involved in NK cell development. Ets-1 appears to regulate CD122 (IL-2Rβ) expression, whereas PU.1 regulates the expression of

CD117 (c-Kit) and CD127 (IL-7Rα) [58]. As these growth factor and cytokine receptors are expressed by most early lymphoid progenitors and only on a fraction of NKP or immature NK cells, it is not clear whether Ets-1 and PU.1 act directly on NKP specification or indirectly via their effects on the early lymphoid progenitor populations. A third member of Ets family, MEF, appears to act later during NK cell differentiation. In the absence of MEF, NK numbers are normal but show decreased effector functions.

Proteins of the basic helix-loop-helix (bHLH) family of TF (also known as E proteins) are involved in the lineage specification of B and T lymphocytes. Interestingly, inhibition of the E protein function through activity of inhibitors of DNA binding (Id family proteins) appears necessary for NK cell development to occur [59]. Id proteins form heterodimers with E proteins to block their activity. Id2 and Id3 are expressed by NKP and while Id2 is expressed during all NK cell stages, Id3 expression decreases at later stages. Compensation by Id3 might explain why mature NK cells are reduced 10-fold in the absence of Id2 whereas NKP develop normally [60]. The hypothesis that the absence of Id function led to increased E protein activity was supported by the finding that deletion of E2A in Id2$^{-/-}$ mice restored peripheral NK cell numbers [60]. However, these ‘rescued’ NK cells were still phenotypically and functionally immature. How E protein activity impacts on mature NK cell functions remains to be determined.

Several TFs appear to be implicated in the maturation of NK cells in the BM and in the migration to and maintenance of NK cells in the periphery. These include T-bet and Eomes (Eomesodermin) that are two members of the family of T-box binding proteins, as well as the TFs GATA-3 and IRF-2 [58]. In the absence of any of these different TFs, the numbers of BM NK cells are normal [61-64]. In contrast, peripheral NK cells in T-bet$^{-/-}$ mice are reduced, whereas splenic NK cells in IRF-2$^{-/-}$ mice and liver NK cells in absence of GATA-3 are reduced. Moreover, the NK cells found in the periphery of these mice appear immature as they display reduced expression of CD11b and CD43. In the absence of GATA-3, the repertoire of Ly49 molecules also appears disturbed. This immaturity appears also to be reflected by selective functional capacities of NK cells from these mutant mice. NK cells from all of these mice show an impaired production of IFN-γ upon stimulation, whereas NK cells from T-bet$^{-/-}$ also demonstrate decreased cytotoxic activity. The similar phenotypic and functional defects of GATA-3, T-bet and IRF-2-deficient NK cells suggests that these different TFs act in parallel or in a cascading fashion [58]. Nevertheless, clear molecular targets of these TFs that could provide a molecular mechanism to account for these deficiencies have not yet been clearly identified.

Soluble Factors

Several soluble factors (growth factors, cytokines, TNF superfamily members) have been implicated in NK cell development and homeostasis. While the mechanism of action for some of these soluble factors is known (promoting cell survival or proliferation), in other cases the mechanism is not clearly defined. For example, lymphotoxin-α appears important at multiple stages of NK cell differentiation [65], although it is not clear whether it directly acts on developing NK cell precursors or on the cellular elements of the microenvironment.

Common gamma chain (γ_c)-dependent cytokines are essential for lymphocyte homeostasis and are critical for generating a normal NK cell pool in both mice and men. In the absence of γ_c, NK cells are often considered 'lacking' since their steady-state numbers are reduced by 1000-fold [20]. γ_c cytokines include interleukins (IL)-2, -4, -7, -9, -15, and -21 and analysis of mice deficient in the IL-15 pathway have demonstrated that this γ_c cytokine is the dominant factor controlling NK cell development, homeostasis and activation [20, 66-69]. Three subunits of the IL-15 receptor are identified, including IL-15Rα, CD122 (IL-2Rβ common to IL-2 and IL-15), and γ_c. The mechanism of IL-15 action is unusual in that IL-15 is 'presented' in '*trans*' by cells via the IL-15Rα chain to IL-15-responsive cells that respond via the CD122/γ_c complex [70]. Several different cell types co-ordinately express IL-15 and IL-15Rα, including both hematopoietic (DC, macrophages) and non-hematopoietic (epithelial, muscle) lineages. These IL-15$^+$ cells play an important role in the development and homeostasis of NK cells [71]. Of IL-15 'presenting' cells, only DCs have been shown to prime resting NK cells, and this has been proposed as an essential step for NK cells activation during immune responses [69, 72].

Mice deficient in IL-15 show a similarly strong reduction in immature and mature NK cell numbers as γ_c-deficient mice. Nevertheless, both these mutant mice harbour normal numbers of NKPs, suggesting that commitment of hematopoietic precursors to the NK lineage and generation of NKPs is not driven by γ_c-dependent cytokines [20]. Nevertheless, IL-15 appears critical in driving further NK cell development and differentiation from NKPs, at least within the BM microenvironment.

So far, no single soluble factor has been identified that is essential for the development of NKP *in vivo*. This includes those cytokines that appear necessary for the generation of NK cells from hematopoietic precursors *in vitro* (stem cell factor, Flt3-ligand, IL-7, and IL-15; [73]. Nevertheless, NK cells developing in vivo in the absence of CD117 [74], or in the absence of Flt3-ligand [75] demonstrate incomplete defects in NK cell homeostasis and function.

NK Cell Development at Different Tissue Sites: Thymus

Although the presence of NK cells within the thymus has been acknowledged for some time [76], it was only recently recognized that the vast majority of thymic NK cells appear phenotypically and functionally distinct from NK cells found in other tissues [77]. Thymic NK cells can be distinguished by their uniform, strong CD127 (IL-7Rα) expression. Furthermore, thymic NK cells are enriched in CD27^{hi}CD11b^{lo} cells that have little or no expression of CD43 and KLRG-1, and bear few (if any) Ly49 molecules. A large subset (30%) of lymph node NK cells share the 'thymic' NK cell phenotype, and it has been shown that thymic NK cells can be exported to peripheral tissues, including the LN [77]. CD127$^+$ NK cells can be detected in lower frequency (around 5%) in spleen, BM and liver.

Thymic NK cells share some phenotypic similarities with iNK cells in the BM and likewise demonstrate a reduced cytotoxic activity towards typical NK cell targets *in vitro*.

Nevertheless – and unlike iNK cells – thymic NK cells have an increased capacity to secrete cytokines such as IFN-γ, GM-CSF, and TNF-α following stimulation *in vitro*. Their developmental requirements also clearly distinguish thymic NK cells from BM-derived NK cells. In addition to their dependence on an intact thymus, the cytokine IL-7, and the TF GATA-3 are essential for thymic NK cell development [77]. It remains to be shown whether GATA-3 is required for transcriptional activation of the CD127 locus in thymic NK cell progenitors.

The developmental origins of thymic NK cells remain poorly defined [78]. It has been hypothesized that uncommitted T cell precursors within the early thymocyte compartment (including $CD4^{-}CD8^{-}$ double-negative (DN)1 and DN2 subsets) that have been previously shown to have NK cell potential may represent the cellular substrate for thymic NK cell development, but other hematopoietic populations that are present in the thymus (including HSC, ETP, or NKP) may also provide precursors. There is no strict rule that thymic NK cells (or developing NK cells at other sites) should derive from a single precursor population. In addition, one can postulate that particular phenotypic and functional repertoires of NK cells in different tissue sites might be imposed by local environmental cues. Still, the local molecular signals that dictate and promote NK cell development within distinct tissue sites remain to be identified.

Interestingly, thymic $CD127^{+}$ NK cells in mice bear some functional characteristics with the $CD56^{hi}$ subset of human NK cells : both express CD127, GATA-3 and show little or expression of inhibitory or activating MHC class I-specific receptors [77]. Moreover, both $CD127^{+}$ mouse NK cells and human $CD56^{hi}$ NK cells are enriched in the LN, suggesting that a proportion of human $CD56^{hi}$ NK cells might have its origins in the thymus. While the functional roles for thymus- versus BM-derived NK cells remain to be defined, their co-existence in secondary lymphoid tissues would suggest that their different functional capacities might serve unique roles during immune responses.

NK Cell Development at Different Tissue Sites: Lymph Nodes

Lymph nodes contain NK cells derived from the bone marrow (70-90%) and from the thymus (10-30%). $CD127^{+}$ NK cells in the LN display the same phenotype and the same functions (at least in terms of cytokine production and cytotoxicity) as NK cells in the thymus. Thus, a fraction of the $CD127^{+}$ NK cells in the LN are actually thymus-derived [77]. However, recent evidence suggests that DN1 and DN2 early thymocyte precursors can also be identified in the LN [79], suggesting that $CD127^{+}$ NK cells in the LN might not only be derived from the thymus but also develop directly *in situ* from LN precursors. Similar findings in humans suggest a local developmental process in the LN for generating the $CD56^{hi}$ NK cell subset [80]. Still, thymus- and LN-derived NK cell developmental pathways need not be mutually exclusive.

During inflammatory responses, NK cell subsets that express high levels of CD27 are strongly recruited to LN. Using adoptive transfer of Toll-like receptor (TLR)-stimulated DC, it was shown that this process was IFN-γ-dependent [81]. However, since $CD27^{hi}$ NK cells

are normally enriched in LN [49], a DC-IFN-γ loop may also be involved in the predominant localization of $CD27^+$ NK cells in LN under steady-state conditions. If so, this might represent the consequence of continual 'physiologic' immune stimulation that occurs in response to environmental antigens.

Interestingly, thymus-derived $CD127^+$ NK cells also express CD27 but only low levels of CD11b. As such, they contribute to the predominance of $CD27^+$ NK cells in the LN. Whether or not these cells are also recruited during inflammatory responses has not been addressed. However, at least under steady-state conditions, thymus-derived NK cells are not (or only at very low frequency) present in the blood.

Another interesting feature of LN (and BM) NK cells is their ability to express MHC class II molecules. Around 20-30% of NK cells in this tissues express class II under steady-state conditions and this frequency increases after TLR stimulation [55, 56]. In contrast, NK cells from the spleen – either naïve or activated – fail to express MHC class II molecules. Thus, the functional relevance of MHC II expression by NK cells remains curious. It has been shown that MHC II^+ NK cells can stimulate naïve CD4 T cells to proliferate *in vitro* (albeit much less efficiently than $CD11c^{hi}$ DCs). However, it is not clear whether this occurs *in vivo* and, if so, with what consequences.

NK Cell Development at Different Tissue Sites: Intestinal Tract

In the intestinal tract, a coordinated system of hematopoietic and non-hematopoietic cell types works in concert to provide mucosal immune defence. Epithelial cells separate the gut lumen harbouring commensal micro-organisms from the body, and elaborate anti-microbial peptides, cytokines and chemokines that recruit and activate hematopoieitic cells. Intestinal dendritic cells (DC) extend trans-epithelial dendrites into the intestinal lumen and sample its contents for signs of infection, whereas specialized M cells provide Peyers' patch DC with gut antigens to initiate adaptive IgA-dominated immune responses. Whereas NK cells have been documented in the intestinal mucosa [82, 83], the developmental pathways that generate gut NK cells and the biological roles for intestinal NK cells in the intestine are not understood. NK cells, by virtue of their rapid cytokine response, might play an important role in intestinal immunity by interfacing with intestinal DC to regulate immune responses. Alternatively, NK cells may eliminate stressed or infected cells within the intestinal lamina propria or epithelium. Finally, intestinal NK cells may contribute to the maintenance of epithelial homeostasis through novel, and perhaps, atypical mechanisms.

We recently characterized distinct subsets of intestinal lymphocytes that expressed the natural cytotoxicity receptor NKp46 [84]. NKp46 has been shown to be highly and specifically expressed in immature and mature NK cells in both mouse and man [22, 85]. Surprisingly, a substantial subset of $NKp46^+$ cells in the intestine lacked perforin and did not transcribe IFN-γ, and thus bore little functional resemblance to classical NK cells. In contrast, these $NKp46^+$ cells expressed the nuclear hormone receptor retinoic acid receptor–related orphan receptor gamma t (RORγt) and interleukin (IL)-22 in response to local micro-environmental signals and were involved in immune defence against the pathogen

Citrobacter rodentium [84]. The fact that intestinal NKp46$^+$ cells have little in common with 'classical' NK cells in terms of phenotype (lacking NK1.1, Ly49, CD11b, CD27 expression) or function (absence of cytolytic capacity, poor IFN-γ secretion, IL-22 expression) cautions the use of CD3$^-$NKp46$^+$ cells as a 'universal definition' of NK cells [86].

Rather, these observations suggest that an intestinal 'niche' conditions the differentiation of diverse NKp46$^+$ cell subsets that appear to play a role in mucosal immunity. What is clear is that NKp46$^+$ cells within the intestine possess properties that are distinct from NKp46$^+$ cells found in other tissues. IL-22 can stimulate epithelial cells to promote secretion of anti-microbial proteins (β-defensins, RegIII family members and lipocalin 2) that reinforce mucosal barrier function [87]. The absence of IL-22 production in mice lacking intestinal NKp46$^+$ cells [84] results in diminished resistance to enteric pathogens and strongly suggests that these innate lymphocytes are involved in a 'cross-talk' that promotes epithelial cell homeostasis. Accordingly, RegIIIβ and RegIIIγ transcripts are strongly reduced in epithelial cells from mice lacking intestinal NKp46$^+$ cells (unpublished observations), and while these mice still maintain the capacity to restrict entry of commensal microflora, their susceptibility to pathogenic micro-organisms is accentuated. In this way, intestinal NKp46$^+$ cells provide a form of 'pre-emptive' immune defence that operates indirectly through strengthening of the epithelial barrier.

Conclusion

We clearly still have much to learn about the developmental pathways and biological roles for diverse NK cells that have been described in both mouse and man. Several outstanding issues that need to be addressed include the identification of the environmental signals that act on hematopoietic precursors to induce particular properties of NKp46$^+$ cell subsets. Are their unique signals that are delivered in the thymus, bone marrow or intestine? Do the same precursors circulate throughout all tissues? Or do unique precursors seed particular tissues to develop into distinct subsets? Can NK cells complete their differentiation following their initial development in a given tissue? NK cells (or maybe it is more accurate to speak of NKp46$^+$ cells) could exhibit some flexibility in their developmental potentials.

Similarly, we are only beginning to learn what are the biological roles for diverse NKp46$^+$ cell subsets. Beyond their classical definition as 'killers', NKp46$^+$ cells can be viewed as cytokine producers that amplify inflammation (IFN-γ, TNF-α) but also act in the arena of tissue remodelling and homeostasis (IL-22). NKp46$^+$ cells may therefore subserve both 'reactive' but also 'pre-emptive' immune functions. Future studies should also address the specificity of immune functions for NKp46$^+$ cells versus their redundant roles with other innate lymphocytes (γδ T cells, NK-T cells) that show rapid reactivity.

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References

[1] Rosenberg, E. B., McCoy, J. L., Green, S. S., Donnelly, F. C., Siwarski, D. F., Levine, P. H. and Herberman, R. B., Destruction of human lymphoid tissue-culture cell lines by human peripheral lymphocytes in 51Cr-release cellular cytotoxicity assays. *J Natl Cancer Inst* 1974. 52: 345-352.

[2] Herberman, R. B., Nunn, M. E., Holden, H. T. and Lavrin, D. H., Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int J Cancer* 1975. 16: 230-239.

[3] Kiessling, R., Klein, E., Pross, H. and Wigzell, H., "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol* 1975. 5: 117-121.

[4] Kiessling, R., Klein, E. and Wigzell, H., "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol* 1975. 5: 112-117.

[5] Karre, K., Ljunggren, H. G., Piontek, G. and Kiessling, R., Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 1986. 319: 675-678.

[6] Ykoyama, W. M., Recognition structures on natural killer cells. *Curr Opin Immunol* 1993. 5: 67-73.

[7] Raulet, D. H., Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol* 2003. 3: 781-790.

[8] Raulet, D. H., Recognition events that inhibit and activate natural killer cells. *Curr Opin Immunol* 1996. 8: 372-377.

[9] Biron, C. A. and Brossay, L., NK cells and NKT cells in innate defense against viral infections. *Curr Opin Immunol* 2001. 13: 458-464.

[10] Hengel, H., Reusch, U., Gutermann, A., Ziegler, H., Jonjic, S., Lucin, P. and Koszinowski, U. H., Cytomegaloviral control of MHC class I function in the mouse. *Immunol Rev* 1999. 168: 167-176.

[11] Maroof, A., Beattie, L., Zubairi, S., Svensson, M., Stager, S. and Kaye, P. M., Posttranscriptional regulation of il10 gene expression allows natural killer cells to express immunoregulatory function. *Immunity* 2008. 29: 295-305.

[12] Shi, F. D., Ljunggren, H. G. and Sarvetnick, N., Innate immunity and autoimmunity: from self-protection to self-destruction. *Trends Immunol* 2001. 22: 97-101.

[13] Di Santo, J. P., Functionally distinct NK-cell subsets: Developmental origins and biological implications. *Eur J Immunol* 2008. 38: 2948-2951.

[14] Di Santo, J. P., Colucci, F. and Guy-Grand, D., Natural killer and T cells of innate and adaptive immunity: lymphoid compartments with different requirements for common gamma chain-dependent cytokines. *Immunol Rev* 1998. 165: 29-38.

[15] Huntington, N. D., Vosshenrich, C. A. and Di Santo, J. P., Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nat Rev Immunol* 2007. 7: 703-714.

[16] Fernandez, N. C., Treiner, E., Vance, R. E., Jamieson, A. M., Lemieux, S. and Raulet, D. H., A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* 2005. 105: 4416-4423.

[17] Anfossi, N., Andre, P., Guia, S., Falk, C. S., Roetynck, S., Stewart, C. A., Breso, V., Frassati, C., Reviron, D., Middleton, D., Romagne, F., Ugolini, S. and Vivier, E., Human NK cell education by inhibitory receptors for MHC class I. *Immunity* 2006. 25: 331-342.

[18] Kim, S., Poursine-Laurent, J., Truscott, S. M., Lybarger, L., Song, Y. J., Yang, L., French, A. R., Sunwoo, J. B., Lemieux, S., Hansen, T. H. and Yokoyama, W. M., Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 2005. 436: 709-713.

[19] Rosmaraki, E. E., Douagi, I., Roth, C., Colucci, F., Cumano, A. and Di Santo, J. P., Identification of committed NK cell progenitors in adult murine bone marrow. *Eur J Immunol* 2001. 31: 1900-1909.

[20] Vosshenrich, C. A., Ranson, T., Samson, S. I., Corcuff, E., Colucci, F., Rosmaraki, E. E. and Di Santo, J. P., Roles for common cytokine receptor gamma-chain-dependent cytokines in the generation, differentiation, and maturation of NK cell precursors and peripheral NK cells in vivo. *J Immunol* 2005. 174: 1213-1221.

[21] Pessino, A., Sivori, S., Bottino, C., Malaspina, A., Morelli, L., Moretta, L., Biassoni, R. and Moretta, A., Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J Exp Med* 1998. 188: 953-960.

[22] Sivori, S., Vitale, M., Morelli, L., Sanseverino, L., Augugliaro, R., Bottino, C., Moretta, L. and Moretta, A., p46, a novel natural killer cell-specific surface molecule that mediates cell activation. *J Exp Med* 1997. 186: 1129-1136.

[23] Lopez-Botet, M., Llano, M., Navarro, F. and Bellon, T., NK cell recognition of non-classical HLA class I molecules. *Semin Immunol* 2000. 12: 109-119.

[24] Vance, R. E., Kraft, J. R., Altman, J. D., Jensen, P. E. and Raulet, D. H., Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1(b). *J Exp Med* 1998. 188: 1841-1848.

[25] Vance, R. E., Jamieson, A. M. and Raulet, D. H., Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. *J Exp Med* 1999. 190: 1801-1812.

[26] Lanier, L. L., NK cell receptors. *Annu Rev Immunol* 1998. 16: 359-393.

[27] Shiow, L. R., Rosen, D. B., Brdickova, N., Xu, Y., An, J., Lanier, L. L., Cyster, J. G. and Matloubian, M., CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* 2006. 440: 540-544.

[28] Raulet, D. H. and Vance, R. E., Self-tolerance of natural killer cells. *Nat Rev Immunol* 2006. 6: 520-531.

[29] Johansson, S., Johansson, M., Rosmaraki, E., Vahlne, G., Mehr, R., Salmon-Divon, M., Lemonnier, F., Karre, K. and Hoglund, P., Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules. *J Exp Med* 2005. 201: 1145-1155.

[30] Held, W., Cado, D. and Raulet, D. H., Transgenic expression of the Ly49A natural killer cell receptor confers class I major histocompatibility complex (MHC)-specific inhibition and prevents bone marrow allograft rejection. *J Exp Med* 1996. 184: 2037-2041.

[31] Held, W. and Mariuzza, R. A., Cis interactions of immunoreceptors with MHC and non-MHC ligands. *Nat Rev Immunol* 2008. 8: 269-278.

[32] Carlyle, J. R., Jamieson, A. M., Gasser, S., Clingan, C. S., Arase, H. and Raulet, D. H., Missing self-recognition of Ocil/Clr-b by inhibitory NKR-P1 natural killer cell receptors. *Proc Natl Acad Sci U S A* 2004. 101: 3527-3532.

[33] Lanier, L. L., Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* 2008. 9: 495-502.

[34] Colucci, F., Schweighoffer, E., Tomasello, E., Turner, M., Ortaldo, J. R., Vivier, E., Tybulewicz, V. L. and Di Santo, J. P., Natural cytotoxicity uncoupled from the Syk and ZAP-70 intracellular kinases. *Nat Immunol* 2002. 3: 288-294.

[35] Sun, J. C. and Lanier, L. L., Tolerance of NK cells encountering their viral ligand during development. *J Exp Med* 2008. 205: 1819-1828.

[36] Tripathy, S. K., Keyel, P. A., Yang, L., Pingel, J. T., Cheng, T. P., Schneeberger, A. and Yokoyama, W. M., Continuous engagement of a self-specific activation receptor induces NK cell tolerance. *J Exp Med* 2008. 205: 1829-1841.

[37] Guerra, N., Tan, Y. X., Joncker, N. T., Choy, A., Gallardo, F., Xiong, N., Knoblaugh, S., Cado, D., Greenberg, N. M. and Raulet, D. H., NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* 2008. 28: 571-580.

[38] Upshaw, J. L., Arneson, L. N., Schoon, R. A., Dick, C. J., Billadeau, D. D. and Leibson, P. J., NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. *Nat Immunol* 2006. 7: 524-532.

[39] Gilfillan, S., Ho, E. L., Cella, M., Yokoyama, W. M. and Colonna, M., NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nature Immunol.* 2002. 3: 1150-1155.

[40] Diefenbach, A., Tomasello, E., Lucas, M., Jamieson, A. M., Hsia, J. K., Vivier, E. and Raulet, D. H., Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nature Immunol.* 2002. 3: 1142-1149.

[41] Oppenheim, D. E., Roberts, S. J., Clarke, S. L., Filler, R., Lewis, J. M., Tigelaar, R. E., Girardi, M. and Hayday, A. C., Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. *Nat Immunol* 2005. 6: 928-937.

[42] Stepp, S. E., Schatzle, J. D., Bennett, M., Kumar, V. and Mathew, P. A., Gene structure of the murine NK cell receptor 2B4: presence of two alternatively spliced isoforms with distinct cytoplasmic domains. *Eur J Immunol* 1999. 29: 2392-2399.

[43] Schatzle, J. D., Sheu, S., Stepp, S. E., Mathew, P. A., Bennett, M. and Kumar, V., Characterization of inhibitory and stimulatory forms of the murine natural killer cell receptor 2B4. *Proc Natl Acad Sci U S A* 1999. 96: 3870-3875.

[44] Veillette, A., Immune regulation by SLAM family receptors and SAP-related adaptors. *Nat Rev Immunol* 2006. 6: 56-66.

[45] Engel, P., Eck, M. J. and Terhorst, C., The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease. *Nat Rev Immunol* 2003. 3: 813-821.

[46] Lee, K. M., Forman, J. P., McNerney, M. E., Stepp, S., Kuppireddi, S., Guzior, D., Latchman, Y. E., Sayegh, M. H., Yagita, H., Park, C. K., Oh, S. B., Wulfing, C., Schatzle, J., Mathew, P. A., Sharpe, A. H. and Kumar, V., Requirement of homotypic NK-cell interactions through 2B4(CD244)/CD48 in the generation of NK effector functions. *Blood* 2006. 107: 3181-3188.

[47] Taniguchi, R. T., Guzior, D. and Kumar, V., 2B4 inhibits NK-cell fratricide. *Blood* 2007. 110: 2020-2023.

[48] Kim, S., Iizuka, K., Kang, H. S., Dokun, A., French, A. R., Greco, S. and Yokoyama, W. M., In vivo developmental stages in murine natural killer cell maturation. *Nat Immunol* 2002. 3: 523-528.

[49] Hayakawa, Y. and Smyth, M. J., CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. *J Immunol* 2006. 176: 1517-1524.

[50] Huntington, N. D., Tabarias, H., Fairfax, K., Brady, J., Hayakawa, Y., Degli-Esposti, M. A., Smyth, M. J., Tarlinton, D. M. and Nutt, S. L., NK cell maturation and peripheral homeostasis is associated with KLRG1 up-regulation. *J Immunol* 2007. 178: 4764-4770.

[51] Hayakawa, Y., Huntington, N. D., Nutt, S. L. and Smyth, M. J., Functional subsets of mouse natural killer cells. *Immunol Rev* 2006. 214: 47-55.

[52] Chan, C. W., Crafton, E., Fan, H. N., Flook, J., Yoshimura, K., Skarica, M., Brockstedt, D., Dubensky, T. W., Stins, M. F., Lanier, L. L., Pardoll, D. M. and Housseau, F., Interferon-producing killer dendritic cells provide a link between innate and adaptive immunity. *Nat Med* 2006. 12: 207-213.

[53] Taieb, J., Chaput, N., Menard, C., Apetoh, L., Ullrich, E., Bonmort, M., Pequignot, M., Casares, N., Terme, M., Flament, C., Opolon, P., Lecluse, Y., Metivier, D., Tomasello, E., Vivier, E., Ghiringhelli, F., Martin, F., Klatzmann, D., Poynard, T., Tursz, T., Raposo, G., Yagita, H., Ryffel, B., Kroemer, G. and Zitvogel, L., A novel dendritic cell subset involved in tumor immunosurveillance. *Nat Med* 2006. 12: 214-219.

[54] Caminschi, I., Ahmet, F., Heger, K., Brady, J., Nutt, S. L., Vremec, D., Pietersz, S., Lahoud, M. H., Schofield, L., Hansen, D. S., O'Keeffe, M., Smyth, M. J., Bedoui, S., Davey, G. M., Villadangos, J. A., Heath, W. R. and Shortman, K., Putative IKDCs are functionally and developmentally similar to natural killer cells, but not to dendritic cells. *J Exp Med* 2007. 204: 2579-2590.

[55] Blasius, A. L., Barchet, W., Cella, M. and Colonna, M., Development and function of murine B220+CD11c+NK1.1+ cells identify them as a subset of NK cells. *J Exp Med* 2007. 204: 2561-2568.

[56] Vosshenrich, C. A., Lesjean-Pottier, S., Hasan, M., Richard-Le Goff, O., Corcuff, E., Mandelboim, O. and Di Santo, J. P., CD11cloB220+ interferon-producing killer dendritic cells are activated natural killer cells. *J Exp Med* 2007. 204: 2569-2578.

[57] Vosshenrich, C. A., Samson-Villeger, S. I. and Di Santo, J. P., Distinguishing features of developing natural killer cells. *Curr Opin Immunol* 2005. 17: 151-158.

[58] Di Santo, J. P., Natural killer cell developmental pathways: a question of balance. *Annu Rev Immunol* 2006. 24: 257-286.

[59] Yokota, Y., Mansouri, A., Mori, S., Sugawara, S., Adachi, S., Nishikawa, S. and Gruss, P., Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature* 1999. 397: 702-706.

[60] Boos, M. D., Yokota, Y., Eberl, G. and Kee, B. L., Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity. *J Exp Med* 2007. 204: 1119-1130.

[61] Lohoff, M., Duncan, G. S., Ferrick, D., Mittrucker, H. W., Bischof, S., Prechtl, S., Rollinghoff, M., Schmitt, E., Pahl, A. and Mak, T. W., Deficiency in the transcription factor interferon regulatory factor (IRF)-2 leads to severely compromised development of natural killer and T helper type 1 cells. *J Exp Med* 2000. 192: 325-336.

[62] Samson, S. I., Richard, O., Tavian, M., Ranson, T., Vosshenrich, C. A., Colucci, F., Buer, J., Grosveld, F., Godin, I. and Di Santo, J. P., GATA-3 Promotes Maturation, IFN-gamma Production, and Liver-Specific Homing of NK Cells. *Immunity* 2003. 19: 701-711.

[63] Townsend, M. J., Weinmann, A. S., Matsuda, J. L., Salomon, R., Farnham, P. J., Biron, C. A., Gapin, L. and Glimcher, L. H., T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity* 2004. 20: 477-494.

[64] Taki, S., Nakajima, S., Ichikawa, E., Saito, T. and Hida, S., IFN regulatory factor-2 deficiency revealed a novel checkpoint critical for the generation of peripheral NK cells. *J Immunol* 2005. 174: 6005-6012.

[65] Iizuka, K., Chaplin, D. D., Wang, Y., Wu, Q., Pegg, L. E., Yokoyama, W. M. and Fu, Y. X., Requirement for membrane lymphotoxin in natural killer cell development. *Proc Natl Acad Sci U S A* 1999. 96: 6336-6340.

[66] Kennedy, M. K., Glaccum, M., Brown, S. N., Butz, E. A., Viney, J. L., Embers, M., Matsuki, N., Charrier, K., Sedger, L., Willis, C. R., Brasel, K., Morrissey, P. J., Stocking, K., Schuh, J. C., Joyce, S. and Peschon, J. J., Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J Exp Med* 2000. 191: 771-780.

[67] Ranson, T., Vosshenrich, C. A., Corcuff, E., Richard, O., Laloux, V., Lehuen, A. and Di Santo, J. P., IL-15 availability conditions homeostasis of peripheral natural killer T cells. *Proc Natl Acad Sci U S A* 2003. 100: 2663-2668.

[68] Jamieson, A. M., Isnard, P., Dorfman, J. R., Coles, M. C. and Raulet, D. H., Turnover and proliferation of NK cells in steady state and lymphopenic conditions. *J Immunol* 2004. 172: 864-870.

[69] Lucas, M., Schachterle, W., Oberle, K., Aichele, P. and Diefenbach, A., Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity* 2007. 26: 503-517.

[70] Dubois, S., Mariner, J., Waldmann, T. A. and Tagaya, Y., IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells. *Immunity* 2002. 17: 537-547.

[71] Koka, R., Burkett, P. R., Chien, M., Chai, S., Chan, F., Lodolce, J. P., Boone, D. L. and Ma, A., Interleukin (IL)-15R[alpha]-deficient natural killer cells survive in normal but not IL-15R[alpha]-deficient mice. *J Exp Med* 2003. 197: 977-984.

[72] Koka, R., Burkett, P., Chien, M., Chai, S., Boone, D. L. and Ma, A., Cutting edge: murine dendritic cells require IL-15R alpha to prime NK cells. *J Immunol* 2004. 173: 3594-3598.

[73] Williams, N. S., Moore, T. A., Schatzle, J. D., Puzanov, I. J., Sivakumar, P. V., Zlotnik, A., Bennett, M. and Kumar, V., Generation of lytic natural killer 1.1+, Ly-49- cells from multipotential murine bone marrow progenitors in a stroma-free culture: definition of cytokine requirements and developmental intermediates. *J Exp Med* 1997. 186: 1609-1614.

[74] Colucci, F. and Di Santo, J. P., The receptor tyrosine kinase c-kit provides a critical signal for survival, expansion, and maturation of mouse natural killer cells. *Blood* 2000. 95: 984-991.

[75] McKenna, H. J., Stocking, K. L., Miller, R. E., Brasel, K., De Smedt, T., Maraskovsky, E., Maliszewski, C. R., Lynch, D. H., Smith, J., Pulendran, B., Roux, E. R., Teepe, M., Lyman, S. D. and Peschon, J. J., Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood* 2000. 95: 3489-3497.

[76] Garni-Wagner, B. A., Witte, P. L., Tutt, M. M., Kuziel, W. A., Tucker, P. W., Bennett, M. and Kumar, V., Natural killer cells in the thymus. Studies in mice with severe combined immune deficiency. *J Immunol* 1990. 144: 796-803.

[77] Vosshenrich, C. A., Garcia-Ojeda, M. E., Samson-Villeger, S. I., Pasqualetto, V., Enault, L., Richard-Le Goff, O., Corcuff, E., Guy-Grand, D., Rocha, B., Cumano, A., Rogge, L., Ezine, S. and Di Santo, J. P., A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat Immunol* 2006. 7: 1217-1224.

[78] Di Santo, J. P. and Vosshenrich, C. A., Bone marrow versus thymic pathways of natural killer cell development. *Immunol Rev* 2006. 214: 35-46.

[79] Veinotte, L. L., Halim, T. Y. and Takei, F., Unique subset of natural killer cells develops from progenitors in lymph node. *Blood* 2008. 111: 4201-4208.

[80] Freud, A. G., Becknell, B., Roychowdhury, S., Mao, H. C., Ferketich, A. K., Nuovo, G. J., Hughes, T. L., Marburger, T. B., Sung, J., Baiocchi, R. A., Guimond, M. and Caligiuri, M. A., A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. *Immunity* 2005. 22: 295-304.

[81] Watt, S. V., Andrews, D. M., Takeda, K., Smyth, M. J. and Hayakawa, Y., IFN-gamma-dependent recruitment of mature CD27(high) NK cells to lymph nodes primed by dendritic cells. *J Immunol* 2008. 181: 5323-5330.

[82] Leon, F., Roldan, E., Sanchez, L., Camarero, C., Bootello, A. and Roy, G., Human small-intestinal epithelium contains functional natural killer lymphocytes. *Gastroenterology* 2003. 125: 345-356.

[83] Tagliabue, A., Befus, A. D., Clark, D. A. and Bienenstock, J., Characteristics of natural killer cells in the murine intestinal epithelium and lamina propria. *J Exp Med* 1982. 155: 1785-1796.

[84] Satoh-Takayama, N., Vosshenrich, C. A. J., Lesjean-Pottier, S., Sawa, S., Lochner, M., Rattis, F., Mention, J.-J., Thiam, K., Cerf-Bensussan, N., Mandelboim, O., Eberl, G. and Di Santo, J. P., Microbial Flora Drives Interleukin 22 Production in Intestinal NKp46+ Cells that Provide Innate Mucosal Immune Defense. *Immunity* 2008. 29.

[85] Biassoni, R., Pessino, A., Bottino, C., Pende, D., Moretta, L. and Moretta, A., The murine homologue of the human NKp46, a triggering receptor involved in the induction of natural cytotoxicity. *Eur J Immunol* 1999. 29: 1014-1020.

[86] Wlzer, T., Blery, M., Chaix, J., Fuseri, N., Chasson, L., Robbins, S. H., Jaeger, S., Andre, P., Gauthier, L., Daniel, L., Chemin, K., Morel, Y., Dalod, M., Imbert, J., Pierres, M., Moretta, A., Romagne, F. and Vivier, E., Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proc Natl Acad Sci U S A* 2007. 104: 3384-3389.

[87] Zheng, Y., Valdez, P. A., Danilenko, D. M., Hu, Y., Sa, S. M., Gong, Q., Abbas, A. R., Modrusan, Z., Ghilardi, N., de Sauvage, F. J. and Ouyang, W., Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med* 2008. 14: 282-289.

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Chapter 8

Thymic Epithelial Cell Development and Function*

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Abstract

The thymus is the primary lymphoid organ responsible for the lifelong generation of T lymphocytes. The ability of the thymus to support T lymphocyte development is intimately linked to specialised functions and architecture of thymic stromal cells, which are mainly comprised of diverse subsets of thymic epithelial cells (TEC). It is the thymic stromal cells that control the homing, expansion, maturation and selection of developing T lymphocytes (thymocytes). While functional and developmental defects of the thymus severely compromise the adaptive immune system and can cause life-threatening immunodeficiency or autoimmunity, a limited understanding of the cellular and molecular mediators of TEC development and function has hindered tissue engineering of the thymus to correct such debilitating defects. Recent insights and new research models have led to advances in understanding of both the origin and lineage relationships of TECs. The identification of key genetic programs that are functional in thymus development and maintenance has set the stage for elucidating mechanisms which may allow control of TEC differentiation and function and support the development of improved approaches for clinical management of immune disorders

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Introduction

Situated in the upper mediastinum, the thymus is the primary site for T lymphocyte development (known as thympoiesis). It is the specialised structure and composition of the thymus stroma which is key to this functional property [1, 2]. Through its unique stromal cell environment, the thymus achieves two functions essential for the generation and maintenance of the adaptive immune system: *(i)* the life-long generation of new T lymphocytes with a diverse repertoire, and *(ii)* the production of regulatory T lymphocyte subsets, which are essential for efficient immune function [3-5]. Thymus-dependent T lymphocytes are critical mediators of the adaptive immune response and are essential for functional immunity to bacteria, viruses and fungi as well as the maintenance of self-tolerance. The thymus functions not only during the fetal and neonatal stages of development but importantly continues to function and export new T lymphocytes throughout life, albeit at a progressively decreasing rate from the post-natal period [6, 7].

The Role of the Thymus

As the thymus does not contain self renewing lymphoid precursor cells, thymus function relies on a continual supply of blood borne, bone marrow derived T lymphocyte progenitors. Once within the thymus stromal microenvironment, progenitor cells become restricted to the T lymphocyte lineage and undergo an ordered maturational process of proliferation and differentiation to subsequently give rise to a functionally and phenotypically mature T lymphocyte population with an appropriately selected repertoire of antigen receptor specificities [2]. The most important factor governing the developmental fate of thymocytes is the random rearrangement of genes encoding the T cell receptor (TCR). It is through the TCR that T lymphocytes recognize and are activated by foreign peptides in the context of polymorphic major histocompatiblity complex (MHC) molecules, which are expressed by the thymic stromal cells [8-10]. Thus, self-MHC:peptide complexes screen the specificity of the TCR during the processes of positive and negative selection. These selection processes cause the apoptosis of those thymocytes with “inappropriate” TCRs (approximately 98% of all thymocytes) ensuring that only the T lymphocytes of potential use to the host are exported. While commitment of lymphoid precursor cells to the T-lymphocyte lineage and limited differentiation can be achieved using OP9 bone-marrow stromal cell lines transfected with the Notch-ligand delta-like 1 or 4 (Dll4) [11], efficient generation of mature, fully functional T lymphocytes is still only possible in cultures based on *ex vivo* thymus tissue [12-14]. Therefore it is the unique properties of the thymic stromal cells that are responsible for efficient thymopoiesis.

As knowledge of T lymphocyte function has grown, so has interest in the mechanisms regulating the development of these cells. An increased awareness of the role of the thymus stroma as the primary mediators of thymopoiesis has sparked research into elucidating their involvement in regulating thymus development. Understanding the composition and lineage relationships between cells of the thymus stromal microenvironment, and importantly the

mechanisms regulating the function of these cells, has potential clinical implications for both transplantation immunology and for regenerative medicine. As such, the identification of mechanisms by which the thymus tissue can be provided, replenished or functionally enhanced may help clinicians rapidly and efficiently restore the T lymphocyte compartment and greatly reduce the dangers associated with T cell lymphopenia.

Thymic Stromal Cells

Definition and Characterization

All non-hematopoietic cellular components of the thymus can be broadly characterised as the thymus stroma. Irrespective of their origin and lineage, the typical feature of thymus stroma cells is that they lack the pan-hematopoietic cell marker CD45. These stromal cells constitute the indigenous microenvironment of the thymus, and together with hematopoietic derived dendritic cells (DC), macrophages and B lymphocytes, they provide the supporting three-dimensional matrix or scaffold on which thymocytes develop. The non-hematopoietic thymus stroma itself is a heterogeneous population of cells: consisting of epithelial cells, as well as various non-epithelial elements that include the thymus capsule and septae forming connective tissue, endothelial cells that form the thymus vasculature, fibroblasts, neural crest derived mesenchyme (NCC) and other mesenchymal elements.

In the mature thymus, separate anatomical regions including the subcapsular area, cortex (outer region) and medulla (inner region) are readily identifiable in a structural organisation that is well conserved throughout evolution. As such, the major component of the thymus stroma, the TECs, which express MHC Class I, MHC Class II, EpCAM and intracellular keratins, can be broadly classified as cortical (cTEC) or medullary (mTEC) based on differential spatial arrangements as well as other morphological and antigenic properties (See Figure 1).

On entry into the thymus and following interaction with Dll4-expressing TEC, T lymphoid progenitor cells initiate their commitment to the T lymphocyte lineage [15, 16], traffic to the outer regions of the thymus cortex and begin to express the αβTCR and co-receptors CD4, CD8 [2]. Newly generated $CD4^{+ve}CD8^{+ve}$ (double positive (DP)) thymocytes interact via their TCR with the MHC:peptide complex that is expressed by cTEC and DCs in the cortex and are selected for survival or death depending on the avidity of this interaction. DP thymocytes that receive weak avidity TCR signals are induced to survive and continue development into mature T lymphocytes. The differential kinetics of TCR interaction with MHC determines whether DP thymocytes become either $CD4^{+ve}CD8^{-ve}$ (CD4) or $CD4^{-ve}CD8^{+ve}$ (CD8) single positive (SP) T lymphocytes [17, 18]. These processes of TCR-mediated developmental progression of DP to SP thymocytes are referred to as positive selection. By contrast, DP thymocytes that receive TCR signals with strong avidity are instructed to die, a process termed negative selection. Positively selected thymocytes not only survive and develop but also relocate to the medulla of the thymus, so that most SP thymocytes accumulate there.

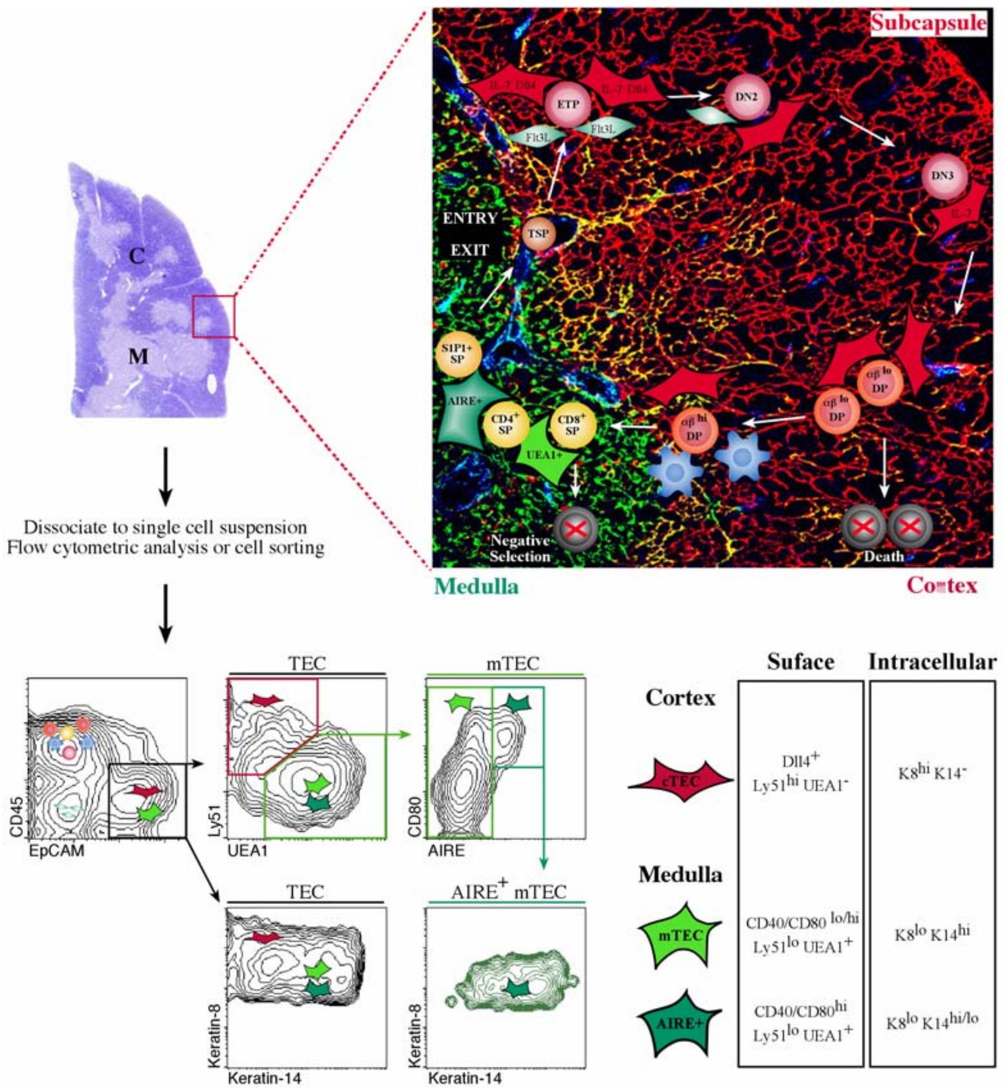

Figure 1. Mature thymus structure and TEC characterization.

This migration is primarily regulated by the chemotaxis of CCR7-expressing, positively selected thymocytes toward CCR7 ligands (CCL19 and CCL21) produced by mTECs [19, 20]. On entering the thymus medulla, positively selected semi-mature thymocytes further interact with self-peptides displayed by mTECs, thymocytes and DCs. Thymic DCs, which can efficiently present both endogenous and acquired peptides, are predominantly localised in the medulla and are, at least in part, derived from the circulation and therefore can ferry peptides into the thymus [21, 22].

It was initially thought that central tolerance induction via negative selection of auto-reactive thymocytes covered only ubiquitous antigens, antigens specific for thymus cells and peripheral self-antigens that have access to the thymus. However, it is now recognised that

the scope of central tolerance is much more diverse, encompassing self-epitopes that represent essentially all peripheral tissues. This insight emerged from the demonstration that a myriad of genes classified as tissue-restricted, based on their cell type specific expression pattern and function, are also transcribed in TEC. This phenomenon, termed promiscuous gene expression, is a particular feature of mTEC [21]. The cellular and molecular regulation of this partially unrestricted gene expression is still largely unknown. So far, only one molecular regulator, the autoimmune regulator (Aire) has been identified, which directs expression of a large subset (a few hundred) of promiscuously expressed genes. Consistent with the pattern of promiscuous gene expression, the Aire protein is highly expressed in a subset of mTECs, concomitant with induction of numerous Aire-dependent and -independent tissue-restricted antigens (TRAs) [23, 24]. The interaction of positively selected thymocytes in thymus medulla with a diverse set of self-peptides, including TRAs presented by mTECs and DCs, is essential for establishing self-tolerance. Consequently, naive T lymphocytes with a diverse yet self-tolerant repertoire are released by the thymus to the circulation via sphingosine-1-phosphate receptor 1- (S1P1) dependent interactions [25]

The thymus is broadly divided into two regions, the cortex (C) and the medulla (M), each of which contains several TEC subtypes. Upper portion of figure: In adults, blood borne thymus settling progenitors (TSP) enter the thymus via vessels at the cotrico-medullary junction [26] and begin a differentiation program, which is linked to migration through the thymus stroma. Different thymocyte subsets are therefore found in spatially restricted regions of the thymus in contact with different stromal cell microenvironments or niches. Uncommitted progenitors ETPs are found close to the CMJ, where they are influenced by Flt-3 ligand (Flt3L), interleukin-7 (IL-7), stem cell factor (SCF) and Dll4 provided by the thymus microenvironment. These cells undergo expansion and differentiate to $CD4^{-ve}CD8^{-ve}$ double-negative (DN2) stage and subsequently have restricted lineage potential. T-lymphocyte lineage commitment and the onset of TCR α-chain rearrangement occur in DN3 cells in the subcapsular region followed by transition from DN to DP. DP cells then migrate back through the cortex and, having differentiated into either $CD4^{+ve}$ or $CD8^{+ve}$ SP cell, into the medulla. SP cells that have completed the differentiation program egress from the medulla to the periphery. Lower portion of figure: Within each of the defined thymus regions TECs may be categorised on the basis of antigenic properties. Immunohistochemical studies have allowed the spatial arrangement of TECs to be characterised by the differential expression of cytokeratins (K), adhesion molecules and lectin reactivity, with the majority of cTECs expressing Ly51 and K8 (red) and mTECs UEA1 and K14 (green). Flow cytometric analysis revealed that all TECs express EPCAM, MHC Class II and K8 (albeit at different levels). Expression of Ly51 and binding of UEA1 lectin can be used to broadly define cTECs and mTECs, respectively. mTECs may be further characterised by expression of high levels of MHCII and the accessory molecules CD40 and CD80. Aire expression is found exclusively within the $UEA1^{+ve}CD80^{+ve}$ mTECs. Aire expressing TECs demonstrate a differential expression of K14, which suggests that intracellular keratin expression alone may not be an accurate guide to identify mTEC subsets by flow cytometric analysis.

Developmental Origins of the Thymic Epithelium

Thymus organogenesis requires a complex cascade of events involving reciprocal interactions between adjacent tissues derived from different germ layers. As such the developmental process is similar to that of other organs, and can be viewed as comprising several distinct stages that must be carefully regulated and coordinated to ensure correct organ formation. Identified stages of this developmental process include: positioning, initiation, outgrowth and differentiation (reviewed in [27]) and results in the bilateral creation of a thymus primordium that is able to attract fetal liver or bone marrow derived progenitors. How the cells that form the lining of the foregut are induced to differentiate and acquire competence to establish the initial thymus rudiment, and the molecular mechanisms that direct their development along this pathway are key questions in the study of TEC biology.

Endodermal Origins

Throughout organogenesis, the endoderm gives rise to the digestive tract and also contributes to the formation of the respiratory system, tympanic cavities, eustachian tubes and the organs that branch from the main digestive tube. These organs include the thymus, thyroid, parathyroids, ultimobrachial body, liver, pancreas, gall bladder and ceacum. In the mouse, as soon as the definitive endoderm is formed at embryonic day (E) 6.5, certain genes are expressed asymmetrically and are subsequently restricted to anterior regions of the endoderm [28-30]. This regional differentiation is progressively refined, and by E9 several transcription factors mark presumptive territories for the esophagus/stomach, liver, pancreas/duodenum, small and large intestines [31-34].

Studies have shown however that while certain genes are located in subsets of the endoderm, commitment of the endoderm to a regional fate or specific tissue lineage is not fully determined at this stage. This suggests that a long term cross talk between endoderm and mesoderm progressively commits endoderm cells to a specific fate. This commitment may be in part mediated by the combination of growth factors produced by mesoderm derived cells which are in close association with, and in part dependant on growth factors provided by the developing endoderm, with fibroblast growth factors (FGF) implicated in this process [35, 36]. This apparent discrepancy between fate determination and lineage commitment has been observed in various tissues, highlighting the symbiotic relationship between endoderm and mesoderm, a recurring theme throughout organogenesis.

Studies that have engrafted pharyngeal region endoderm of E8.5-E9 embryos under the kidney capsule of nude mice have demonstrated that purified endoderm is sufficient to generate functional thymus tissue, independent of a physical contribution from the pharyngeal ectoderm [37]. This result indicates either that factors provided by the kidney mesenchyme may be sufficient for thymus organogenesis to occur, or that at this early stage, cells in the pharyngeal endoderm are already specified to enter the TEC lineage before overt signs of thymus organogenesis. However no molecular markers have yet been identified in the early endoderm that may be used to specifically distinguish definitive endodermal cells specified to give rise to the thymus epithelium.

The Cellular Basis of Thymus Organogenesis

Thymocyte Independent TEC Patterning

In order to establish the microenvironment necessary for thympoiesis, the epithelial primordium of the thymus expands from a transient outpocketing of the pharyngeal endoderm, the pharyngeal pouch. In mice, the epithelial-mesenchymal interaction between the third pharyngeal pouch endoderm and NCC from the third and fourth branchial arches creates a first visible parathyroid-thymus anlage as early as E10.5. At this stage each of the bilateral primordia contains the precursors to one thymus lobe and one parathyroid gland (reviewed in [27]).

Budding and outgrowth of the anlage is coincident with dorsal and ventral specification, which may be identified by the onset of transcription factors Gcm2 and Foxn1, which are essential for parathyroid [38] and thymus development [39, 40], respectively. Foxn1, the gene that is mutated in nude mice, is the earliest identified transcription factor in the pharyngeal region that is specifically associated with and has an obligate and cell autonomous role in TEC development [41]. Analysis of mice bearing a hypomorphic allele of Foxn1 also suggests an ongoing role for this transcription factor in the later stages of TEC maturation and maintenance [42, 43]. As transplantation experiments suggest that the endoderm at E8.5-E9 is specified to a thymus fate before organ formation and Foxn1 expression is detectible, and no phenotype has been found in Foxn1 mutants before E11.25, it is unlikely that Foxn1 is responsible for specifying thymus identity during initial organogenesis. As such, nude mice do undergo the initial stages of thymus organogenesis, the primordium forms, but fails to differentiate or be colonised by lymphocyte progenitors [44-47]. Therefore thymus organogenesis may be separated into two phases. An early Foxn1-independent phase consists of conversion of endoderm cells to a proto-differentiated state that culminates in TEC specific protein expression (such as IL-7 [45]) and progenitor TEC (pTEC) specification. A secondary Foxn1 dependent transition of the proto-differentiated pTEC to differentiated cells is associated with protein synthesis of MHC and Dll4 [44, 48]. At this stage, the prospective thymus epithelium appears homogeneous in terms of cell phenotype and morphology. The epithelial cells display ubiquitous expression of EpCAM, Plet-1 (MTS24), intracellular K8 and K19 and lack expression of markers associated with differentiated cortical and medullary TEC [1]. Therefore using currently defined reagents, the phenotype of the early thymus primordium is essentially indistinguishable from that of the endodermal epithelium lining the pharyngeal region.

After day E11.5, the organization of TECs differs from most other epithelial organs in the body. Rather than forming a sheet of cells positioned on a basement membrane, TECs form a three-dimensional meshwork. The initial patterning of this thymus primordium is dependent on mesenchyme-derived inductive signals but independent of hematopoietic cells [49, 50], which seed into the avascular thymus anlage at E11.5. This active migration toward the thymus anlage is influenced by the expression of chemokines CCL21 and CCL25 on the parathyroid primordium, with their cognate receptors (CCR7 CCR9 respectively) on immigrating hematopoietic cells [47, 51, 52]. Coincident with hematopoietic cell colonization, immature TECs undergo further patterning and differentiation into distinct

subtypes, characterised by an alteration in intracellular cytokeratin expression within a rare population of centrally located cells. These cells express uniform levels of EpCAM, Plet-1 and K8 and co-express K5 and K14, which subsequently are primarily associated with mature mTECs [53, 54]. At about E12.5, the shared primordia separate from the pharynx and migrate towards the anterior thoracic cavity under the influence of NCCs. By E13.5 the parathyroid and thymus domains are physically separated and resolved into separate organs localised in their respective adult associated positions. TECs present at the E13.5 stage and beyond develop further heterogeneity, which may be characterised by alterations in intracellular cytokeratin distribution, variable expression of adhesion molecules and binding of UEA1 lectin.

The Role of Neural Crest Cell Derived Mesenchyme

Early in thymus organogenesis, a mesenchyme derived capsule surrounds each thymus primordia. Initially derived both from pharyngeal mesoderm and the NCC, the cells eventually establish an intra-thymus network of fibroblasts and also contribute to the thymus capsule and septae [55]. While the physical contribution of NCC derived fibroblasts to processes beyond initial organogenesis had been questioned, recently it has been convincingly demonstrated that the majority (if not all) fibroblast tissue in the post-natal thymus are actually NCC derived [56, 57], and these cells have an ongoing role in thymus function. A functional role for NCCs in the establishment and differentiation of the thymus has previously been inferred from deficiencies in thymus development following NC ablation in chick embryos, from mutation of the Pax3 and the endothelin 1 genes in mice [58, 59], and from physiological alterations seen in human DiGeorge patients [60]. A possible molecular link between NCCs and thymus epithelium is provided via both insulin-like growth factor (IGF) and FGF and their associated receptors. IGF1, IGF2, FGF7 and FGF10 are expressed by the mesenchyme surrounding the embryonic thymus epithelium, which expresses IGF1R and FGFR2-IIIb [61-63]. Defects in these signaling pathways disrupt both thymus size and function, demonstrating a growth-promoting role for mesenchyme on thymus epithelium [63]. Another important role of thymus mesenchyme in regulating thymocyte cellularity is the provision of other growth factors such as Wnts or BMPs to TECs and the provision of Flt3L, IL-7 or c-kit ligand to developing thymocytes [64-66]. However, it is clear that the signals provided from cells of mesenchyme origin are necessary but not sufficient for the subsequent development of a regularly structured and normally functioning thymus, which is also dependent on inductive interactions with developing hematopoietic elements.

Thymocyte Dependent TEC Patterning

The transition from an immature, essentially homogeneous primordium to a diverse microenvironment with adult associated cortex-medulla organization is perturbed in mice in which T lymphocyte development is blocked at immature stages. The influence of the presence or absence of different stages of thymocyte development on TEC structure and

composition has been viewed as interdependence between thymocytes and stroma [67], and is referred to as thymus crosstalk [68].

Studies investigating the heterogeneity of fetal TECs have demonstrated that beyond E14, the composition of TECs changes significantly, with the emergence of a population with low expression of Ly51. These cells bind UEA1 and also express CD80 and Aire, and may be characterised as *bone fide* mTECs. Small numbers of Aire expressing mTECs are detectable even in RAG2-deficient mice that lack DP thymocytes [69, 70], demonstrating that mTECs are generated independently of mature thymocytes. Aire expressing mTEC appearance in ontogeny is however coincident with the presence in the thymus of a population of $CD4^{+ve}CD3^{-ve}$ lymphoid tissue inducer (LTi) like cells, which also express among other markers CD127, CD117 and RANKL [70, 71]. Although the origins and lineage relationships of these thymus resident LTi-like cells are at present unknown, it is believed these cells represent a population of fetal-liver derived hematopoietic cells, which may be characterised by expression of the retinoic-acid-receptor-related orphan receptor-γt (RORγt) and the transcriptional repressor Id2. Phenotypically similar cells have been demonstrated to influence the maturation of lymphoid environments of the spleen and lymph nodes. It has been proposed that LTi-like cells in the embryonic fetal thymus have an important inductive role in the development and maintenance of the Aire expressing mTECs. At present, although they are sufficient to do so in *in vitro* models, it is unclear whether LTi cells are essential for mTEC development. However as no significant impairment in the development of thymus medulla or Aire expressing mTECs are evident in adult mice lacking Id2 or RoRγt, where LTi cells are rarely detectable, it is likely that Id2- or RORγt-dependent LTi cells are dispensable for mTEC development and medulla formation in postnatal mice [71, 72].

Whilst cells characterised phenotypically as cortical and medullary TECs are present from the early stages of thymus ontogeny and the initial patterning of the TEC compartment does not depend on inductive signals from T lineage cells, the expansion, differentiation and maintenance of the epithelial cells of the medullary region is critically dependent on the presence of positively selected thymocytes within the thymus medulla. This role is evident in mice that lack positive selection ($TCR\alpha^{-/-}$, $Zap70^{-/-}$, and $Rag2^{-/-}$) or in mice that have deficiencies in CCR7 or CCR7 ligands and hence defective thymocyte migration. Consequently, these mice have severely defective or reduced numbers of mTECs [20][72-74]. Recent, detailed studies have demonstrated a role for $CD4^{+ve}$ but not $CD8^{+ve}$ SP thymocytes in the proliferation and differentiation of mTECs [74]. This role was dependent on direct MHC Class II-TCR interaction and not soluble factors, with autoantigen-specific interaction (autoreactivity) of $CD4^{+ve}$ SP thymocytes critical for the number of Aire expressing mTECs present in the thymus medulla. It remains to be established whether the signals delivered by $CD4^{+ve}$ SP thymocytes promote the differentiation of mature $Aire^{+ve}$ mTECs from proliferating precursors in the $CD80^{lo}$ population, the $Aire^{-}CD80^{hi}$ population, or both. Together, these results indicate that positive selection, via the provision of $CD4^{+ve}$ SP cells, promotes the proliferation rather than the functional maturation of mTECs and thereby nurtures the formation and maintenance of the thymus medulla. Investigation of molecules differentially expressed by $CD4^{+ve}$ and $CD8^{+ve}$ SP thymocytes has recently provided insights into some of the molecular mediators of TEC function and will be discussed in a later section. Given that the thymus microenvironment is quite heterogeneous and varies considerably over

time, questions remain as to whether the same molecular pathways operate in both the embryonic and postnatal thymus, or do they exert their roles in different stages, differentially effecting TEC differentiation, proliferation or organization?

Establishing Lineage Relationships between TEC Subsets

Resident tissue-specific progenitor cells have been described for several somatic tissues, and their asymmetric self-renewal has been linked to homeostatic tissue maintenance [75-77]. The observations that in the adult, cortical and medullary TECs proliferate and that post-mitotic TECs are continuously replaced throughout life, have been taken as circumstantial evidence for the existence of such progenitors within the postnatal thymus. However, while the phenotypic characteristics of the early embryonic progenitors that give rise to mature cortical and medullary TECs have now been identified and lineage relationships established in ontogeny, the equivalent cell type and its cellular and developmental features in the postnatal thymus still await discovery.

Lineage Model of TEC Differentiation

In contrast to the lymphoid components of the thymus, the stromal elements are relatively poorly understood. This is primarily due to inherent difficulties in TEC isolation and the fact that techniques for *in vitro* culture or *in vivo* lineage analysis to probe the stromal cell subsets functionally or phenotypically have not been fully established. However, recent advances in experimental techniques and development of new animal models have allowed for a better understanding of lineage relationships among TEC subsets.

Techniques that are currently utilised for stromal cell isolation and analysis are dependent on successful enzymatic digestion and stromal cell dissociation, followed by phenotype-based cell sorting on the basis of the reactivity of monoclonal antibodies and other reagents that specifically recognize subtypes of stromal cells (see Figure 1 and [78]). Isolated TECs may then be utilised in assays aimed at probing the functional or developmental capacity of TEC subsets *in vitro* and *in vivo* [48, 53, 79-81]. The observation that when dissociated and allowed to reaggregate *in vitro* and subsequently engrafted under the kidney capsule, suspensions of purified fetal thymus stroma are able to re-form a functional thymus, has allowed examination of lineage potential and function of embryonic TEC subsets. This capacity however has not been demonstrated beyond E18 in reaggregate thymus organ culture (RTOC) [48].

Subsequent studies have utilised RTOC to examine the lineage potential of TEC subsets and have established that, in the embryo, TECs that express Aire are contained within the $CD80^{+ve}$ subpopulation, and in *in vitro* models arise from $CD80^{-ve}$ progenitors [70]. Heterogeneity of the $CD80^{-ve}$ TEC population was evident at E13.5, when by flow cytometry subpopulations could be identified and separated based on Claudin-3, 4 (Cld) expression and

UEA-1 binding [81]. In an RTOC setting, which allowed tracing of introduced cells by MHC expression, Cld3,4^{lo} cells gave rise to both mTECs and cTECs following engraftment under the kidney capsule. Cld3, 4^{hi} cells however gave rise only to mTECs suggesting the existence of committed mTEC progenitors. This finding reflects earlier studies which demonstrated that the thymus medulla is comprised of aggregations, or islets, of clonally derived cells that vary in size [82] and suggests that commitment to the mTEC lineage can take place as early as E13.5, at least in cells defined by Cld3, 4 expression.

A Progenitor Sufficient to Generate Both Ctec cTEC and MtecmTEC

A characteristic of progenitor cell function throughout development and tissue homeostasis is that a small pool of progenitor cells generates a larger pool of mature downstream cells through proliferation and differentiation. This process is concurrent with a progressive loss of developmental potential, and under normal conditions, once completed, differentiation steps are believed to be irreversible. For a long time, steady-state adult TECs were considered end-stage, post mitotic cells. However, several reports have found substantial TEC proliferation, indicating rapid epithelial turnover in the adult thymus [83, 84] raising the possibility that maintenance of the thymus microenvironment may also involve expansion of mTEC- and cTEC-committed cells, analogous to transit amplifying cells, which have been described in various tissues. In adulthood, thymus atrophy (or involution) results in a loss of normal cortical and medullary architecture. Under certain conditions, this atrophy is reversible, and normal thymus architecture and T-lymphocyte output can be restored [85-87]. These findings further demonstrate plasticity of the TEC microenvironment and support the idea that there are TEC progenitors that may persist and that can be activated to proliferate and differentiate later in life. However, whether the development and maintenance of TECs within the adult thymus occurs in the same way as in the embryonic thymus and if these cells are generated from bi-potent progenitors are currently unknown.

To attempt to address these issues, bulk RTOC techniques using heterogeneous TECs were refined to a clonal basis by mixing in a single, genetically marked (EYFP) E12 fetal TEC, into a donor lobe that was then transplanted under the kidney capsule and then allowed to develop for 4 weeks [88]. Subsequent visualization of single cell–derived TEC progeny demonstrated that in 4 of 13 transplanted thymuses, fluorescent cells were detected and that the progeny of single cells had contributed to both cTEC and mTEC lineages. Bleul and colleagues [89] reported similar findings when using a transgenic mouse line based on the Foxn1-deficient nude mouse strain. In this mouse line, TEC development is blocked at an early stage. However, when Foxn1 expression was randomly induced by Cre-LoxP recombination in TECs, functional thymus tissue with defined cortical and medullary areas was generated. These data suggest that at least some of the primordial epithelial cells in the thymus remnant of the nude mouse retain the capacity to commit to a TEC fate.

In the same report [89], a separate experiment that addressed progenitor activity in TECs was based on epithelial cell tracing using genetic *in situ* labeling. Random and very rare ‘leakiness’ in Cre recombinase under the control of the human Keratin 14 promoter (K14Cre) acted as a switch that turned on YFP expression in TECs. Although no YFP^{+ve} cells were

found in the thymus at birth mice, YFP^{+ve} TEC in the thymus increased with age. At all times analyzed, labeled progeny remained very rare and patterns of progeny were either; mTEC or cTEC clusters only or mTEC and cTEC clusters. These results tentatively suggest that bipotent and committed cTEC and mTEC progenitors may co-exist in the postnatal thymus. However, due to the unknown activity of the transgenic construct supplying Cre to the different TECs, it cannot be unequivocally demonstrated that clusters of cTEC and mTEC either arise from a single progenitor or separately from pre-committed progenitors. Taken together [88, 89] these reports establish that in cells of the early thymus rudiment, or its equivalent (the thymic promordium arrested prior to expression of Foxn1), at least a population of cells possesses bipotent-pTEC potential and that this activity is dependent on Foxn1 function. These studies together with observations made throughout ontogeny and in mutant mouse models have allowed a preliminary lineage model of TEC development to be established (Figure 2).

Any capacity for self-renewal of cells with bi-potent pTEC potential is currently unknown, and the existence and phenotype of a bi-potent progenitor in adult mice has not yet been fully established. Based on limited data, two models have been proposed to address the question as to whether in the adult steady-state thymus cTECs and mTECs are derived from pTECs or from two separate, unipotent precursors [90]. The first model is that a self-renewing pTECs gives rise to two distinct, transient amplifying TEC populations without self-renewing capacity from which the diverse populations of cTECs and mTECs arise. The second model predicts that for each epithelial lineage there are separate cTEC and mTEC progenitors with self-renewing capacity. This second model could further accommodate the possibility that these lineage-committed precursors have only a limited life span and hence need to be replenished from bi-potent precursors. However, until new experimental systems are developed, it is unlikely that information on the self-renewal potential of specific TEC subpopulations will be forthcoming.

At present the data on TEC development in ontogeny are consistent with this simplified model of TEC development. Uncommitted endoderm cells in the developing primordium become specified to a thymic epithelial cell fate, forming a proto-differentiated pTEC that, subsequent to expression of Foxn1, become thymus committed bi-potent pTECs. These cells undergo maturation to give rise to a range of more mature cTECs and mTECs. The various stages of TEC differentiation arise sequentially through ontogeny and may be characterised by the progressive initiation (green) or loss (red) of associated phenotypes, lower panel. Although a committed mTEC progenitor has been identified in the embryo, no equivalent cell population has been determined for the cTEC lineage.

The relationship between the various subpopulations of mature MHC Class II^{hi} TECs also are not fully characterised, indeed whether under steady state conditions Aire induction represents a terminally differentiated TEC destined to undergo cell death, or a separate lineage is still unclear, as is any plasticity in lineages, such as a potential mTEC contribution to cTECs in the postnatal thymus. Solid arrows represent the proposed lineage progression based on experimental observation, while those indicated by grey arrows remain to be experimentally determined.

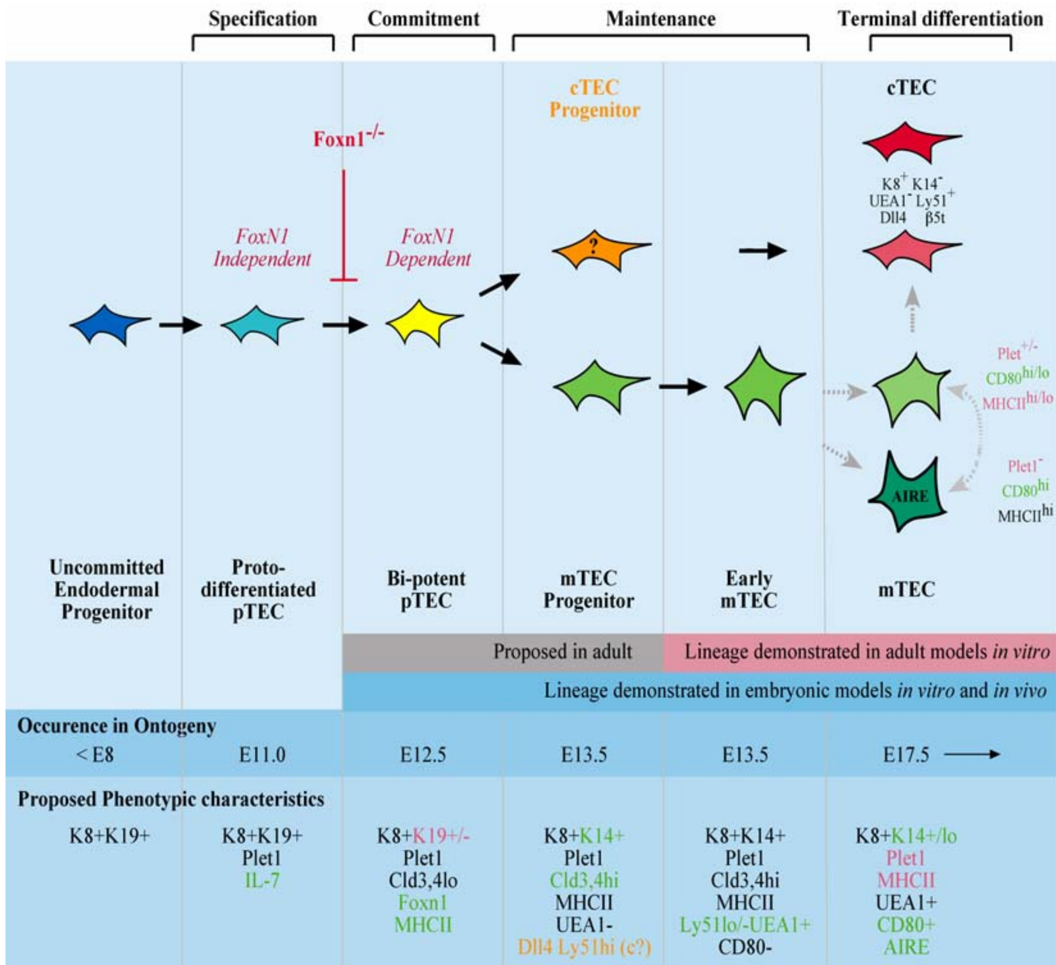

Figure 2. Lineage model of TEC development.

Molecular Mediators of TEC Development and Function

With knowledge of the cellular requirements for thymus development and the identification of lineage relationships pertaining to TEC differentiation, we are now in a position to identify the potential molecular mechanisms that control the generation of the embryonic pTECs and that subsequently direct their differentiation and organization into cortical and medullary regions. To this point Foxn1 is the earliest identified transcription factor in the pharyngeal region that is specifically associated with and that has an obligate role in TEC development. Therefore expression of Foxn1 may divide thymus organogenesis into an early Foxn1-independent phase and ensuing Foxn1-dependent stage.

Transcription Factors

The initiation of thymus organogenesis is dependent on the correct initial formation of the pharyngeal region, therefore gene mutant mice with defective patterning of the pharyngeal region and defective pouch formation, such as that observed in Chordin mutants [91], will consequently demonstrate a failure in development of pouch-derived structures. However, no direct role in thymus initiation, or Foxn1 regulation, can be inferred from analysis of such severe and early occurring mutations.

The analysis of mutant phenotypes and gene-expression patterns by *in situ*-hybridization has identified a transcription factor regulatory network that is required for the initial formation and patterning of the thymus rudiment. At present, this consists of five factors: Hoxa3-Pax9-Eya1-Six1-Pax1. (Reviewed and referenced in [27, 92] and summarised in Table 1). In mice, these transcription factors are expressed in multiple cell lineages, but are co-expressed and presumed to act predominantly within the pharyngeal endoderm of the third pharyngeal pouch. Mutation studies have shown these factors to have variable but important roles in establishing the thymus primordiumal. However, with the exception of Pax1 and Pax9, these factors are also expressed in the ectoderm and NCCs, complicating interpretation of the indicated phenotypes. NCCs do however densely populate the third pharyngeal arches independently of Hoxa3, although loss of Hoxa3 may affect the capacity of these cells to induce the correct differentiation of the third pharyngeal pouch, which is required for subsequent Foxn1 expression.

Foxn1 mRNA expression was not detected by *in situ* hybridization in Hoxa3, Eya1 or Six1 mutants. However Foxn1 can only be detected in the third pharyngeal pouch by RT-PCR at E10.5, or by *in situ* hybridization after E11.25 [119], which is well after the block in thymus organogenesis observed in these mutants. As such, it is not clear which transcription factors initiate and maintain the expression of Foxn1 in the prospective thymus anlage, and downstream targets of Hoxa3-controlled gene transcription in third pharyngeal pouch endoderm, which directly promote Foxn1 expression, have yet to be identified. These and other early acting genes may function in both initial patterning of the pouch as well as in thymus organogenesis and function, however it is difficult to determine a direct role in thymus initiation and later function from examination of these mutant models. TEC specific and temporally restricted deletion of such widely expressed and early acting genes will be required to resolve their function specifically in thymus development.

The T-box transcription factor Tbx1 is normally expressed in the pharyngeal endoderm and the arch mesenchyme as well as in the developing thymus at later stages [94]. Mutations in Tbx1 cause thymus and parathyroid defects and have been implicated in the complex phenotype of the DiGeorge syndrome [60, 120, 121], while expression in the pharyngeal region appears to be regulated by sonic hedgehog (Shh) signaling [94, 95]. Analysis of gene expression patterns in wildtype and Tbx1-deficient mice and experiments *in vitro* have identified several downstream targets of Tbx1 transcriptional activation including FGF8 and FGF10 [122, 123]. During development, FGF8 is secreted by epithelial cells and provides survival, mitogenic and patterning signals to adjacent mesenchyme [111].

Table 1. Early acting molecular mediators of TEC development and function

Gene	Expression in pharyngeal region		Disrupted expression		References
	Initiated	Lineage	Foxn1	Thymus	
Chordin	9.5 – 10.5	Mes, End	N	No pouch	[91]
Shh	9.5	Mes, End (excluded from pouch)	N	No pouch	[93-95]
Pbx1	10.5	Ect, NCC, End	Delayed*	Hypoplasia	[96]
Tbx1	7.5 - 9.5	Ect, NCC, End	N	No pouch	[94, 97]
Hoxa3	9.5 – 10.5	Ect, NCC, End	N	Absent	[96, 98-100]
Eya1	9.5 – 10.5	Ect, NCC, End	N	Absent	[101, 102]
Six1	9.5 – 10.5	Ect, NCC, End	Y**	Absent beyond E12.5	[101, 103]
Foxn1	10.5 – 11.25	End	-	Primordium arrested	[40-43, 104, 105]
Pax1	8.5 – 10.5	End	Y	Hypoplasia	[98, 106, 107]
Pax9	8.5 – 10.5	End	Y	Hypoplasia	[108-110]
FGF8	9.5	End		Hypoplasia-aplasia	[95, 111]
FGF7/10	E10.5	NCC	Y	Hypoplasia	[63, 112]
FGFR2iiib	E13 E14	TEC Mes	Y	Hypoplasia	[62, 113, 114]
Noggin	9.5 10.5	Mes, End (dorsal)	Y***	Normal***	[115-117]
BMP4	9.5 10.5 12.5	Mes, End (ventral) End, Mes	Y	Y	[115, 116, 118]

Abbreviations: End; endoderm, Ect; ectoderm, Mes; mesenchyme, NCC; neural crest cell,

*Foxn1 expression in Pbx1 mutant mice is not detected by *in situ* until E12.5.

** Six1 mutant have reduced expression of Foxn1 at E11.5, increased apoptosis and loss of common parathyroid/thymus primordia by E12.5

***Noggin mutants have no thymus phenotype, Tg expression of Xnoggin under control of the Foxn1 promoter results in a severely hypoplastic mosaic thymus, with partial loss of Foxn1 expression.

While mice deficient either for FGF10 or for the FGF10-specific receptor, FGFR2IIIb have, among other anomalies, a hypoplastic thymus, a phenotype similar to that of mice where a Tbx1 deletion was induced at E10.5 [63, 112, 124]. A similar thymic hypoplasia and reduced proliferative potential has been reported for mice deficient in p63 [125], which is also upstream of FRFR2IIIb in the thymus [126]. However p63 expression in the thymus is independent of Foxn1[125] and a direct relationship between Tbx-p63-Foxn1-FGFR is yet to be established.

Eya1, which encodes a transcriptional co-activator, is expressed early in the pharyngeal endoderm, mesenchyme and ectoderm. Eya1 knockout mice have no thymus or parathyroid and fail to express Wnt5b in the endoderm [101, 102], which has been shown to regulate Foxn1 expression in TECs *in vitro* [127]. Although together these studies have started to define specific genetic programs controlling early thymus/parathyroid development, the identity of the regulatory pathways and the molecular basis of epithelial–mesenchymal interactions remain largely unknown.

Signaling Molecules

Although Foxn1 is dispensable for the initial formation of the thymus anlage, subsequent differentiation and functional maintenance of the TEC is dependent on Foxn1. The molecular mechanisms that regulate Foxn1 expression and activity are only incompletely understood, however Wnt, Shh and TGFβ family proteins have been implicated in the transcriptional control of Foxn1.

Wnts

Wnts are a large family of secreted glycoproteins, which regulate many aspects of cellular development including fate specification, migration, proliferation and death. Wnt expression can be detected as early as E10.5 in the developing pharyngeal pouch by *in-situ* studies, while Wnts and downstream signaling components are expressed and maintained by both TECs and developing thymocytes from early ontogeny. Transfection and *in vitro* TEC–thymocyte co-culture studies have established that Wnts can induce Foxn1 expression in cultured TECs [127]. Studies *in vivo* have demonstrated that Cre- mediated deletion of the Wnt signaling intermediate protein APC under control of the human-K14 promoter results in severe TEC disorganization and development [128]. As these mice demonstrate stunted growth and die before weaning, the thymus phenotype could not be dissociated from the poor condition of the animals, and the expression level of Foxn1 in TEC was not reported. Mice that lack KREMEN1 a negative regulator of Wnt signaling have abnormal TEC architecture suggesting a role for Wnt signaling in cTEC [129], however whether this role was in the initiation, expansion or maintenance of TEC was not addressed in this study. Taken together these findings suggest regulation of Wnt signaling is important for either the correct development or maintenance of the thymus. At least in part this may be through the regulation of Foxn1 expression in TECs. However any direct involvement of canonical or

alternative Wnt signaling in TEC development and function *in vivo* remains to be fully addressed.

BMPs

BMPs belong to the transforming growth factor (TGF) β family of cytokines that encompass multiple ligands and receptors. Upon ligand binding, two transmembrane receptor serine/threonine protein kinases (receptor types I and II) activate specific receptor-regulated Smad proteins. Activated Smads form a multi-subunit complex with a common partner, Smad4, which then translocates from the cytoplasm to the nucleus where they interact with additional nuclear factors to regulate transcription. Several proteins have been identified that may interfere with BMP signaling by either blocking ligand binding to the receptor (Noggin or Chordin) or by altering the cytoplasmic signal transduction by competing with Smad4 thus antagonizing BMP signaling (Smad7).

BMPs are expressed very early in thymus development. BMP4 is expressed in the ventral prospective thymus domain in the third pharyngeal pouch at E10.5 prior to the onset of Foxn1 [93, 116], and appears to act directly on thymus stroma leading to the up-regulation of Foxn1 [115]. In the third pharyngeal pouch, a dorsal domain of Shh expression opposes BMP4 expression (See Figure 3). In the absence of Shh, the domain of BMP4 expression extends dorsally with Foxn1 expression at E11.5 also expanded throughout the entire pouch [93]. Gcm2 expression was absent, suggesting that a BMP signal may be responsible for the induction of Foxn1, and hence the induction of a bipotent pTEC within the thymus primordia. This was consistent with a previous report suggesting a role for BMP4 in the induction of Foxn1 in TECs *in vitro* [118].

In contrast, at E10.5 Noggin expression overlaps with those cells that express Gcm2 and that will become the parathyroids [119]. Noggin expression in the presumptive parathyroid domain of the third pharyngeal pouch initiates after, but is independent of, the expression of Gcm2 [38]. Thus, the timing of Noggin expression within the pouch corresponds to that of BMP4, possibly as part of a negative feedback loop where epithelial BMP inhibits its own expression to allow specification of the Gcm2 expressing parathyroid domain [116]. This suggests that Foxn1 expression is the default pathway for the pouch endoderm and that Foxn1 requires active suppression to allow the establishment of an alternative parathyroid fate [117]. Another possibility is that Foxn1 controls the development of TECs in order to prevent the activation of a default pathway, which enforces a respiratory cell fate to epithelium of the ventral aspect of the third pharyngeal pouch [105, 130]. If this conclusion is correct, Foxn1 takes part in the specification of the third pharyngeal endoderm beyond a mere role as a differentiation factor for epithelial cells that are already committed to a thymus fate.

Direct evidence for a role of BMP in the development of the thymus stroma, has been demonstrated through analysis of a mouse in which transgenic expression in TECs of *Xenopus* Noggin (Xnoggin) is controlled by the Foxn1 promoter.

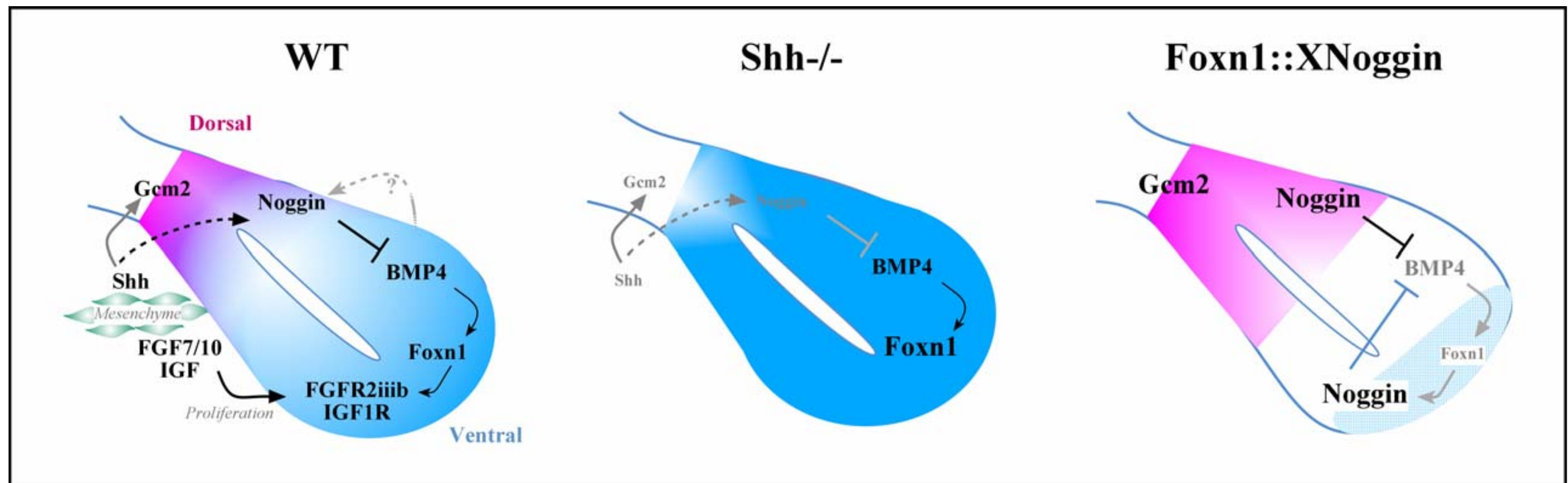

Figure 3. Regulation of the Foxn1 expression domain in the third pharyngeal pouch.

At E10.5 the future thymus anlage is marked by BMP expression, while that of the future parathyroid is marked by Noggin expression. Signaling through BMP within the ventral region of the third pharyngeal pouch endoderm at E10.5-11.5 results in the expression of Foxn1 and downstream products, such as FGFR. Ligation of FGFR by specific mesenchyme derived ligands, FGF7 or 10 results in TEC proliferation. Expression of the BMP antagonist Noggin in the dorsal region, possibly regulated by Shh, restricts the BMP signaling domain and allows for the maintenance of Gcm2 expression which is essential for parathyroid development. In mice lacking Shh, loss of Noggin expression results in broadened expression of Foxn1. In the Foxn1:Xnoggin transgenic mouse, decreased BMP signaling results in the loss of Foxn1 expression and impaired thymus development suggesting that BMP signaling might be involved at least in maintaining the reciprocal specification of parathyroid and thymus anlagen in the pharyngeal pouch.

In this model, interfering with BMP signals from E11.5 [116] may act on TECs either directly, though action on presumptive TECs, or indirectly, via an effect on the surrounding mesenchyme which expresses BMP at E12 and beyond, thus regulating the initiation, differentiation or maintenance of the thymus rudiment. Such inhibition of BMP signaling results in drastically impaired thymus development, with an epithelial mosaic, which may be due to insufficient or incomplete blocking of BMP signaling in this transgenic line. Abnormal TEC development was characterised by subsets of TECs failing to maintain Foxn1 expression and an associated loss of characteristics of proto-differentiated or differentiated TEC, such as IL-7, with a reversion to a phenotype characteristic of foregut epithelium [116, 117]. This finding suggests that a prolonged threshold of BMP signaling from E11.5 onward is required for the maintenance of Foxn1 expression and the irreversible commitment of pharyngeal pouch endoderm to the TEC lineage. Furthermore FoxN1 expression is required not only for the induction, but the maintenance of the thymus epithelium. In support of this hypothesis, recent analysis of Foxn1 mutant mice suggests an ongoing role for Foxn1 in thymus function [43].

An ongoing role for TGFβ/BMP signaling in TEC function has been investigated by transgenic expression of the inhibitory Smad7 in a subset of TECs under control of the K5 promoter, which resulted in a mild thymus phenotype, with an organised TEC structure but reduced thymus cellularity [131]. This mild phenotype is surprising as Smad7 inhibits TGFβ, activin and BMP signaling as well as affecting functions independent of TGFβ/BMP signaling, such as JNK activation and β-catenin degradation [132]. Compared to the transgenic Foxn1:Xnoggin model, the decreased severity of this phenotype may be due to the promoter used, which may not be expressed across all TEC and the cell autonomous effect does not directly impair BMP signaling in cells of mesenchymal origin. However, as this mouse is neonatally lethal, their reduced thymus cellularity could be a consequence of their general ill health and therefore, an unambiguous, ongoing role for BMP signaling in the thymus function could not be directly established. To address this, canonical TGFβ signaling in TEC was specifically inhibited beyond E12.5, by Cre-mediated deletion of Smad4 under control of the Foxn1 promoter [133]. The thymus of these mice demonstrate that Smad4 is not required for TEC differentiation, but disruption of the TGFβ pathway resulted in progressive structural disorganization of the microenvironment with a loss of thymopoietic potential and significant peripheral T cell lymphopenia. Such observations are characteristic of age associated thymus involution and support the possibility that canonical TGFβ/BMP signaling in TEC is required to maintain the postnatal thymus. Although these results indicate that BMP signaling is necessary for the maintenance of Foxn1 expression in the embryonic thymus, it may not be sufficient for Foxn1 initiation and such is likely to require additional factors, such as activation of Wnt or FGF signaling pathways [134].

Late Mediators of TEC Differentiation and Function

The expansion, differentiation and maintenance of the epithelial cells of the thymus medulla, in particular Aire expressing mTEC, is critically dependent on the presence of LTi like cells or positively selected $CD4^{+ve}$ but not $CD8^{+ve}$ thymocytes within the thymus

medulla. Until recently, however, it remained unclear precisely how these cells maintain the TEC compartment and what the regulators of TEC functions are at a molecular level. Analysis of the cellular regulators of the mTEC compartment, together with observations made from mutant models that demonstrate an abnormal mTEC development, has resulted in a number of molecules being recognised as specifically required for the differentiation of a normal medulla, and hence provided insights into some of the molecular mediators of mTEC function (Figure 4).

Nuclear Factor-κB (NFκB)-Mediated Signals

NFκB activation is mediated by either the canonical or alternate NFκB pathways, which trigger signal transduction events that lead to the translocation of NFκB subunits to into the nucleus, predominantly in association with RelB (Recently reviewed in [135]. In the thymus, NFκB-mediated signaling, through both TNFR-associated factor 6 (TRAF6) dependent and independent pathways [136], plays a central role in the development and organization of the thymus medulla. The severe reduction of the mTEC compartment in the RelB$^{-/-}$ thymus includes a dramatic loss of Aire expression and a paucity of UEA1^{+ve} mTEC [137]. Inactivation of signaling pathways that regulate RelB, namely TRAF6 [138], NFκB-inducing kinase (NIK) [139] or inhibitor of NFκB-inducing kinase–α [140], leads to mTEC abnormalities that variably recapitulate the RelB$^{-/-}$ thymus phenotype. In all of these mice, central tolerance induction is invariably compromised with development of organ specific autoimmunity consistent with a deficiency in the role of TECs, and in particular Aire expressing mTECs, in safeguarding tolerance to peripheral antigens. These observations suggest that activation of the alternate NFκB pathway is essential for full TEC function. The receptors upstream of the signal transducers of NFκB that activate the alternate NFκB signaling pathway include lymphotoxin β receptor (LTβR), Receptor Activator of NFκB (RANK) and CD40.

Lymphotoxin-β receptor

Targeted disruption of the LTβR signaling in TEC results in abnormal medullary organization with reduced numbers of mTECs and Aire-independent TRA expression, which leads to autoimmunity. However the loss of central tolerance in these mice is not caused by either the lack of differentiation or Aire expression in mTEC [141-143]. These findings show that LTβR signals have an important role in formation of the thymus medulla, but not at the level of Aire^{+ve} mTEC maturation or maintenance. Importantly, signaling through LTβR does not involve TRAF6 [144], which is essential for the development of Aire^{+ve} mTECs.

Receptor Activator of Nuclear Factor kappa B (RANK) and CD40

TRAF6 interacts with a variety of TNFRs expressed by TEC including RANK and CD40. RANK ligand (RANKL) and CD40L are expressed in the thymus by LTi-like cells [70, 72] and CD4 SP thymocytes from as early as E18 [72, 74, 145], suggesting that RANK–RANKL and CD40-CD40L interactions between TECs and lymphoid cells might regulate mTEC development and Aire expression. *In vivo* analysis of mice deficient for RANKL or CD40 has revealed a marked reduction in Aire^{+ve} mTECs [72, 74, 145], while double deficiency of RANKL and CD40 resulted in the abolishment of mature mTEC development,

Aire and TRA expression, with exacerbated symptoms of autoimmunity [145]. The RANKL decoy receptor OPG is strongly expressed in mTECs and deficiency in OPG results in an increase in the number of mTEC and enlargement of the thymus medulla, suggesting that OPG-mediated fine-tuning of RANKL availability at the mTEC surface crucially regulates RANK-mediated signals in mTECs [72].

Studies *in vitro* have demonstrated that stimulation of embryonic TEC with recombinant RANKL or CD40L is sufficient to induce TEC proliferation and the induction of Aire and TRA expression, independent of the presence of hematopoietic elements. Furthermore RANK or CD40 ligation induces the expression and translocation of RelB into the nucleus of mTECs, which was dependent on TRAF6 and NIK [72, 74, 145]. Collectively the data demonstrate that two TNF-family receptors RANK and CD40 cooperatively regulate the development of mTECs through the TRAF6- and NIK-dependent alternate NFκB activation pathway to establish self-tolerance (Figure 4). However, many questions still remain. A primary question is how these signals work to activate Aire expression in TEC and do they exert their roles in different stages of TEC development? Does the duration or strength of interaction between auto-antigen specific TCR bearing thymocytes and MHC:peptide bearing mTEC affect the activation of TRAF6-dependant pathways in addition to NFκB, such as JNK, p38, and Akt? If so, what is the threshold of affinity that allows for prolonged interaction that is sufficient to activate the alternate NF-κB pathway, which has significantly slower kinetics that the canonical pathway?

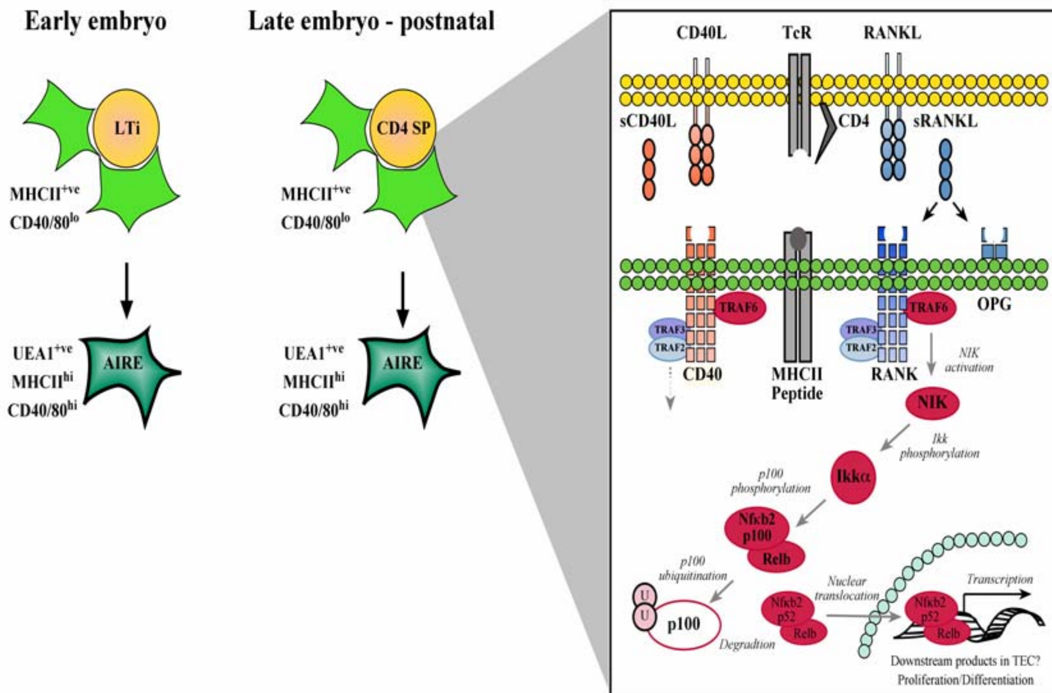

Figure 4. mTEC differentiation and proliferation is mediated by NFκB dependent thymus crosstalk.

LTi-like cells or CD4^{+ve} SP thymocytes bear TNFsf molecules, which interact with specific receptors on mTECs resulting in activation of the non-canonical NFκB signaling pathway that culminates in RelB activation. Ligand engagement activates NIK, which phosphorylates the downstream IKKα. Activated IKKα in turn phosphorylates the NFκB2/p100 leading to ubiquitin-dependent degradation and release of the N terminal polypeptide, p52. The formation of RelB/p52 heterodimers permits shuttling of RelB from the cytoplasm into the nucleus where it functions as a transcriptional regulator. Red symbols denote intracellular NFκB pathway intermediates that have been shown by functional inactivation *in vivo* to have an essential role in mTEC differentiation and proliferation. Abbreviations: NIK, NFκB-inducing kinase; IKK, inhibitor of κB kinase; p100/p52, NFκB2; p50, NFκB1; Rank, Receptor Activator of NFκB.

The Thymus and Regenerative Medicine

Loss and Regeneration of Thymus Function

Progressive, age related changes in the immune system contribute to an overall reduction in immune responsiveness, leading to increased susceptibility to infections [146, 147]. Age associated thymic senescence (or involution) is an important contributor to this immune dysfunction. The precise cellular and molecular mechanisms that underlie thymic senescence are only incompletely understood but are likely to affect hematopoietic as well as thymic stromal compartments [83, 85, 148, 149]. While the aged thymus may be sufficient to maintain some level of immune competence, these changes have important clinical implications, particularly in delayed immune reconstitution with minimal recovery which is likely following damaging immuno-depletive regimes for cancer treatment, such as chemotherapy and irradiation, or severe viral infections such as HIV where prolonged immunodeficiency can contribute to increased morbidity and mortality.

A number of methods to improve immune function by activating the regenerative capacity of the thymus can be revealed in a variety of experimental models (recently reviewed in [150]). Inhibition of sex steroid hormones can induce recovery in thymus function and return immune competence to young levels [85, 151, 152]. Signaling via FGFs enhances thymopoiesis via induction of post-natal TEC proliferation and also protects TECs from injury by irradiation or by graft versus-host disease [86, 152-154], while IGF administration has also been demonstrated to enhance thymopoiesis, primarily through TEC expansion [155]. As such, the use of inhibitors of sex steroid hormones, FGFs, GH and IGFs represent the first candidates in thymus-based regenerative therapies. However, further studies are clearly required to understand TEC development and function in more detail and to explore potential therapeutic interventions to overcome TEC senescence.

New Beginnings

Developments in the rapidly emerging field of stem cell biology combined with the identification of pTECs in the embryonic thymus has raised the possibility of generating thymus tissues from extrathymic sources such as embryonic stem (ES) cells. ES cells are derived from the inner cell mass of the blastocyst of both human and the mouse and can be maintained in a pluripotent state under defined conditions [156, 157]. The capacity of ES cells to differentiate and generate diverse cell types in culture, together with the access to virtually unlimited numbers of potential tissue-specific progenitors in these differentiation

cultures, provides a novel source of cells for cell replacement therapy [158]. Furthermore, the recent discovery that adult, somatic cells can be reprogrammed using a relatively simple procedure to an induced pluripotent state (iPS) [159, 160] opens the way for the generation of patient-specific ES cells [161]. This may overcome the current rejection problems associated with transplantation of ES-derived tissues [162]. As such ES cells are a potential source of replacement TEC that could be used to generate an immune system in people with congenital thymic deficiencies (such as in DiGeorge or velocardiofacial syndromes), or to improve acquired thymic dysfunction, such as occurs with aging or after immuno-depletive regimes for cancer treatment, or severe viral infections such as HIV.

However, in order to implement such a strategy, research on how to direct the development of TEC from ES cells is necessary. The identification of factors that govern the differentiation of ES cells towards the endoderm lineage are still relatively new [163, 164], and currently protocols for the derivation of thymus-specified progenitors, have not yet been elucidated. A central element of this research will involve the identification and investigation of factors that act upstream of Foxn1, (see Table 1) which have a role in directing the transition from ES cells to endoderm and subsequently to pTEC. In addition, as expression of Foxn1 and Dll4 are highly dependent on the three-dimensional organization of the thymic stroma [127] and the ability to support thymopoiesis is lost when TECs are cultured in a monolayer [165], techniques to overcome this dependence or the establishment of matrices/scaffolding to preserve a three-dimensional structure in *in vitro* cultured cells may be required for efficient generation of pTEC. ES cell technology is in its infancy, but knowledge is advancing rapidly, and has clear prospects for use in the future treatment of states of clinical immunodeficiency.

New Animal Models to Study TEC Development and Function

It is clear that there are large gaps in our understanding of the sequence of events occurring during differentiation from endodermal progenitors to mature TEC. Specifically, factors responsible for regulating TEC proliferation, death, differentiation and function are incompletely understood. To address the current lack of knowledge new animal models have recently been designed and as a result, mutations can now be introduced specifically into TEC, either by gene targeting using the FoxN1 promoter [116] or by employing a Cre/lox strategy with expression of the Cre-recombinase in TEC under the control of the Foxn1 promoter [15, 133, 166, 167]. These recently established models will prove useful in studying gene function in thymus organogenesis and for fate and functional mapping of TEC subsets. However, care must be taken in the interpretation of the resulting phenotypes of these mutation or deletion systems, as the interdependence of the thymus microenvironment results in difficulties in demonstrating function of specific TEC subpopulations in a physiological context. In addition, the identification of direct from indirect TEC phenotypes is not easily distinguishable and may be relative to the experimental settings used. For example, timing of gene deletion and cessation of function may vary between experimental strains, and needs to

be clearly determined for each line used. Differential timing of deletion may result in ambiguous findings or to the creation of chimeric tissues [117].

Additionally without knowledge of early transcriptional mediators, which act prior to Foxn1 induction, the current models may shed limited information on genes functional in the earliest inductive stages of anlage formation (< E10.5). Models in which gene deletion may be specifically targeted to developing pharyngeal regions prior to the onset of Foxn1 (Foxg1-[168], FGF15-[124] or Hoxa3-Cre [169]) may reveal information on early gene function, although the ability to determine direct from indirect TEC phenotypes may prove difficult due to strain-dependent variable Cre activity or wide distribution of Cre expression. Conversely, deletion of a gene required in early thymus ontogeny may result in severe thymus hyoplasia making it difficult to study any effect of the gene in adult thymus function. These difficulties in determining the differential gene function in embryonic from adult TEC may eventually be overcome by a new generation of mouse lines, which promise TEC specific expression of Cre by controlled transcriptional activation, utilizing either Cre-ERT or Cre-TET off systems. The use of these new and proposed models promise to provide insights into TEC development and maintenance and to reveal temporal and spatial changes in gene expression patterns in TECs.

Conclusion

As the primary mediators of T lymphocyte development, a better understanding of the cellular and molecular mechanisms underlying TEC specification and maintenance will be of interest both for basic and clinical immunology. As thymus function deteriorates with age or under certain congenital, disease or treatment-associated conditions, it would be highly desirable to improve thymus function and *de novo* T lymphocyte production in these situations. Recent research has provided novel insight into thymus organogenesis and function. The observation that the generation of a thymus is the result of pTEC activity, subsequent establishment of lineage relationships among TEC subsets and the identification of key molecular mediators of mature TEC function ensures that new approaches to clinical management of immune disorders will continue to evolve. As such, research on TEC development and function stands to offer significant clinical contributions to both transplantation immunology and for regenerative medicine in the coming years.

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References

[1] Gill J, Malin M, Sutherland J, Gray D, et al. (2003). Thymic generation and regeneration. *Immunol Rev,* 195,28-50.

[2] Petrie HT, Zúñiga-Pflücker JC. (2007). Zoned Out: Functional Mapping of Stromal Signaling Microenvironments in the Thymus. *Annu Rev Immunol,* 25,649-679.

[3] Hogquist KA, Baldwin TA, Jameson SC. (2005). Central tolerance: learning self-control in the thymus. *Nat Rev Immunol,* 5,772-782.

[4] Kronenberg M, Rudensky A. (2005). Regulation of immunity by self-reactive T cells. *Nature,* 435,598-604.

[5] Bluestone JA, Tang Q. (2005). How do CD4(+)CD25(+) regulatory T cells control autoimmunity? *Curr Opin Immunol,* 17,638-642.

[6] Haynes BF, Markert ML, Sempowski GD, Patel DD, Hale LP. (2000). The role of the thymus in immune reconstitution in aging, bone marrow transplantation, and HIV-1 infection. *Annu Rev Immunol,* 18,529-560.

[7] Gruver AL, Hudson LL, Sempowski GD. (2007). Immunosenescence of ageing. *J Pathol,* 211,144-156.

[8] Murata S, Sasaki K, Kishimoto T, Niwa S, et al. (2007). Regulation of CD8+ T cell development by thymus-specific proteasomes. *Science,* 316,1349-1353.

[9] Hogquist KA, Bevan MJ. (1996). The nature of the peptide/MHC ligand involved in positive selection. *Semin Immunol,* 8,63-8..

[10] Marrack P, McCormack J, Kappler J. (1989). Presentation of antigen, foreign major histocompatibility complex proteins and self by thymus cortical epithelium. *Nature,* 338,503-505.

[11] Schmitt TM, Ciofani M, Petrie HT, Zúñiga-Pflücker JC. (2004). Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J Exp Med,* 200,469-479.

[12] Hare KJ, Jenkinson EJ, Anderson G. (1999). In vitro models of T cell development. *Semin Immunol,* 11,3-12.

[13] de Pooter RF, Cho SK, Carlyle JR, Zúñiga-Pflücker JC. (2003). In vitro generation of T lymphocytes from embryonic stem cell-derived prehematopoietic progenitors. *Blood,* 102,1649-1653.

[14] Rodewald HR. (2000). Thymus epithelial cell reaggregate grafts. *Curr Top Microbiol Immunol,* 251,101-108.

[15] Hozumi K, Mailhos C, Negishi N, Hirano K, et al. (2008). Delta-like 4 is indispensable in thymic environment specific for T cell development. *J Exp Med,* 205,2507-2513.

[16] Koch U, Fiorini E, Benedito R, Besseyrias V, et al. (2008). Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *J Exp Med,* 205,2515-2523.

[17] Goldrath AW, Bevan MJ. (1999). Selecting and maintaining a diverse T-cell repertoire. *Nature,* 402,255-62..

[18] Singer A, Adoro S, Park JH. (2008). Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat Rev Immunol,* 8,788-801.

[19] Kurobe H, Liu C, Ueno T, Saito F, et al. (2006). CCR7-Dependent Cortex-to-Medulla Migration of Positively Selected Thymocytes Is Essential for Establishing Central Tolerance. *Immunity,* 24,165-177.

[20] Ueno T, Saito F, Gray DH, Kuse S, et al. (2004). CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *J Exp Med,* 200,493-505.

[21] Kyewski B, Klein L. (2006). A central role for central tolerance. *Annu Rev Immunol,* 24,571-606.

[22] Gallegos AM, Bevan MJ. (2006). Central tolerance: good but imperfect. *Immunol Rev,* 209,290-296.

[23] Derbinski J, Gäbler J, Brors B, Tierling S, et al. (2005). Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J Exp Med,* 202,33-45.

[24] Anderson MS, Venanzi ES, Klein L, Chen Z, et al. (2002). Projection of an immunological self shadow within the thymus by the aire protein. *Science,* 298,1395-1401.

[25] Schwab SR, Cyster JG. (2007). Finding a way out: lymphocyte egress from lymphoid organs. *Nat Immunol,* 8,1295-1301.

[26] Mori K, Itoi M, Tsukamoto N, Kubo H, Amagai T. (2007). The perivascular space as a path of hematopoietic progenitor cells and mature T cells between the blood circulation and the thymic parenchyma. *Int Immunol,* 19,745-753.

[27] Blackburn CC, Manley NR. (2004). Developing a new paradigm for thymus organogenesis. *Nat Rev Immunol,* 4,278-289.

[28] Grapin-Botton A, Melton DA. (2000). Endoderm development: from patterning to organogenesis. *Trends Genet,* 16,124-130.

[29] Kumar M, Jordan N, Melton D, Grapin-Botton A. (2003). Signals from lateral plate mesoderm instruct endoderm toward a pancreatic fate. *Dev Biol,* 259,109-122.

[30] Moore-Scott BA, Opoka R, Lin SC, Kordich JJ, Wells JM. (2007). Identification of molecular markers that are expressed in discrete anterior-posterior domains of the endoderm from the gastrula stage to mid-gestation. *Dev Dyn,* 236,1997-2003.

[31] Nyeng P, Norgaard GA, Kobberup S, Jensen J. (2007). FGF10 signaling controls stomach morphogenesis. *Dev Biol,* 303,295-310.

[32] Bort R, Signore M, Tremblay K, Martinez Barbera JP, Zaret KS. (2006). Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development. *Dev Biol,* 290,44-56.

[33] Cardoso WV, Lü J. (2006). Regulation of early lung morphogenesis: questions, facts and controversies. *Development,* 133,1611-1624.

[34] Zaret KS. (2002). Regulatory phases of early liver development: paradigms of organogenesis. *Nat Rev Genet,* 3,499-512.

[35] Shannon JM, Hyatt BA. (2004). Epithelial-mesenchymal interactions in the developing lung. *Annu Rev Physiol,* 66,625-645.

[36] Crump JG, Maves L, Lawson ND, Weinstein BM, Kimmel CB. (2004). An essential role for Fgfs in endodermal pouch formation influences later craniofacial skeletal patterning. *Development,* 131,5703-5716.

[37] Gordon J, Wilson VA, Blair NF, Sheridan J, et al. (2004). Functional evidence for a single endodermal origin for the thymic epithelium. *Nat Immunol,* 5,546-553.

[38] Liu Z, Yu S, Manley NR. (2007). Gcm2 is required for the differentiation and survival of parathyroid precursor cells in the parathyroid/thymus primordia. *Dev Biol,* 305,333-346.

[39] Nehls M, Kyewski B, Messerle M, Waldschütz R, et al. (1996). Two genetically separable steps in the differentiation of thymic epithelium. *Science,* 272,886-889.

[40] Nehls M, Pfeifer D, Schorpp M, Hedrich H, Boehm T. (1994). New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature,* 372,103-107.

[41] Blackburn CC, Augustine CL, Li R, Harvey RP, et al. (1996). The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors. *Proc Natl Acad Sci U S A,* 93,5742-5746.

[42] Chen L, Xiao S, Manley NR. (20082009). Foxn1 is required to maintain the postnatal thymic microenvironment in a dosage-sensitive manner. *Blood,* 113, 567-574.

[43] Su DM, Navarre S, Oh WJ, Condie BG, Manley NR. (2003). A domain of Foxn1 required for crosstalk-dependent thymic epithelial cell differentiation. *Nat Immunol,* 4,1128-1135.

[44] Tsukamoto N, Itoi M, Nishikawa M, Amagai T. (2005). Lack of Delta like 1 and 4 expressions in nude thymus anlages. *Cell Immunol,* 234,77-80.

[45] Zamisch M, Moore-Scott B, Su DM, Lucas PJ, et al. (2005). Ontogeny and regulation of IL-7-expressing thymic epithelial cells. *J Immunol,* 174,60-67.

[46] Bleul CC, Boehm T. (2000). Chemokines define distinct microenvironments in the developing thymus. *Eur J Immunol,* 30,3371-3379.

[47] Liu C, Saito F, Liu Z, Lei Y, et al. (2006). Coordination between CCR7- and CCR9-mediated chemokine signals in pre-vascular fetal thymus colonization. *Blood,* 108,2531-2539.

[48] Rossi SW, Chidgey AP, Parnell SM, Jenkinson WE, et al. (2007). Redefining epithelial progenitor potential in the developing thymus. *Eur J Immunol,* 37,2411-2418.

[49] Klug DB, Carter C, Gimenez-Conti IB, Richie ER. (2002). Cutting edge: thymocyte-independent and thymocyte-dependent phases of epithelial patterning in the fetal thymus. *J Immunol,* 169,2842-2845.

[50] Itoi M, Tsukamoto N, Yoshida H, Amagai T. (2007). Mesenchymal cells are required for functional development of thymic epithelial cells. *Int Immunol,* 19,953-964.

[51] Liu C, Ueno T, Kuse S, Saito F, et al. (2005). The role of CCL21 in recruitment of T-precursor cells to fetal thymi. *Blood,* 105,31-39.

[52] Jenkinson WE, Rossi SW, Parnell SM, Agace WW, et al. (2007). Chemokine receptor expression defines heterogeneity in the earliest thymic migrants. *Eur J Immunol,* 37,2090-2096.

[53] Gill J, Malin M, Holländer GA, Boyd R. (2002). Generation of a complete thymic microenvironment by MTS24(+) thymic epithelial cells. *Nat Immunol,* 3,635-642.

[54] Klug DB, Carter C, Crouch E, Roop D, et al. (1998). Interdependence of cortical thymic epithelial cell differentiation and T-lineage commitment. *Proc Natl Acad Sci U S A,* 95,11822-11827.

[55] Suniara RK, Jenkinson EJ, Owen JJ. (2000). An essential role for thymic mesenchyme in early T cell development. *J Exp Med,* 191,1051-1056.

[56] Foster K, Sheridan J, Veiga-Fernandes H, Roderick K, et al. (2008). Contribution of neural crest-derived cells in the embryonic and adult thymus. *J Immunol,* 180,3183-3189.

[57] Müller SM, Stolt CC, Terszowski G, Blum C, et al. (2008). Neural crest origin of perivascular mesenchyme in the adult thymus. *J Immunol,* 180,5344-5351.

[58] Conway SJ, Henderson DJ, Copp AJ. (1997). Pax3 is required for cardiac neural crest migration in the mouse: evidence from the splotch (Sp2H) mutant. *Development,* 124,505-14..

[59] Kurihara Y, Kurihara H, Maemura K, Kuwaki T, et al. (1995). Impaired development of the thyroid and thymus in endothelin-1 knockout mice. *J Cardiovasc Pharmacol,* 26,S13-S6..

[60] Greenberg F. (1993). DiGeorge syndrome: an historical review of clinical and cytogenetic features. *J Med Genet,* 30,803-86..

[61] Jenkinson WE, Rossi SW, Parnell SM, Jenkinson EJ, Anderson G. (2006). PDGFR{alpha}-expressing mesenchyme regulates thymus growth and the availability of intrathymic niches. *Blood,* 109,954-960.

[62] Dooley J, Erickson M, Larochelle WJ, Gillard GO, Farr AG. (2007). FGFR2IIIb signaling regulates thymic epithelial differentiation. *Dev Dyn,* 236,3459-3471.

[63] Revest JM, Suniara RK, Kerr K, Owen JJ, Dickson C. (2001). Development of the thymus requires signaling through the fibroblast growth factor receptor r2-iiib. *J Immunol,* 167,1954-161..

[64] Kenins L, Gill JW, Boyd RL, Holländer GA, Wodnar-Filipowicz A. (2008). Intrathymic expression of Flt3 ligand enhances thymic recovery after irradiation. *J Exp Med,* 205,523-531.

[65] Rodewald HR, Kretzschmar K, Swat W, Takeda S. (1995). Intrathymically expressed c-kit ligand (stem cell factor) is a major factor driving expansion of very immature thymocytes in vivo. *Immunity,* 3,313-319.

[66] Balciunaite G, Ceredig R, Fehling HJ, Zúñiga-Pflücker JC, Rolink AG. (2005). The role of Notch and IL-7 signaling in early thymocyte proliferation and differentiation. *Eur J Immunol,* 35,1292-1300.

[67] Ritter MA, Boyd RL. (1993). Development in the thymus: it takes two to tango. *Immunol Today,* 14,462-469.

[68] van Ewijk W, Holländer G, Terhorst C, Wang B. (2000). Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. *Development,* 127,1583-1591.

[69] Derbinski J, Schulte A, Kyewski B, Klein L. (2001). Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol,* 2,1032-109..

[70] Rossi SW, Kim MY, Leibbrandt A, Parnell SM, et al. (2007). RANK signals from CD4(+)3(-) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla. *J Exp Med,* 204,1267-1272.

[71] White AJ, Withers DR, Parnell SM, Scott HS, et al. (2008). Sequential phases in the development of Aire-expressing medullary thymic epithelial cells involve distinct cellular input. *Eur J Immunol,* 38,942-947.

[72] Hikosaka Y, Nitta T, Ohigashi I, Yano K, et al. (2008). The Cytokine RANKL Produced by Positively Selected Thymocytes Fosters Medullary Thymic Epithelial Cells that Express Autoimmune Regulator. *Immunity,* 29,438-450.

[73] Shores EW, van Ewijk W, Singer A. (1991). Disorganisation and restoration of thymic medullary epithelial cells in T cell receptor-negative scid mice: evidence that receptor-bearing lymphocytes influence maturation of the thymic microenvironment. *Eur. J. Immunol.,* 21,1657-1661.

[74] Irla M, Hugues S, Gill J, Nitta T, et al. (2008). Autoantigen-specific interactions with CD4+ thymocytes control mature medullary thymic epithelial cell cellularity. *Immunity,* 29,451-463.

[75] Fuchs E. (2007). Scratching the surface of skin development. *Nature,* 445,834-842.

[76] Sharma AD, Cantz T, Manns MP, Ott M. (2006). The role of stem cells in physiology, pathophysiology, and therapy of the liver. *Stem Cell Rev,* 2,51-58.

[77] Xu X, D'Hoker J, Stangé G, Bonné S, et al. (2008). Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. *Cell,* 132,197-207.

[78] Gray DH, Fletcher AL, Hammett M, Seach N, et al. (2008). Unbiased analysis, enrichment and purification of thymic stromal cells. *J Immunol Methods,* 329,56-66.

[79] Gray D, Abramson J, Benoist C, Mathis D. (2007). Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. *J Exp Med,* 204,2521-2528.

[80] Bennett AR, Farley A, Blair NF, Gordon J, et al. (2002). Identification and characterization of thymic epithelial progenitor cells. *Immunity,* 16,803-814.

[81] Hamazaki Y, Fujita H, Kobayashi T, Choi Y, et al. (2007). Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin. *Nat Immunol,* 8,304-311.

[82] Rodewald HR, Paul S, Haller C, Bluethmann H, Blum C. (2001). Thymus medulla consisting of epithelial islets each derived from a single progenitor. *Nature,* 414,763-78..

[83] Gray DH, Seach N, Ueno T, Milton MK, et al. (2006). Developmental kinetics, turnover and stimulatory capacity of thymic epithelial cells. *Blood,* 108,3777-3785.

[84] Gillard GO, Farr AG. (2006). Features of medullary thymic epithelium implicate postnatal development in maintaining epithelial heterogeneity and tissue-restricted antigen expression. *J Immunol,* 176,5815-5824.

[85] Sutherland JS, Goldberg GL, Hammett MV, Uldrich AP, et al. (2005). Activation of Thymic Regeneration in Mice and Humans following Androgen Blockade. *J Immunol,* 175,2741-2753.

[86] Rossi SW, Jeker LT, Ueno T, Kuse S, et al. (2007). Keratinocyte growth factor (KGF) enhances postnatal T- cell development via enhancements in proliferation and function of thymic epithelial cells. *Blood,* 109,3803-3811.

[87] Min D, Panoskaltsis-Mortari A, Kuro-O M, Hollander GA, et al. (2006). Sustained thymopoiesis and improvement in functional immunity induced by exogenous KGF administration in murine models of aging. *Blood,* 109,2529-2537.

[88] Rossi SW, Jenkinson WE, Anderson G, Jenkinson EJ. (2006). Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium. *Nature,* 441,988-991.

[89] Bleul CC, Corbeaux T, Reuter A, Fisch P, et al. (2006). Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. *Nature,* 441,992-996.

[90] Anderson G, Lane PJ, Jenkinson EJ. (2007). Generating intrathymic microenvironments to establish T-cell tolerance. *Nat Rev Immunol,* 7,954-963.

[91] Bachiller D, Klingensmith J, Shneyder N, Tran U, et al. (2003). The role of chordin/Bmp signals in mammalian pharyngeal development and DiGeorge syndrome. *Development,* 130,3567-3578.

[92] Holländer G, Gill J, Zuklys S, Iwanami N, et al. (2006). Cellular and molecular events during early thymus development. *Immunol Rev,* 209,28-46.

[93] Moore-Scott BA, Manley NR. (2005). Differential expression of Sonic hedgehog along the anterior-posterior axis regulates patterning of pharyngeal pouch endoderm and pharyngeal endoderm-derived organs. *Dev Biol,* 278,323-335.

[94] Garg V, Yamagishi C, Hu T, Kathiriya IS, et al. (2001). Tbx1, a DiGeorge syndrome candidate gene, is regulated by sonic hedgehog during pharyngeal arch development. *Dev Biol,* 235,62-73..

[95] Abu-Issa R, Smyth G, Smoak I, Yamamura KI, Meyers EN. (2002). Fgf8 is required for pharyngeal arch and cardiovascular development in the mouse. *Development,* 129,4613-4625..

[96] Manley NR, Selleri L, Brendolan A, Gordon J, Cleary ML. (2004). Abnormalities of caudal pharyngeal pouch development in Pbx1 knockout mice mimic loss of Hox3 paralogs. *Dev Biol,* 276,301-312.

[97] Ivins S, Lammerts van Beuren K, Roberts C, James C, et al. (2005). Microarray analysis detects differentially expressed genes in the pharyngeal region of mice lacking Tbx1. *Dev Biol,* 285,554-569.

[98] Su D, Ellis S, Napier A, Lee K, Manley NR. (2001). Hoxa3 and pax1 regulate epithelial cell death and proliferation during thymus and parathyroid organogenesis. *Dev Biol,* 236,316-329.

[99] Su DM, Manley NR. (2000). Hoxa3 and pax1 transcription factors regulate the ability of fetal thymic epithelial cells to promote thymocyte development. *J Immunol,* 164,5753-5760.

[100] Manley NR, Capecchi MR. (1995). The role of Hoxa-3 in mouse thymus and thyroid development. *Development,* 121,1989-2003.

[101] Zou D, Silvius D, Davenport J, Grifone R, et al. (2006). Patterning of the third pharyngeal pouch into thymus/parathyroid by Six and Eya1. *Dev Biol,* 293,499-512.

[102] Xu PX, Zheng W, Laclef C, Maire P, et al. (2002). Eya1 is required for the morphogenesis of mammalian thymus, parathyroid and thyroid. *Development,* 129,3033-3044.

[103] Laclef C, Souil E, Demignon J, Maire P. (2003). Thymus, kidney and craniofacial abnormalities in Six 1 deficient mice. *Mech Dev,* 120,669-679.

[104] Nehls M, Kyewski B, Messerle M, Waldschutz R, et al. (1996). Two genetically separable steps in the differentiation of thymic epithelium. *Science,* 272,886-889.

[105] Dooley J, Erickson M, Roelink H, Farr AG. (2005). Nude thymic rudiment lacking functional foxn1 resembles respiratory epithelium. *Dev Dyn,* 233,1605-1612.

[106] Su D, Manley NR. (2000). Hoxa3 and Pax1 transcription factors regulate the ability of fetal thymic epithelial cells to promote thymocyte development. *J. Immunol.,* 164,5753-5760.

[107] Wallin J, Eibel H, Neubuser A, Wilting J, et al. (1996). Pax1 is expressed during development of the thymus epithelium and is required for normal T-cell maturation. *Development,* 122,23-30.

[108] Fraser GJ, Bloomquist RF, Streelman JT. (2008). A periodic pattern generator for dental diversity. *BMC Biol,* 6,32.

[109] Hetzer-Egger C, Schorpp M, Haas-Assenbaum A, Balling R, et al. (2002). Thymopoiesis requires Pax9 function in thymic epithelial cells. *Eur J Immunol,* 32,1175-1181.

[110] Peters H, Neubüser A, Kratochwil K, Balling R. (1998). Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes Dev,* 12,2735-2747.

[111] Frank DU, Fotheringham LK, Brewer JA, Muglia LJ, et al. (2002). An Fgf8 mouse mutant phenocopies human 22q11 deletion syndrome. *Development,* 129,4591-4603..

[112] Ohuchi H, Hori Y, Yamasaki M, Harada H, et al. (2000). FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. *Biochem Biophys Res Commun,* 277,643-649.

[113] Erickson M, Morkowski S, Lehar S, Gillard G, et al. (2002). Regulation of thymic epithelium by keratinocyte growth factor. *Blood,* 100,3269-3278.

[114] Revest JM, Spencer-Dene B, Kerr K, De Moerlooze L, et al. (2001). Fibroblast growth factor receptor 2-IIIb acts upstream of Shh and Fgf4 and is required for limb bud maintenance but not for the induction of Fgf8, Fgf10, Msx1, or Bmp4. *Dev Biol,* 231,47-62..

[115] Patel SR, Gordon J, Mahbub F, Blackburn CC, Manley NR. (2006). Bmp4 and Noggin expression during early thymus and parathyroid organogenesis. *Gene Expr Patterns,* 6,794-799.

[116] Bleul CC, Boehm T. (2005). BMP Signaling Is Required for Normal Thymus Development. *J Immunol,* 175,5213-5221.

[117] Soza-Ried C, Bleul CC, Schorpp M, Boehm T. (2008). Maintenance of thymic epithelial phenotype requires extrinsic signals in mouse and zebrafish. *J Immunol,* 181,5272-5277.

[118] Tsai PT, Lee RA, Wu H. (2003). BMP4 acts upstream of FGF in modulating thymic stroma and regulating thymopoiesis. *Blood,* 102,3947-3953.

[119] Gordon J, Bennett AR, Blackburn CC, Manley NR. (2001). Gcm2 and Foxn1 mark early parathyroid- and thymus-specific domains in the developing third pharyngeal pouch. *Mech Dev,* 103,141-143.

[120] Arnold JS, Werling U, Braunstein EM, Liao J, et al. (2006). Inactivation of Tbx1 in the pharyngeal endoderm results in 22q11DS malformations. *Development,* 133,977-987.

[121] Merscher S, Funke B, Epstein JA, Heyer J, et al. (2001). TBX1 is responsible for cardiovascular defects in velo-cardio- facial/DiGeorge syndrome. *Cell,* 104,619-29..

[122] Brown CB, Wenning JM, Lu MM, Epstein DJ, et al. (2004). Cre-mediated excision of Fgf8 in the Tbx1 expression domain reveals a critical role for Fgf8 in cardiovascular development in the mouse. *Dev Biol,* 267,190-202.

[123] Vitelli F, Taddei I, Morishima M, Meyers EN, et al. (2002). A genetic link between Tbx1 and fibroblast growth factor signaling. *Development,* 129,4605-4611.

[124] Zhang Z, Cerrato F, Xu H, Vitelli F, et al. (2005). Tbx1 expression in pharyngeal epithelia is necessary for pharyngeal arch artery development. *Development,* 132,5307-5315.

[125] Senoo M, Pinto F, Crum CP, McKeon F. (2007). p63 Is essential for the proliferative potential of stem cells in stratified epithelia. *Cell,* 129,523-536.

[126] Candi E, Rufini A, Terrinoni A, Giamboi-Miraglia A, et al. (2007). {Delta}Np63 regulates thymic development through enhanced expression of FgfR2 and Jag2. *Proc Natl Acad Sci U S A,* 104,11999-12004.

[127] Balciunaite G, Keller MP, Balciunaite E, Piali L, et al. (2002). Wnt glycoproteins regulate the expression of FoxN1, the gene defective in nude mice. *Nat Immunol,* 3,1102-1108.

[128] Kuraguchi M, Wang XP, Bronson RT, Rothenberg R, et al. (2006). Adenomatous Polyposis Coli (APC) Is Required for Normal Development of Skin and Thymus. *PLoS Genet,* 2,e146.

[129] Osada M, Ito E, Fermin HA, Vazquez-Cintron E, et al. (2006). The Wnt signaling antagonist Kremen1 is required for development of thymic architecture. *Clin Dev Immunol,* 13,299-319.

[130] Dooley J, Erickson M, Farr AG. (2005). An organized medullary epithelial structure in the normal thymus expresses molecules of respiratory epithelium and resembles the epithelial thymic rudiment of nude mice. *J Immunol,* 175,4331-4337.

[131] He W, Li AG, Wang D, Han S, et al. (2002). Overexpression of Smad7 results in severe pathological alterations in multiple epithelial tissues. *Embo J,* 21,2580-290..

[132] Nakao A, Afrakhte M, Moren A, Nakayama T, et al. (1997). Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature,* 389,631-635.

[133] Jeker LT, Barthlott T, Keller MP, Zuklys S, et al. (2008). Maintenance of a normal thymic microenvironment and T cell homeostasis require Smad4-mediated signalling in thymic epithelial cells. *Blood,* 112, 3688-3695.

[134] Boehm T. (2008). Thymus development and function. *Curr Opin Immunol,* 20,178-184.

[135] Sun SC, Ley SC. (2008). New insights into NF-kappaB regulation and function. *Trends Immunol,* 29,469-478.

[136] Dickson KM, Bhakar AL, Barker PA. (2004). TRAF6-dependent NF-kB transcriptional activity during mouse development. *Dev Dyn,* 231,122-127.

[137] Heino M, Peterson P, Sillanpää N, Guérin S, et al. (2000). RNA and protein expression of the murine autoimmune regulator gene (Aire) in normal, RelB-deficient and in NOD mouse. *Eur J Immunol,* 30,1884-1893.

[138] Akiyama T, Maeda S, Yamane S, Ogino K, et al. (2005). Dependence of self-tolerance on TRAF6-directed development of thymic stroma. *Science,* 308,248-251.

[139] Kajiura F, Sun S, Nomura T, Izumi K, et al. (2004). NF-kappa B-inducing kinase establishes self-tolerance in a thymic stroma-dependent manner. *J Immunol,* 172,2067-2075.

[140] Kinoshita D, Hirota F, Kaisho T, Kasai M, et al. (2006). Essential role of IkappaB kinase alpha in thymic organogenesis required for the establishment of self-tolerance. *J Immunol,* 176,3995-4002.

[141] Seach N, Ueno T, Fletcher AL, Lowen T, et al. (2008). The lymphotoxin pathway regulates Aire-independent expression of ectopic genes and chemokines in thymic stromal cells. *J Immunol,* 180,5384-5392.

[142] Martins VC, Boehm T, Bleul CC. (2008). Ltbetar signaling does not regulate Aire-dependent transcripts in medullary thymic epithelial cells. *J Immunol,* 181,400-407.

[143] Venanzi ES, Gray DH, Benoist C, Mathis D. (2007). Lymphotoxin pathway and Aire influences on thymic medullary epithelial cells are unconnected. *J Immunol,* 179,5693-5700.

[144] Ishida T, Mizushima S, Azuma S, Kobayashi N, et al. (1996). Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region. *J Biol Chem,* 271,28745-28748.

[145] Akiyama T, Shimo Y, Yanai H, Qin J, et al. (2008). The tumor necrosis factor family receptors RANK and CD40 cooperatively establish the thymic medullary microenvironment and self-tolerance. *Immunity,* 29,423-437.

[146] Aspinall R, Andrew D. (2000). Thymic involution in aging. *J Clin Immunol,* 20,250-26..

[147] Prelog M. (2006). Aging of the immune system: A risk factor for autoimmunity? *Autoimmun Rev,* 5,136-139.

[148] Heng TS, Goldberg GL, Gray DH, Sutherland JS, et al. (2005). Effects of castration on thymocyte development in two different models of thymic involution. *J Immunol,* 175,2982-2993.

[149] Zediak VP, Maillard I, Bhandoola A. (2007). Multiple prethymic defects underlie age-related loss of T progenitor competence. *Blood,* 110,1161-1167.

[150] Chidgey AP, Seach N, Dudakov J, Hammett MV, Boyd RL. (2008). Strategies for reconstituting and boosting T cell-based immunity following haematopoietic stem cell transplantation: pre-clinical and clinical approaches. *Semin Immunopathol,* 30, 457-477.

[151] Sutherland JS, Spyroglou L, Muirhead JL, Heng TS, et al. (2008). Enhanced immune system regeneration in humans following allogeneic or autologous hemopoietic stem cell transplantation by temporary sex steroid blockade. *Clin Cancer Res,* 14,1138-1149.

[152] Kelly RM, Highfill SL, Panoskaltsis-Mortari A, Taylor PA, et al. (2008). Keratinocyte growth factor and androgen blockade work in concert to protect against conditioning regimen-induced thymic epithelial damage and enhance T-cell reconstitution after murine bone marrow transplantation. *Blood,* 111,5734-5744.

[153] Min D, Taylor PA, Panoskaltsis-Mortari A, Chung B, et al. (2002). Protection from thymic epithelial cell injury by keratinocyte growth factor: a new approach to improve thymic and peripheral T-cell reconstitution after bone marrow transplantation. *Blood,* 99,4592-4600.

[154] Rossi S, Blazar BR, Farrell CL, Danilenko DM, et al. (2002). Keratinocyte growth factor preserves normal thymopoiesis and thymic microenvironment during experimental graft-versus-host disease. *Blood,* 100,682-691.

[155] Chu YW, Schmitz S, Choudhury B, Telford W, et al. (2008). Exogenous insulin-like growth factor 1 enhances thymopoiesis predominantly through thymic epithelial cell expansion. *Blood,* 112,2836-2846.

[156] Rossant J. (2001). Stem cells from the Mammalian blastocyst. *Stem Cells,* 19,477-482.

[157] Nichols J, Zevnik B, Anastassiadis K, Niwa H, et al. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell,* 95,379-391.

[158] Semb H. (2008). Definitive endoderm: a key step in coaxing human embryonic stem cells into transplantable beta-cells. *Biochem Soc Trans,* 36,272-275.

[159] Okita K, Ichisaka T, Yamanaka S. (2007). Generation of germline-competent induced pluripotent stem cells. *Nature,* 448,313-317.

[160] Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. (2008). Induced pluripotent stem cells generated without viral integration. *Science,* 322,945-949.

[161] Hanna J, Wernig M, Markoulaki S, Sun CW, et al. (2007). Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science,* 318,1920-1923.

[162] Swijnenburg RJ, Schrepfer S, Govaert JA, Cao F, et al. (2008). Immunosuppressive therapy mitigates immunological rejection of human embryonic stem cell xenografts. *Proc Natl Acad Sci U S A,* 105,12991-12996.

[163] Takenaga M, Fukumoto M, Hori Y. (2007). Regulated Nodal signaling promotes differentiation of the definitive endoderm and mesoderm from ES cells. *J Cell Sci,* 120,2078-2090.

[164] Séguin CA, Draper JS, Nagy A, Rossant J. (2008). Establishment of endoderm progenitors by SOX transcription factor expression in human embryonic stem cells. *Cell Stem Cell,* 3,182-195.

[165] Mohtashami M, Zúñiga-Pflücker JC. (2006). Cutting Edge: Three-Dimensional Architecture of the Thymus Is Required to Maintain Delta-Like Expression Necessary for Inducing T Cell Development. *J Immunol,* 176,730-734.

[166] Hauri-Hohl MM, Zuklys S, Keller MP, Jeker LT, et al. (2008). TGF-{beta} signaling in thymic epithelial cells regulates thymic involution and post-irradiation reconstitution. *Blood,* 112, 626-634.

[167] Gordon J, Xiao S, Hughes B, Su DM, et al. (2007). Specific expression of lacZ and cre recombinase in fetal thymic epithelial cells by multiplex gene targeting at the Foxn1 locus. *BMC Dev Biol,* 7,69.

[168] Hébert JM, McConnell SK. (2000). Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures. *Dev Biol,* 222,296-306.

[169] Macatee TL, Hammond BP, Arenkiel BR, Francis L, et al. (2003). Ablation of specific expression domains reveals discrete functions of ectoderm- and endoderm-derived FGF8 during cardiovascular and pharyngeal development. *Development,* 130,6361-6374.

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Chapter 9

The Biology of Lymphoid Tissue Inducer Cells*

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Abstract

Lymphoid tissue inducer (LTi) cells are a unique set of hematopoietic $CD4^+$ IL-$7R\alpha^+$ $lineage^-$ cells, which are required for the development of lymph nodes (LNs) and Peyer's patches (PPs) in mice. The differentiation of fetal liver (FL) progenitors into LTi cells is regulated by transcription factors and by cytokines provided by non-hematopoietic cells. During fetal development, LTi cells colonize the intestine and putative sites of LN formation, where they associate with vascular endothelial cells and mesenchymal stromal cells. They express a number of tumor necrosis factor super-family (TNFSF) members, amongst them lymphotoxin (LT) $\alpha 1\beta 2$ engages LTβR expressed by stromal cells. Activation of the LTβR signaling pathway within stromal cells induces the NFκB-dependent transcription of genes that is required for the development of lymphoid tissues. LTβR signals also contribute to the formation of the splenic white pulp during fetal development. Fetal LTi cells not only migrate to putative primordia of secondary lymphoid organs, but also to the fetal thymus, where they are proposed to promote the differentiation of medullary thymic epithelial cells (mTECs). In adult mice, LTi cells persist in lymphoid organs and intestinal follicles, where they have a function in lymphoid tissue organization and adaptive immune responses.

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Introduction

In mice, the development of secondary lymphoid organs (SLOs), such as lymph nodes (LNs) and Peyer's patches (PPs), is coordinated by the collaboration between $CD45^+$ hematopoietic and mesenchymal cells (1-3). In fetal mice, SLO anlagen and the splenic white pulp are in place before they become colonized with mature lymphocytes. The first sign of SLO anlagen is the formation of cell clusters composed of hematopoietic "lymphoid tissue inducer (LTi)" and mesenchymal "organizer cells". Signals mediated by tumor necrosis factor receptor super-family (TNFRSF) members that are expressed by LTi cells trigger the expression of adhesion molecules and chemokines by the organizer cells. This further enhances hematopoietic-mesenchymal interactions and cell cluster formation. In addition, TNFRSF engagement induces the differentiation of stromal cells into T and B cell zone stroma and the specification of post-capillary high endothelial venules (HEVs), the entry site for naïve lymphocytes. In mice lacking LTi cells, the formation of LN and PP primordia is completely blocked indicating that these cells are indispensable for lympho-organogenesis (4). After birth, other TNFSF-expressing lymphocytes, such as B lymphocytes, play a role in maintaining the architecture of lymphoid tissues and the generation of germinal centers containing follicular dendritic cells (5). Hence, lymphoid tissue formation requires a coordinated sequence of interactions between hematopoietic and mesenchymal cells, thereby leading to the maturation of microenvironments, where adaptive immune responses can be generated.

It has been recently shown that TNFRSF member signals, induced by interactions with ligands such as receptor activator of nuclear factor κB ligand (RANKL) and CD40L, are required for mTEC development in the thymus (6-9), and that LTi cells, in addition to their role in SLO development and organization, can trigger the development of autoimmune regulator (AIRE)-expressing mTECs (9). These new findings indicate a more general role of LTi cells in the development and function of the immune system than previously thought. In addition, LTi cells expressing a similar genetic profile as fetal LTi cells are still detectable in adult mice (10, 11). They are found in SLO and in specialized intestinal compartments called isolated lymphoid follicles (ILF) and cryptopatches (CP) (10, 12). In these sites, tThey function as bystander cells for adaptive immune responses and promote *de novo* ILF formation (13) and lymphoid tissue organization after viral infection (14).

The molecular requirements for generating LTi cells during fetal and adult life remain largely unknown. Studies in knockout mice, chimeric mice and analysis of *in vitro* hematopoietic lineage commitment revealed that precursor cells derived from the fetal liver (FL) and adult bone marrow (BM) may exist that can give rise to fetal and adult LTi cells, respectively. The LTi precursor cells appear to exert multi-potent lineage potential with the capacity to differentiate into various lymphoid lineages except B lymphocytes (15, 16).

Cytokines are essential for the differentiation, homeostasis and function of hematopoietic cells. We have recently shown that interleukin 7 (IL-7) is a crucial cytokine for the generation and survival of LTi cells and its FL progenitor (17). Other cytokines may have overlapping functions or collaborate with IL-7 in regulating the differentiation, homing and maintenance of LTi cells. Dependent on the anatomical site, the repertoire of cytokines that is produced differs amongst various subsets of stromal and epithelial cells.

In this review, we summarize our current knowledge on the origin of LTi cells and on the pathways which regulate LTi cell biology. We discuss recent advances in understanding the regulation of LTi cell numbers by cytokines, and the function of LTi cells in adult animals.

Characterization and Origin of Fetal LTi Cells

Although numerous studies in the last 10 years have improved our knowledge on regulation of lympho-organogenesis and have highlighted the role of fetal LTi cells for LN and PP development in mice, little is known about the origin and the molecular requirements for differentiation and maintenance of LTi cells. In addition, it is currently unknown if there is a relationship between LTi cells and other hematopoietic lineages.

LTi cells are a unique population of IL-7Rα $(CD127)^+$ cells (18, 19), which do not express any lineage marker with the exception of various levels of CD4 (19). Fetal $CD4^+$ and $CD4^-$ LTi cells share an anatomical location, and both express CD25, CD44, CD90, CD122, CD127, CD117, CD132 and TNF family member molecules (LTα1β2, LIGHT, TNF-α, RANK, DR3, 4-1BB), suggesting that both subsets are developmentally linked (20). In addition, both express the nuclear hormone receptor RORγt (12). This transcription factor is expressed in double positive (DP) thymocytes (4) and in pro-inflammatory $IL\text{-}17^+$ T helper cells (21), but not in any other lineages.

The function of $CD4^+$ LTi cells was identified by their capacity to restore PP and nasopharyngeal-associated lymphoid tissue (NALT) development after adoptive transfer into $CXCR5^{-/-}$ and $Id\text{-}2^{-/-}$ mice, respectively (22, 23). It was previously shown that the level of CD4 expression varied depending on the presence of IL-7 (16), whereas other workers described a constant ratio of $CD4^+$ and $CD4^-$ cells in cultures containing IL-7 (20). Since an inducer capacity was only shown for CD4-expressing cells, the LTi cell population discussed in this report refers to $CD4^+$ $IL\text{-}7R\alpha^+$ lin^- cells.

In the absence of RORγ, LTi cells, LNs and PP were undetectable (4). In the same study, it was shown that RORγ was essential for the survival of DP thymocytes (4). We generated Bcl-2tg $ROR\gamma^{-/-}$ mice in order to test if LTi cell numbers and peripheral LN could be restored through over-expression of an anti-apoptotic gene. Bcl-2tg $ROR\gamma^{-/-}$ mice were completely devoid of LTi cells and LNs indicating that RORγ is unlikely to act exclusively as a survival factor, but rather promotes the generation of the cells.

In the context of lymphoid organ development, disruption of the gene encoding the transcriptional repressor Id2 results in a phenotype similar to that of $ROR\gamma^{-/-}$ mice (24). Id2 null mice lack LNs, PPs, LTi cells and natural killer (NK) cells suggesting the existence of a common LTi/NK cell precursor. In a more recent study, it was reported that by deleting Id2 and E2A, an E protein transcription factor required for B lymphocyte development and a target molecule of the Id2 repressor, mature NK cells developed in the BM (25). In addition, in $Id2^{-/-}E2A^{-/-}$ double mutant mice LTi cells developed normally during fetal life, and LNs and PPs were readily visible in adult mice. These data suggest that Id2 inhibited E protein activity in a lymphoid precursor subset thereby allowing LTi cell and NK cell development but simultaneously limiting B lymphocyte differentiation. A partial decrease in the E2A protein in $Id2^{-/-}E2A^{+/-}$ mice rescued only mesenteric and cervical LN development indicating

that there is a dose effect of E protein activity. This strongly suggests that there is a threshold of LTi cell numbers required for LN differentiation that varies with anatomic site.

LTi cells develop normally in RAG-$2^{-/-}$, *scid/scid* and *nu/nu* mice and these animals have small but detectable LNs and PPs (18). In addition, LTi cells and LNs are found in spleen-deficient Hox$11^{-/-}$ mice (18). Thus, LTi cells do not require gene rearrangement, and a functional thymus or spleen for their development. Ikaros$^{-/-}$ mice and animals, which express a dominant negative form of Ikaros, are completely devoid of LNs and PPs (26, 27). Using a GFP reporter-gene expression cassette, Ikaros was found to act on both erythroid-myeloid progenitors (EMP) and lymphoid-myeloid progenitors (LMP) in the BM as a "fate decision" factor (28). In the absence of Ikaros, EMPs developed into erythroid lineages whereas LMPs developed into myeloid cells. Whether LTi cells failed to develop in Ikaros null mice has not been studied, and such may explain the lack of LNs and PPs. Ikaros null lymphoid-primed multipotent progenitors (LMPP) fail to express IL-7Rα (28), a receptor that is clearly involved in the generation of T, B and LTi cells. Since the phenotype of Ikaros$^{-/-}$ mice is more severe than in IL-7Rα$^{-/-}$ mice, it is likely that Ikaros has a crucial role in generating LTi cells from early LMPs.

In the mouse embryo, hematopoietic stem cell (HSC) activity is detectable from E11 in the FL (29). Two independent studies have shown that a progenitor cell exists in the FL which could give rise to LTi cells (E12.5) (16, 30). In one of the studies, the cells were reported as α4β7^+ c-Kit$^+$ IL-7Rα$^+$ lin$^-$ cells, and upon *in vitro* stimulation with IL-7 they differentiated towards CD4^+ LTi cells.

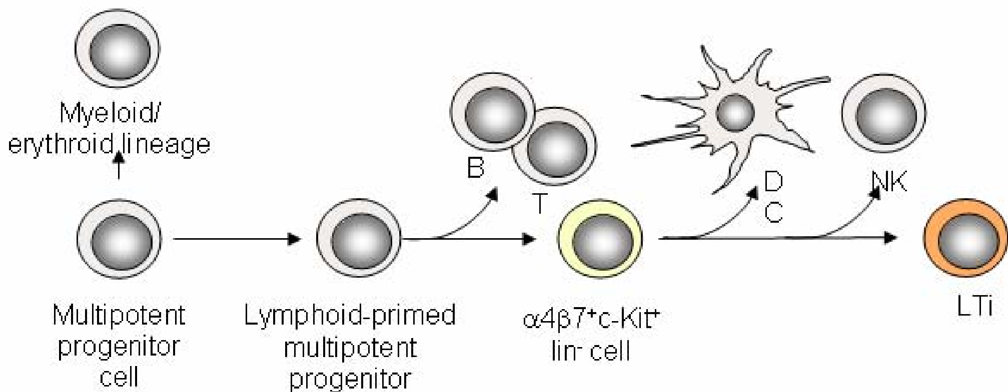

Figure 1: Origin of fetal LTi cells. .Multipotent progenitor cells colonize the FL between E10 and 12. They can give rise to α4β7^+ c-Kit$^+$ IL-7Rα$^+$ lin$^-$ LTi precursor cells (yellow), which have lost the potential to differentiate into B and T cells and retained the capacity to differentiate into DCs, NK and LTi (orange) cells (16).

This is in agreement with the observation that the increased availability of IL-7, through transgene expression, promoted the survival and commitment of α4β7^+ c-Kit$^+$ lin$^-$ LTi precursor cells, thereby increasing the pool of peripheral LTi cells (17). Interestingly, CD4^{hi} LTi cells were not found in the FL (15, 16, 30). In contrast, LTi cells and α4β7^+ c-Kit$^+$ lin$^-$ LTi precursor cells could be isolated from the fetal gut (16). Since intestinal epithelial cells

are a major source of IL-7 (Thomas Schueler, personal communication) and high levels of IL-7mRNA were found in the fetal intestine (31), this organ may serve as a site for differentiation of LTi precursors into LTi cells.

In addition to their capacity to differentiate into LTi cells, $\alpha4\beta7^+$ c-Kit$^+$ IL-7Rα^+ lin$^-$ cells were capable of generating NK cells and DCs, but had lost the potential to differentiate into B or T lymphocytes. *In vitro* differentiation assays further suggested that in WT mice, lin$^-$ IL-7Rα^+ $\alpha4\beta7^-$ cells in the FL were progenitor cells of $\alpha4\beta7^+$ c-Kit$^+$ IL-7Rα^+ lin$^-$ LTi precursor cells (16). They have characteristic features of FL multi-potent progenitor cells (MPP) with the capacity to differentiate into both lymphoid and myeloid lineages (32) (Figure 1).

There has been a debate about the potential of LTi cells to differentiate into other lineages. On the one hand, *in vitro* studies revealed that LTi cells could give rise to NK and DC-like cells (18). On the other hand, fate mapping experiments using RORγt$^{+/GFP}$-reported mice ruled out this possibility (33). We cannot fully exclude that LTi cells harbor lineage commitment capacity that is only detectable in the absence of competition with lymphoid cells. Therefore, the adoptive transfer of sorted LTi cells isolated from fetal spleen or LNs into RAG$^{-/-}\gamma_c^{-/-}$ mice will allow this question to be addressed further.

LTi Cell Localization and Cell-Cell Interactions in Developing Lymphoid Organs

LTi cells are detectable in the peripheral blood, spleen, fetal LN anlagen, stomach, gut and thymus between E12.5 and 14.5. The requirements for migration of LTi precursors from the FL to these sites are unknown. It is likely that, analogous to other hematopoietic lineages, they enter tissues via blood vessels, which proceed the development of lymphatic vessels and LNs (34). In SLO anlagen, the first LTi cells are found as single cells in close vicinity to vessels further supporting the idea that they enter tissues from the blood vasculature (12, 22, 35-37). The perivascular distribution of LTi cells could be mediated by the chemokines CCL19, CCL21, CXCL12 and CXCL13, which all are highly expressed by endothelial cells (38).

In LNs, specialized post-capillary venules, termed HEV, mediate the entry of circulating naïve lymphocytes. LTi cells are negative for L-selectin (CD62L), an adhesion molecule that can mediate the transient attachment and rolling of naïve lymphocytes to CD34 expressed by HEV. In contrast to adults, HEV of fetal LNs express the vascular addressin MAdCAM-1 (39). As LTi cells are present in fetal blood (18, 40), and homogenously express the corresponding integrin $\alpha4\beta7$ (18), MAdCAM-1/$\alpha4\beta7$ interactions might allow the exit of LTi cells from the circulation. Indeed, treatment with antibodies (Abs) against MAdCAM-1, $\alpha4$ or $\beta7$ partially prevented the entry of LTi cells into LN anlagen (39). The fact that in contrast to $\gamma\delta$ T cell entry, which was completely inhibited, LTi cell colonization was not completely abrogated by Ab treatments suggest that either LTi cells can use other adhesion molecules to enter the LN anlage, and/or some of them enter tissue via venules that are different from MAdCAM-1$^+$ HEV. These hypotheses are in line with the fact that in fetal

mice, some LTi cells were found in LN anlagen prior to the presence of MAdCAM-1^{+} HEV (35). In addition, $\beta7^{-/-}$ mice have normal LN and PP development (41), ruling out that $\alpha4\beta7$ is essential for LTi cell colonization and lympho-organogenesis.

In tissues, LTi cells form cellular clusters with VCAM-1-expressing stromal cells (42, 43). Nishikawa and colleagues were the first to identify this stromal cell subset in the fetal gut, and termed them "PP organizer cells" because of their capacity to produce chemokines that were able to recruit mature lymphocytes (3). Later, similar subsets of stromal cells were found in LN anlagen, but their expression profile was dependent on the anatomical site from which they were isolated (35, 44). Since they are of mesenchymal origin (45), they may originate from cellular subsets present in the lymphoid anlagen. The aggregates of LTi and organizer cells indicate the PP anlagen. Within the primitive anlagen, cells first form diffuse aggregates, before they develop into segregated sub-regions (12, 45). A recently described CD11c^{+} c-Kit^{+} cell population was apparent in PP anlagen as early as LTi and organizer cells formed clusters (46). By deleting CD11c^{+} cells, the number of PPs was significantly reduced suggesting that PP organogenesis requires the presence of these cells. CD11c^{+} c-Kit^{+} cells were positive for both GR-1 and NK1.1, markers which are not expressed by adult DCs. In addition, a substantial proportion of these cells expresses RET, a tyrosine kinase receptor, which is important for formation of the enteric nerve system (47). In mice deficient for RET, PP primordia were absent although the relative frequency of LTi cells was normal. This strongly suggests that RET is required for cluster formation by of LTi cells.

Adhesion molecules expressed during early embryonic development are essential not only for homing but also for the retention of cells in developing organs, thereby leading to a cluster of cells. LTi cells express $\alpha4\beta1$ and $\alpha4\beta7$ integrin; both these molecules mediate adhesion to organizer cells that express the Ig super-family adhesion molecules VCAM-1 and MAdCAM-1, respectively. In addition, LTi cells express ICAM-1 (35), but its role in interactions with mesenchymal organizer cells is unknown. In RORγt$^{-/-}$ mice, VCAM-1^{+} cell clusters are undetectable in the fetal and neonatal gut, clearly demonstrating that the cluster formation of organizer cells is strictly dependent on the presence of LTi cells. Amongst other TNFSF members, LTi cells express lymphotoxin $\alpha_1\beta_2$ (LT$\alpha_1\beta_2$), which engages the LTβR expressed by the organizer cells. The critical role for LT signaling has been clearly demonstrated by the discovery that LT$\alpha^{-/-}$, LT$\beta^{-/-}$ and LTβR$^{-/-}$ mice lack peripheral LNs and that administration of a LTβR inhibitor (LTβR:Ig) during pregnancy leads to defects in both LN and PP development (48-51). LTβR engagement activates NFκB, which then induces the transcription of genes required for lymphoid organ formation, such as adhesion molecules and chemokines (5, 45, 52). Chemokine production by organizer cells may further recruit LTi cells and B and T lymphocytes. Despite the unequivocal role of LT$\alpha_1\beta_2$ in organizer cell clustering and lymphoid organ development, LTi cells can colonize fetal LN anlagen independently of LTα (12, 37). In addition, the early LN anlagen appear to develop independently of LT$\alpha_1\beta_2$, as iLNs were readily found in LT$\alpha^{-/-}$ mice showing normal numbers of LTi and CD45^{-} stromal cells (53).

There is evidence that the colonization of SLO anlagen with B lymphocytes precedes T lymphocyte colonization. In E 18.5 PP anlagen LTi cells aggregate in follicles whereas B cells remain diffusely localized in the inter-follicular region (46) (Figure 2). .

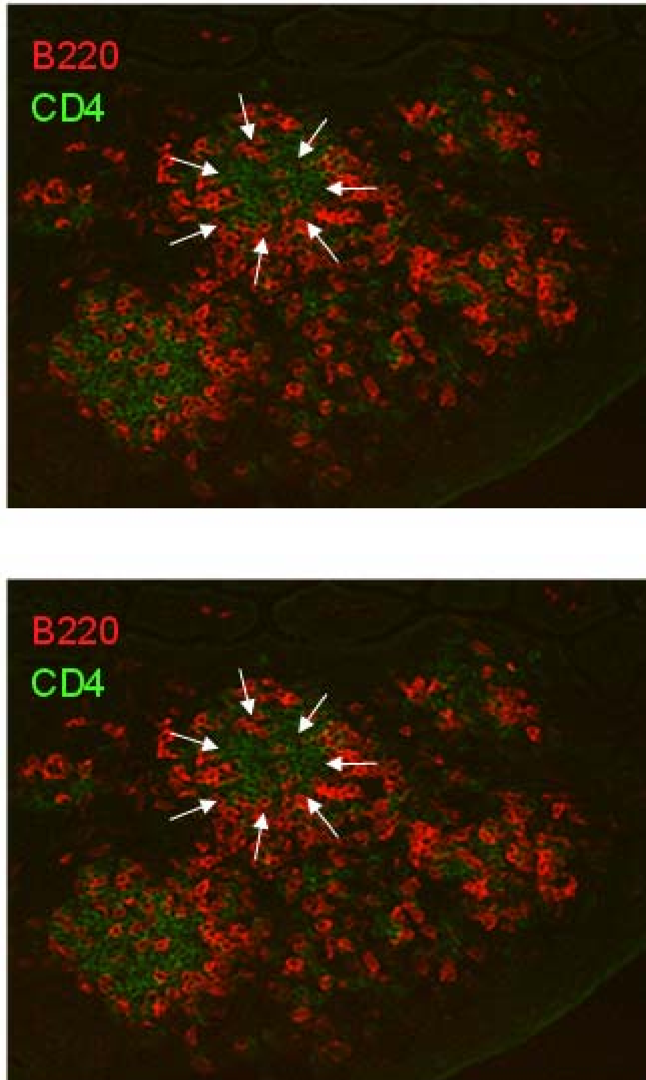

Figure 2. Peyer's Patch of fetal wild type mouse. Immunostaining with anti-CD4 Ab (green) and anti-B220 Ab (red) illustrates the formation of $CD4^+$ follicles surrounded by $B220^+$ B cells. $CD4^+$ cells were negative for CD3 and CD11c (data not shown).

In LNs of 2 day old severe combined immunodeficient (SCID) or wild type (WT) mice, LTi cells were found in the cortical region (18). Studies in $CXCL13^{-/-}$ mice revealed that the predominant cortical localization of these cells occurs independently of CXCL13 (54). At this time point B cell follicles were not observed in LNs. It is likely that through the engagement of the LTβR, stromal cells are activated to produce chemokines allowing the entry of B lymphocytes to putative follicle zones. Notably, studies in WT and $CXCL13^{-/-}$ mice have shown that neonatal B lymphocytes express low levels of CXCR5, are unresponsive to CXCL13 *in vitro*, and can colonize the outer cortex of d4 LNs in the absence of CXCL13 (54). However, the subsequent formation of B cell follicles is dependent on CXCL13.

Altogether, these data strongly suggest that LTi cells play a substantial role in B cell follicle formation by instructing stromal cells to differentiate into B cell zones.

Independently of mature lymphocytes, the splenic white pulp containing LTi cells is already in place at E15.5 (38). The transfer of *in vitro* IL-7- activated fetal LTi cells was shown to restore the B and T segregation in the spleen of $LT\alpha^{-/-}$ mice suggesting a role of LTi cells in the development of the splenic white pulp. (55). However, $ROR\gamma^{-/-}$ mice have a normal splenic architecture. One explanation could be that a RORγ-independent cell population exists which can contribute to the organization of the developing spleen.

$CD4^{+}$ $c\text{-}Kit^{+}$ Lin^{-} cells are detectable in the fetal thymus (9), and these thymic cells express the tumor necrosis factor-related activation induced cytokine TRANCE (RANKL). This cytokine was originally found to play a role in osteoclastogenesis and LN development (56). The corresponding receptor RANK is expressed by mTECs and signals via TRAF6. $TRAF6^{-/-}$ mice exhibit a defective thymic medulla development, which results in severe autoimmune disorders (57). RANK signaling was recently shown to regulate the development of AIRE-expressing mTECs during fetal life (9, 58). Since LTi cells, but no other SP or DP thymocytes were shown to express TRANCE in fetal thymus, LTi cells may have a major role in mTEC maturation during fetal life. In adult mice, however, RANK signals were mediated by single positive (SP) thymocytes, and occurred independently of LTi cells (6-8).

Box 1. The role of TRANCE in mTEC Development

Amongst other TNFSF signals, the cellular cross-talk between $TRANCE^{+}$ hematopoietic cells and RANK-expressing mTEC plays an essential role in establishing a normal medulla in the thymus, where central tolerance induction occurs. Fetal LTi cells express both TRANCE and RANK (11, 59), suggesting that there might be *cis* activation of RANK signaling in LTi cells. In addition, TRANCE signals by LTi cells promote the *in vitro* generation of mTECs that express AIRE (9), which is essential for the presentation of self-tissue-restricted antigens by mTECs (60). $Aire^{-/-}$ mice fail to eliminate self-reactive thymocytes and hence develop systemic autoimmune diseases (61). $Id2^{-/-}$ mice completely lack LTi cells, but have normal numbers of $AIRE^{+}$ mTECs cells in the adult thymus (7). In contrast to fetal mice, TRANCE is expressed by SP thymocytes in the adult thymus suggesting that these cells could induce RANK signaling in mTECs. Indeed, in $H2\text{-}Aa^{-/-}$ mice, mature mTEC subsets are substantial reduced demonstrating a pivotal role of $CD4^{+}$ thymocytes in generating a normal medulla in the adult thymus (8). Altogether, these findings suggest that $CD4^{+}$ thymocytes-driven mTEC development after birth can compensates for the lack of fetal LTi cells. Alternatively, other Id2-independent subsets may provide TRANCE signals during fetal life.

The Roles of Cytokines and Chemokines in LtiLTi Cell Biology

Stromal cells in SLO produce cytokines, which are crucial for the survival of hematopoietic cells and for the generation of adaptive immune responses. During fetal life,

organizer cells from mesenteric LN anlagen produce TRANCE (35, 44), and organizer cells from both mesenteric LN and PP anlagen express IL-7 mRNA (44, 62). Studies in knockout mice (see below) revealed that cytokines are essential for LN and PP development, and there is strong evidence that the main function of cytokines that are produced during fetal development is to control the generation and function of LTi cells.

IL-7 is required for LN development, as IL-7$^{-/-}$, IL-7R$\alpha^{-/-}$ and $\gamma_c^{-/-}$ mice lack several peripheral LN (17, 40). The lack of LN is identical in $\gamma_c^{-/-}$ mice and IL-7R$\alpha^{-/-}$ mice (40), suggesting that IL-7 is the main γ_c cytokine for LN formation. The reasons why IL-7$^{-/-}$, $\gamma_c^{-/-}$ and IL-7R$\alpha^{-/-}$ mice have a reduced number of LNs remain unclear, but several studies indicate that IL-7 has pleiotropic effects on LTi cells and their putative FL precursors. Firstly, IL-7 directly affects the function of LTi cells by up-regulating LT expression *in vitro* (63, 64) and *in vivo* in IL-7tg mice (17). Secondly, IL-7 over-expression increases the size of the FL $\alpha4\beta7^+$ population that contains LTi cell progenitors (17). Thirdly, IL-7 is required for the generation of LTi cells from FL precursors *in vitro* (17, 65, 66), and regulates the size of the LTi cell pool *in vivo* (17). In line with this, mesenteric LNs from IL-7R$\alpha^{-/-}$ newborn mice contain decreased numbers of LTi cells (40). Finally, LTi cells are not in rapid cell cycle (12) and we found that IL-7 promoted the survival rather than the proliferation of LTi cells (17).

IL-7$^{-/-}$ mice have normal numbers of VCAM-1$^+$ PP anlagen, while such clusters are absent from the gut of newborn IL-7R$\alpha^{-/-}$ mice (62, 67). These results indicate the existence of an unidentified alternative ligand for IL-7Rα, which is instrumental for PP formation. IL-7R$\alpha^{-/-}$, JAK3$^{-/-}$ and $\gamma_c^{-/-}$ mice have no VCAM-1$^+$ clusters indicating that this ligand may be dependent on γ_c and JAK3. Thymic stromal lymphopoietin (TSLP) is a cytokine which binds to a receptor composed of the IL-7Rα chain and a specific subunit called TSLPR (68, 69). The fact that TSLP signals independently of γ_c and JAK3 (69, 70) make it an unlikely candidate for triggering PP development (62). Consistent with this hypothesis, IL-7$^{-/-}$ TSLPR$^{-/-}$ mice develop PP anlage similar in size and number to IL-7$^{-/-}$ and WT controls (our unpublished data). An alternative ligand for IL-7Rα is likely to act specifically in the intestine, as IL-7$^{-/-}$ and IL-7R$\alpha^{-/-}$ mice have very similar defects in LN development (17, 40).

Mice deficient in TRANCE, RANK or in the downstream signaling molecule TNF receptor associated factor 6 (TRAF6) lack all LNs but have normal numbers of PPs (64, 71, 72). The effect of TRANCE on LN development is reflected by its expression pattern, as TRANCE mRNA is found at higher levels in organizer cells of mesenteric as compared to intestinal origin (44). Except for a sub-epithelial population of PPs, intestinal stromal cells in PPs appear to be TRANCE negative (73).

LTi cells express both TRANCE and RANK (59) (Figure 3), and TRANCE expression levels increase following stimulation with IL-7 or TL1A (VEGI, TNFRSF12), a TNF family member produced by endothelial cells (74). The close proximity of LTi cells to endothelial cells after entry into lymphoid anlagen may help to increase TRANCE production by LTi cells beyond the threshold required for *cis* activation of RANK signaling in LTi cells. TRANCE can induce LT expression by LTi cells *in vitro* and *in vivo* (64), suggesting that the failure of optimal LT expression in TRANCE$^{-/-}$ mice might prevent LTi cells from properly inducing organizer cell clustering, thereby preventing the formation of LNs. However, the fact that agonist anti-LTβR antibody failed to restore LN development in TRANCE$^{-/-}$ mice (72) indicates that TRANCE has additional LT-independent functions. LTi cells can migrate

to LN anlagen of TRAF6$^{-/-}$ mice demonstrating that, analogous to LT, the TRANCE/TRAF6 axis is not absolutely required for the colonization of LN anlagen with LTi cells (37). The anlagen, however, involute after birth, most likely because LTi cells and VCAM-1 organizer cells fail to form aggregates. Considering that mesenteric LNs from newborn TRANCE$^{-/-}$ mice (72) and from E17.5 TRAF6$^{-/-}$ embryos (64) contain lower percentage of LTi cells than WT mice, TRANCE was proposed to promote LTi cell differentiation and/or survival in LN anlage (35, 72). Hence, the number of LTi cells might be the limiting factor for the further maturation of LN anlage. LN formation in TRAF6$^{-/-}$ mice can be partially rescued by intraembryonic injection of IL-7 (64), but it is not clear yet whether in this experimental setting IL-7 restores LN development by up-regulating LT on LTi cells and/or by promoting LTi cell accumulation within LN anlagen.

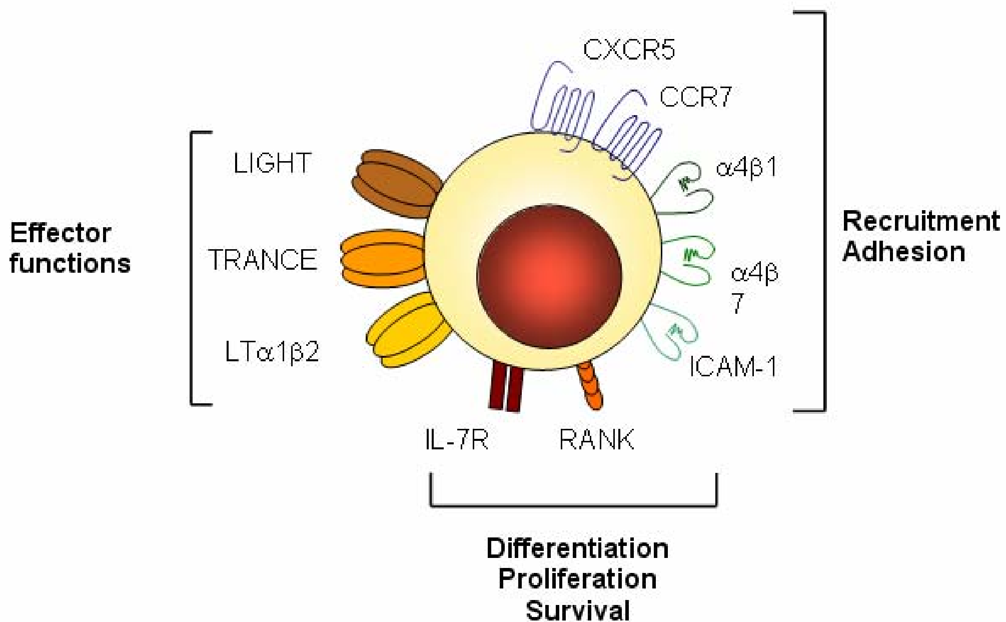

Figure 3: Receptor expression by LTi cells. Activation of cytokine receptors and adhesion molecules controls the development, homing and the effector functions of LTi cells. Shown are the chemokine receptors, TNFR super-family members and adhesion molecules with a known function for LTi cells.

Increased IL-7 availability leads to a significant increase in LTi cell numbers, and to the formation of ectopic LNs and additional PPs, which is a LTi cell-dependent process (17). These findings further support the idea that the availability of cytokines controlling LTi cell numbers is mandatory for the formation of lymphoid organs. Mice doubly deficient for Flt3 Ligand (Flt3L) and IL-7Rα lack all LNs (75), indicating that cytokine synergism might participate in the homeostasis, function and differentiation of LTi cells. Other molecules, such as thrombopoietin and TNF, might partially contribute to LN organogenesis by inducing LT expression by LTi cells (64).

PP and mesenteric LN organizer cells express the lymphoid chemokines CXCL13, CCL19 and CCL21 (44, 63). LTi cells express the cognate receptors CXCR5 and CCR7 (40, 63, 76) (Figure 3), and migrate in response to each of these chemokines (63). Chemokines are likely to play a central role in the function of LTi cells by attracting LTi cells to the LN anlage. Ectopic expression of CXCL13 in the pancreas is sufficient to recruit LTi cells (40), while mice deficient for the chemokine CXCL13 (77) or its receptor CXCR5 (78) lack several peripheral LNs. Furthermore, peripheral LNs are completely absent in mice deficient for lymphoid chemokines or chemokine receptors, such as CXCL13$^{-/-}$ plt/plt (40) and CXCR5$^{-/-}$ CCR7$^{-/-}$ mice (76). Altogether, these results suggest that the recruitment of LTi cells via chemotaxis is a crucial step for LN formation. Chemokines not only recruit LTi cells to the LN anlage, but also modulate LTi cell adhesion. The CXCL13-CXCR5 interaction activates the $\alpha4\beta1$ integrin expressed by LTi cells and, in turn, allows LTi cells to interact with VCAM-1 molecules expressed by intestinal organizer cells (22). This chemokine-mediated integrin activation is a crucial event in PP organogenesis, as suggested by the fact that CXCR5$^{-/-}$ mice have greatly reduced PP numbers (78). These findings illustrate the importance of adhesion molecules in LN development lympho-organogenesis. Altogether, the results indicate that the chemokines might play a crucial role in LN and PP organogenesis by recruiting LTi cells to the LN and PP anlage and by modulating their capacity to interact with organizer cells.

LTi Cells in the Adult

In adult mice, CD4$^+$CD3$^-$ cells were identified in B cell follicles and the B-T interface of the spleen (10). They are localized in close association with VCAM-1$^+$ stromal cells, DCs and, like fetal LTi cells, around central arterioles of the spleen (55). Following *in vitro* stimulation with IL-7, adult splenic CD4$^+$CD3$^-$ cells express OX40L and CD30L. Both these molecules are important to the promotion of T-cell help for memory antibody responses (79). Adult CD4$^+$CD3$^-$ cells share a similar gene transcriptional profile with fetal LTi cells and were hence termed LTi-like cells (11). However, mRNA levels for RORγt are significantly lower in adult cells. Our explanation is that the pool of adult CD4$^+$CD3$^-$ cells may be heterogeneous, consisting of both RORγt$^+$ and RORγt$^-$ cells. Adult BM-derived LTi-like cells adoptively transferred into LT$\alpha^{-/-}$ mice were proposed to play a role in B/T segregation of the splenic white pulp (55). BM chimera experiments also revealed that adult LTi-like cells helped reorganizing the splenic architecture following lymphocytic choriomeningitis virus infection (14). Altogether, there is clear evidence that adult CD4$^+$CD3$^-$ cells exist which have similar properties to fetal LTi cells. We have experimental evidence that adult CD4$^+$CD3$^-$ cells are able to induce PP development following adoptive transfer into PP-deficient CXCR5$^{-/-}$ mice, thus confirming their function as inducers of lymphoid tissue formation.

Likewise, RORγt$^+$CD4$^+$CD3$^-$ cells were observed in the lamina propria of the gut in lymphoid aggregates termed isolated lymphoid follicles (ILF) and cryptopatches (CP) (33). Unlike PPs, which form during embryogenesis, ILF are inducible lymphoid aggregates, which develop after colonization of the intestine with bacteria (80), and are highly dynamic structures, which can vary in size and number. ILF localize to the antimesenteric side of the

intestine. They are composed of B lymphocytes, DCs, few T lymphocytes and RORγt^{+} c-Kit^{+} IL-7Rα^{+} cells, the latter closely localizing to the sub-epithelial dome. Interestingly, TRANCE was found to be exclusively expressed by a small subset of sub-epithelial stromal cells in ILF and PPs of adult WT mice (73). It remains to be investigated whether local TRANCE production in this dome region plays a role in the local persistence of intestinal RORγt^{+} c-Kit^{+} IL-7Rα^{+} cells.

CP and ILF appear at approximately the same time after birth (between 7 and 25 days after birth). CP are smaller than ILF, localize to the intestinal crypts and contain fewer B lymphocytes (81). Morphological intermediates between CP and ILF suggest that CP can differentiate into ILF depending on the extrinsic environmental signals (82). LT signals provided by hematopoietic cells are involved in the formation and maturation of ILF and CP (80, 83, 84). Although ILF are not absolutely required for adaptive immune responses to oral antigens (85), ILF can generate IgA plasma cells in the absence of PPs (13). Hence, ILF may contribute to specific immune responses by sensing the intestinal microflora.

RORγ$^{-/-}$ mice lack CP suggesting that adult LTi-like cells are involved in the development of these structures (33). RORγt^{+} cells are often seen in close contact with DCs. They may indirectly help B lymphocytes to colonize the lamina propria, since RORγ$^{-/-}$ mice were reported to have fewer B lymphocytes at this anatomical site (86). In line with this, the adoptive transfer of adult LTi-like cells induced the *de novo* formation of ILF together with a considerable increase in IgA plasma cell numbers (13). The effect of adult LTi-like cells on lymphoid stromal cell differentiation was further augmented by Toll-like receptor stimulation, thereby suggesting a cross-talk between intestinal LTi-like cells, stromal cells and bacteria.

Altogether, these data clearly demonstrate that LTi cells are found during adult life. They may function in preserving lymphoid architecture and in promoting adaptive immune responses. There are several questions that remain to be addressed. Firstly, it will be important to identify the stimuli that trigger the activation of adult LTi cells during infection and the accumulation or expansion at sites of infection. Secondly, a role of adult LTi cells in the development of ectopic lymphoid follicles in chronically inflamed non-lymphoid organs has to be studied. Finally, it is unclear whether LTi cells persist from fetal to adult life or are continuously produced from BM precursor cells. Reconstitution experiments in mice lacking CP and ILF have shown that CP and ILF can be reconstituted by the adoptive transfer of WT BM (13, 84, 87) and that B and T lymphocytes are dispensable for the induction of follicle formation. These data suggest that the adult BM can be the source of precursors for adult LTi cells.

Conclusion

Approximately 20 years after the discovery of CD4^{+}CD3^{-} cells in neonatal LNs (88), our knowledge on the function of LTi cells in the immune system has been substantially extended at both the cellular and the molecular level. Challenging recent data indicate that LTi cells are not only found in SLO but also in the thymus, where they may play a role in fetal mTEC development, a process that leads to central tolerance induction. Another striking observation

is that in lymphoid organs and the intestine of adult mice, LTi-like cells continue to act as inducers of lymphoid tissue formation and organization. Additionally, LTi cells may provide signals that promote immune responses during infection. The data summarized here further support the idea that LTi cells are responsive to inflammatory signals produced during pathogen-host interactions.

An important question that remains to be answered is the nature of the specific transcription factors downstream of Ikaros and Id2, which control lineage commitment towards LTi cells. Moreover, the specific niches where the LTi cell pool can persist or become replenished from hematopoietic progenitor cells are still to be identified. The function of LTi cell cross-talk with other cell subsets such as stromal cells, TECs, vascular endothelial cells, $CD11c^+$ cells and lymphocytes will be subject of future research in order to better understand the function of LTi cells in the fetal and adult immune system.

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References

[1] Finke, D., and J.P. Kraehenbuhl. 2001. Formation of Peyer's patches. *Curr Opin Genet Dev* 11:561-567.

[2] Mebius, R.E. 2003. Organogenesis of lymphoid tissues. *Nat Rev Immunol* 3:292-303.

[3] Nishikawa, S., K. Honda, P. Vieira, and H. Yoshida. 2003. Organogenesis of peripheral lymphoid organs. *Immunol Rev* 195:72-80.

[4] Sun, Z., D. Unutmaz, Y.R. Zou, M.J. Sunshine, A. Pierani, S. Brenner-Morton, R.E. Mebius, and D.R. Littman. 2000. Requirement for RORgamma in thymocyte survival and lymphoid organ development. *Science* 288:2369-2373.

[5] Ngo, V.N., H. Korner, M.D. Gunn, K.N. Schmidt, D.S. Riminton, M.D. Cooper, J.L. Browning, J.D. Sedgwick, and J.G. Cyster. 1999. Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J. Exp. Med.* 189:403-412.

[6] Akiyama, T., Y. Shimo, H. Yanai, J. Qin, D. Ohshima, Y. Maruyama, Y. Asaumi, J. Kitazawa, H. Takayanagi, J.M. Penninger, M. Matsumoto, T. Nitta, Y. Takahama, and J. Inoue. 2008. The tumor necrosis factor family receptors RANK and CD40 cooperatively establish the thymic medullary microenvironment and self-tolerance. *Immunity* 29:423-437.

[7] Hikosaka, Y., T. Nitta, I. Ohigashi, K. Yano, N. Ishimaru, Y. Hayashi, M. Matsumoto, K. Matsuo, J.M. Penninger, H. Takayanagi, Y. Yokota, H. Yamada, Y. Yoshikai, J. Inoue, T. Akiyama, and Y. Takahama. 2008. The cytokine RANKL produced by

positively selected thymocytes fosters medullary thymic epithelial cells that express autoimmune regulator. *Immunity* 29:438-450.

[8] Irla, M., S. Hugues, J. Gill, T. Nitta, Y. Hikosaka, I.R. Williams, F.X. Hubert, H.S. Scott, Y. Takahama, G.A. Hollander, and W. Reith. 2008. Autoantigen-specific interactions with CD4+ thymocytes control mature medullary thymic epithelial cell cellularity. *Immunity* 29:451-463.

[9] Rossi, S.W., M.Y. Kim, A. Leibbrandt, S.M. Parnell, W.E. Jenkinson, S.H. Glanville, F.M. McConnell, H.S. Scott, J.M. Penninger, E.J. Jenkinson, P.J. Lane, and G. Anderson. 2007. RANK signals from CD4(+)3(-) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla. *J Exp Med* 204:1267-1272.

[10] Kim, M.Y., F.M. Gaspal, H.E. Wiggett, F.M. McConnell, A. Gulbranson-Judge, C. Raykundalia, L.S. Walker, M.D. Goodall, and P.J. Lane. 2003. CD4(+)CD3(-) accessory cells costimulate primed CD4 T cells through OX40 and CD30 at sites where T cells collaborate with B cells. *Immunity* 18:643-654.

[11] Kim, M.Y., K.M. Toellner, A. White, F.M. McConnell, F.M. Gaspal, S.M. Parnell, E. Jenkinson, G. Anderson, and P.J. Lane. 2006. Neonatal and adult CD4+ CD3- cells share similar gene expression profile, and neonatal cells up-regulate OX40 ligand in response to TL1A (TNFSF15). *J Immunol* 177:3074-3081.

[12] Eberl, G., S. Marmon, M.J. Sunshine, P.D. Rennert, Y. Choi, and D.R. Littman. 2004. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol* 5:64-73.

[13] Tsuji, M., K. Suzuki, H. Kitamura, M. Maruya, K. Kinoshita, Ivanov, II, K. Itoh, D.R. Littman, and S. Fagarasan. 2008. Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin a generation in the gut. *Immunity* 29:261-271.

[14] Scandella, E., B. Bolinger, E. Lattmann, S. Miller, S. Favre, D.R. Littman, D. Finke, S.A. Luther, T. Junt, and B. Ludewig. 2008. Restoration of lymphoid organ integrity through the interaction of lymphoid tissue-inducer cells with stroma of the T cell zone. *Nat Immunol* 9:667-675.

[15] Mebius, R.E., T. Miyamoto, J. Christensen, J. Domen, T. Cupedo, I.L. Weissman, and K. Akashi. 2001. The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3- cells, as well as macrophages. *J. Immunol.* 166:6593-6601.

[16] Yoshida, H., H. Kawamoto, S. Santee, H. Hashi, K. Honda, S. Nishikawa, C. Ware, Y. Katsura, and S. Nishikawa. 2001. Expression of alpha(4)beta(7) integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. *J. Immunol.* 167:2511-2521.

[17] Meier, D., C. Bornmann, S. Chappaz, S. Schmutz, L.A. Otten, R. Ceredig, H. Acha-Orbea, and D. Finke. 2007. Ectopic lymphoid-organ development occurs through interleukin 7-mediated enhanced survival of lymphoid-tissue-inducer cells. *Immunity* 26:643-654.

[18] Mebius, R.E., P. Rennert, and I.L. Weissman. 1997. Developing lymph nodes collect CD4+CD3- LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. *Immunity* 7:493-504.

[19] Yoshida, H., K. Honda, R. Shinkura, S. Adachi, S. Nishikawa, K. Maki, K. Ikuta, and S.I. Nishikawa. 1999. IL-7 receptor alpha+ CD3(-) cells in the embryonic intestine induces the organizing center of Peyer's patches. *Int. Immunol.* 11:643-655.

[20] Kim, M.Y., S. Rossi, D. Withers, F. McConnell, K.M. Toellner, F. Gaspal, E. Jenkinson, G. Anderson, and P.J. Lane. 2008. Heterogeneity of lymphoid tissue inducer cell populations present in embryonic and adult mouse lymphoid tissues. *Immunology*

[21] Ivanov, II, B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelley, J.J. Lafaille, D.J. Cua, and D.R. Littman. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126:1121-1133.

[22] Finke, D., H. Acha-Orbea, A. Mattis, M. Lipp, and J. Kraehenbuhl. 2002. CD4+CD3- cells induce Peyer's patch development: role of alpha4beta1 integrin activation by CXCR5. *Immunity* 17:363-373.

[23] Fukuyama, S., T. Hiroi, Y. Yokota, P.D. Rennert, M. Yanagita, N. Kinoshita, S. Terawaki, T. Shikina, M. Yamamoto, Y. Kurono, and H. Kiyono. 2002. Initiation of NALT organogenesis is independent of the IL-7R, LTbetaR, and NIK signaling pathways but requires the Id2 gene and CD3(-)CD4(+)CD45(+) cells. *Immunity* 17:31-40.

[24] Yokota, Y., A. Mansouri, S. Mori, S. Sugawara, S. Adachi, S. Nishikawa, and P. Gruss. 1999. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature* 397:702-706.

[25] Boos, M.D., Y. Yokota, G. Eberl, and B.L. Kee. 2007. Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity. *J Exp Med* 204:1119-1130.

[26] Georgopoulos, K., K. Bigby, M. Wang, J.-H. Molnar, A. Wu, S. Winandy, and A. Sharpe. 1994. The Ikaros gene is required for the development of all lymphoid lineages. *Cell* 79:143-156.

[27] Wang, J.H., A. Nichogiannopoulou, L. Wu, L. Sun, A.H. Sharpe, M. Bigby, and K. Georgopoulos. 1996. Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity* 5:537-549.

[28] Yoshida, T., S.Y. Ng, J.C. Zuniga-Pflucker, and K. Georgopoulos. 2006. Early hematopoietic lineage restrictions directed by Ikaros. *Nat Immunol* 7:382-391.

[29] Medvinsky, A., and E. Dzierzak. 1996. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* 86:897-906.

[30] Mebius, R., and K. Akashi. 2000. Precursors to neonatal lymph nodes: LT beta+CD45+CD4+CD3- cells are found in fetal liver. *Curr Top Microbiol Immunol* 251:197-201.

[31] Murray, A.M., B. Simm, and K.W. Beagley. 1998. Cytokine gene expression in murine fetal intestine: potential for extrathymic T cell development. *Cytokine* 10:337-345.

[32] Lacaud, G., L. Carlsson, and G. Keller. 1998. Identification of a fetal hematopoietic precursor with B cell, T cell, and macrophage potential. *Immunity* 9:827-838.

[33] Eberl, G., and D.R. Littman. 2004. Thymic origin of intestinal αβ T cells revealed by fate mapping of RORγt$^+$ cells. *Science* 305:248-251.

[34] Wigle, J.T., and G. Oliver. 1999. Prox1 function is required for the development of the murine lymphatic system. *Cell* 98:769-778.

[35] Cupedo, T., M.F. Vondenhoff, E.J. Heeregrave, A.E. De Weerd, W. Jansen, D.G. Jackson, G. Kraal, and R.E. Mebius. 2004. Presumptive lymph node organizers are differentially represented in developing mesenteric and peripheral nodes. *J Immunol* 173:2968-2975.

[36] Withers, D.R., M.Y. Kim, V. Bekiaris, S.W. Rossi, W.E. Jenkinson, F. Gaspal, F. McConnell, J.H. Caamano, G. Anderson, and P.J. Lane. 2007. The role of lymphoid tissue inducer cells in splenic white pulp development. *Eur J Immunol* 37:3240-3245.

[37] Yoshida, H., A. Naito, J. Inoue, M. Satoh, S.M. Santee-Cooper, C.F. Ware, A. Togawa, and S. Nishikawa. 2002. Different cytokines induce surface lymphotoxin-alphabeta on IL-7 receptor-alpha cells that differentially engender lymph nodes and Peyer's patches. *Immunity* 17:823-833.

[38] Vondenhoff, M.F., G.E. Desanti, T. Cupedo, J.Y. Bertrand, A. Cumano, G. Kraal, R.E. Mebius, and R. Golub. 2008. Separation of splenic red and white pulp occurs before birth in a LTalphabeta-independent manner. *J Leukoc Biol* 84:152-161.

[39] Mebius, R.E., P.R. Streeter, S. Michie, E.C. Butcher, and I.L. Weissman. 1996. A developmental switch in lymphocyte homing receptor and endothelial vascular addressin expression regulates lymphocyte homing and permits CD4+ CD3- cells to colonize lymph nodes. *Proceedings of the National Academy of Sciences of the United States of America* 93:11019-11024.

[40] Luther, S.A., K.M. Ansel, and J.G. Cyster. 2003. Overlapping roles of CXCL13, interleukin 7 receptor alpha, and CCR7 ligands in lymph node development. *The Journal of experimental medicine* 197:1191-1198.

[41] Wagner, N., J. Lohler, E.J. Kunkel, K. Ley, E. Leung, G. Krissansen, K. Rajewsky, and W. Muller. 1996. Critical role for beta7 integrins in formation of the gut-associated lymphoid tissue. *Nature* 382:366-370.

[42] Adachi, S., H. Yoshida, K. Honda, K. Maki, K. Saijo, K. Ikuta, T. Saito, and S. Nishikawa. 1998. Essential role of IL-7 receptor alpha in the formation of Peyer's patch anlage. *Int. Immunol.* 10:1-6.

[43] Adachi, S., H. Yoshida, H. Kataoka, and S. Nishikawa. 1997. Three distinctive steps in Peyer's patch formation of murine embryo. *Int. Immunol.* 9:507-514.

[44] Okuda, M., A. Togawa, H. Wada, and S. Nishikawa. 2007. Distinct activities of stromal cells involved in the organogenesis of lymph nodes and Peyer's patches. *J Immunol* 179:804-811.

[45] Honda, K., H. Nakano, H. Yoshida, S. Nishikawa, P. Rennert, K. Ikuta, M. Tamechika, K. Yamaguchi, T. Fukumoto, T. Chiba, and S.I. Nishikawa. 2001. Molecular basis for hematopoietic/mesenchymal interaction during initiation of Peyer's patch organogenesis. *J. Exp. Med.* 193:621-630.

[46] Hashi, H., H. Yoshida, K. Honda, S. Fraser, H. Kubo, M. Awane, A. Takabayashi, H. Nakano, Y. Yamaoka, and S. Nishikawa. 2001. Compartmentalization of Peyer's patch anlagen before lymphocyte entry. *J. Immunol.* 166:3702-3709.

[47] Veiga-Fernandes, H., M.C. Coles, K.E. Foster, A. Patel, A. Williams, D. Natarajan, A. Barlow, V. Pachnis, and D. Kioussis. 2007. Tyrosine kinase receptor RET is a key regulator of Peyer's patch organogenesis. *Nature* 446:547-551.

[48] Fu, Y.X., and D.D. Chaplin. 1999. Development and maturation of secondary lymphoid tissues. *Annu Rev Immunol* 17:399-433.

[49] Futterer, A., K. Mink, A. Luz, M.H. Kosco-Vilbois, and K. Pfeffer. 1998. The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity* 9:59-70.

[50] Rennert, P.D., J.L. Browning, and P.S. Hochman. 1997. Selective disruption of lymphotoxin ligands reveals a novel set of mucosal lymph nodes and unique effects on lymph node cellular organization. *Int. Immunol.* 9:1627-1639.

[51] Rennert, P.D., J.L. Browning, R. Mebius, F. Mackay, and P.S. Hochman. 1996. Surface lymphotoxin alpha/beta complex is required for the development of peripheral lymphoid organs. *J. Exp. Med.* 184:1999-2006.

[52] Dejardin, E., N.M. Droin, M. Delhase, E. Haas, Y. Cao, C. Makris, Z.W. Li, M. Karin, C.F. Ware, and D.R. Green. 2002. The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways. *Immunity* 17:525-535.

[53] White, A., D. Carragher, S. Parnell, A. Msaki, N. Perkins, P. Lane, E. Jenkinson, G. Anderson, and J.H. Caamano. 2007. Lymphotoxin a-dependent and -independent signals regulate stromal organizer cell homeostasis during lymph node organogenesis. *Blood* 110:1950-1959.

[54] Cupedo, T., F.E. Lund, V.N. Ngo, T.D. Randall, W. Jansen, M.J. Greuter, R. de Waal-Malefyt, G. Kraal, J.G. Cyster, and R.E. Mebius. 2004. Initiation of cellular organization in lymph nodes is regulated by non-B cell-derived signals and is not dependent on CXC chemokine ligand 13. *J Immunol* 173:4889-4896.

[55] Kim, M.Y., F.M. McConnell, F.M. Gaspal, A. White, S.H. Glanville, V. Bekiaris, L.S. Walker, J. Caamano, E. Jenkinson, G. Anderson, and P.J. Lane. 2007. Function of CD4+CD3- cells in relation to B- and T-zone stroma in spleen. *Blood* 109:1602-1610.

[56] Kong, Y.Y., H. Yoshida, I. Sarosi, H.L. Tan, E. Timms, C. Capparelli, S. Morony, A.J. Oliveira-dos-Santos, G. Van, A. Itie, W. Khoo, A. Wakeham, C.R. Dunstan, D.L. Lacey, T.W. Mak, W.J. Boyle, and J.M. Penninger. 1999. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 397:315-323.

[57] Akiyama, T., S. Maeda, S. Yamane, K. Ogino, M. Kasai, F. Kajiura, M. Matsumoto, and J. Inoue. 2005. Dependence of self-tolerance on TRAF6-directed development of thymic stroma. *Science* 308:248-251.

[58] White, A.J., D.R. Withers, S.M. Parnell, H.S. Scott, D. Finke, P.J. Lane, E.J. Jenkinson, and G. Anderson. 2008. Sequential phases in the development of Aire-expressing medullary thymic epithelial cells involve distinct cellular input. *Eur J Immunol* 38:942-947.

[59] Kim, D., R.E. Mebius, J.D. MacMicking, S. Jung, T. Cupedo, Y. Castellanos, J. Rho, B.R. Wong, R. Josien, N. Kim, P.D. Rennert, and Y. Choi. 2000. Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. *J. Exp. Med.* 192:1467-1478.

[60] Kyewski, B., and L. Klein. 2006. A central role for central tolerance. *Annual reviews of immunology* 24:571-606.

[61] Anderson, M.S., E.S. Venanzi, L. Klein, Z. Chen, S.P. Berzins, S.J. Turley, H. von Boehmer, R. Bronson, A. Dierich, C. Benoist, and D. Mathis. 2002. Projections of an immunological self shadow within the thymus by the aire protection. *Science* 298:1395-1401.

[62] Nishikawa, S., K. Honda, P. Vieira, and H. Yoshida. 2003. Organogenesis of peripheral lymphoid organs. *Immunological reviews* 195:72-80.

[63] Honda, K., H. Nakano, H. Yoshida, S. Nishikawa, P. Rennert, K. Ikuta, M. Tamechika, K. Yamaguchi, T. Fukumoto, T. Chiba, and S.I. Nishikawa. 2001. Molecular basis for hematopoietic/mesenchymal interaction during initiation of Peyer's patch organogenesis. *The Journal of experimental medicine* 193:621-630.

[64] Yoshida, H., A. Naito, J. Inoue, M. Satoh, S.M. Santee-Cooper, C.F. Ware, A. Togawa, S. Nishikawa, and S. Nishikawa. 2002. Different cytokines induce surface lymphotoxin-alphabeta on IL-7 receptor-alpha cells that differentially engender lymph nodes and Peyer's patches. *Immunity* 17:823-833.

[65] Mebius, R.E., T. Miyamoto, J. Christensen, J. Domen, T. Cupedo, I.L. Weissman, and K. Akashi. 2001. The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3- cells, as well as macrophages. *J Immunol* 166:6593-6601.

[66] Yoshida, H., H. Kawamoto, S.M. Santee, H. Hashi, K. Honda, S. Nishikawa, C.F. Ware, Y. Katsura, and S.I. Nishikawa. 2001. Expression of alpha(4)beta(7) integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. *J Immunol* 167:2511-2521.

[67] Adachi, S., H. Yoshida, K. Honda, K. Maki, K. Saijo, K. Ikuta, T. Saito, and S.I. Nishikawa. 1998. Essential role of IL-7 receptor alpha in the formation of Peyer's patch anlage. *International immunology* 10:1-6.

[68] Pandey, A., K. Ozaki, H. Baumann, S.D. Levin, A. Puel, A.G. Farr, S.F. Ziegler, W.J. Leonard, and H.F. Lodish. 2000. Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. *Nature immunology* 1:59-64.

[69] Park, L.S., U. Martin, K. Garka, B. Gliniak, J.P. Di Santo, W. Muller, D.A. Largaespada, N.G. Copeland, N.A. Jenkins, A.G. Farr, S.F. Ziegler, P.J. Morrissey, R. Paxton, and J.E. Sims. 2000. Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: Formation of a functional heteromeric complex requires interleukin 7 receptor. *The Journal of experimental medicine* 192:659-670.

[70] Levin, S.D., R.M. Koelling, S.L. Friend, D.E. Isaksen, S.F. Ziegler, R.M. Perlmutter, and A.G. Farr. 1999. Thymic stromal lymphopoietin: a cytokine that promotes the development of IgM+ B cells in vitro and signals via a novel mechanism. *J Immunol* 162:677-683.

[71] Dougall, W.C., M. Glaccum, K. Charrier, K. Rohrbach, K. Brasel, T. De Smedt, E. Daro, J. Smith, M.E. Tometsko, C.R. Maliszewski, A. Armstrong, V. Shen, S. Bain, D. Cosman, D. Anderson, P.J. Morrissey, J.J. Peschon, and J. Schuh. 1999. RANK is essential for osteoclast and lymph node development. *Genes & development* 13:2412-2424.

[72] Kim, D., R.E. Mebius, J.D. MacMicking, S. Jung, T. Cupedo, Y. Castellanos, J. Rho, B.R. Wong, R. Josien, N. Kim, P.D. Rennert, and Y. Choi. 2000. Regulation of

peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. *The Journal of experimental medicine* 192:1467-1478.

[73] Taylor, R.T., S.R. Patel, E. Lin, B.R. Butler, J.G. Lake, R.D. Newberry, and I.R. Williams. 2007. Lymphotoxin-independent expression of TNF-related activation-induced cytokine by stromal cells in cryptopatches, isolated lymphoid follicles, and Peyer's patches. *J Immunol* 178:5659-5667.

[74] Migone, T.S., J. Zhang, X. Luo, L. Zhuang, C. Chen, B. Hu, J.S. Hong, J.W. Perry, S.F. Chen, J.X. Zhou, Y.H. Cho, S. Ullrich, P. Kanakaraj, J. Carrell, E. Boyd, H.S. Olsen, G. Hu, L. Pukac, D. Liu, J. Ni, S. Kim, R. Gentz, P. Feng, P.A. Moore, S.M. Ruben, and P. Wei. 2002. TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. *Immunity* 16:479-492.

[75] Sitnicka, E., C. Brakebusch, I.L. Martensson, M. Svensson, W.W. Agace, M. Sigvardsson, N. Buza-Vidas, D. Bryder, C.M. Cilio, H. Ahlenius, E. Maraskovsky, J.J. Peschon, and S.E. Jacobsen. 2003. Complementary signaling through flt3 and interleukin-7 receptor alpha is indispensable for fetal and adult B cell genesis. *The Journal of experimental medicine* 198:1495-1506.

[76] Ohl, L., G. Henning, S. Krautwald, M. Lipp, S. Hardtke, G. Bernhardt, O. Pabst, and R. Forster. 2003. Cooperating mechanisms of CXCR5 and CCR7 in development and organization of secondary lymphoid organs. *The Journal of experimental medicine* 197:1199-1204.

[77] Ansel, K.M., V.N. Ngo, P.L. Hyman, S.A. Luther, R. Forster, J.D. Sedgwick, J.L. Browning, M. Lipp, and J.G. Cyster. 2000. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 406:309-314.

[78] Forster, R., A.E. Mattis, E. Kremmer, E. Wolf, G. Brem, and M. Lipp. 1996. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* 87:1037-1047.

[79] Gaspal, F.M., M.Y. Kim, F.M. McConnell, C. Raykundalia, V. Bekiaris, and P.J. Lane. 2005. Mice deficient in OX40 and CD30 signals lack memory antibody responses because of deficient CD4 T cell memory. *J Immunol* 174:3891-3896.

[80] Hamada, H., T. Hiroi, Y. Nishiyama, H. Takahashi, Y. Masunaga, S. Hachimura, S. Kaminogawa, H. Takahashi-Iwanaga, T. Iwanaga, H. Kiyono, H. Yamamoto, and H. Ishikawa. 2002. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J. Immunol.* 168:57-64.

[81] Kanamori, Y., K. Ishimaru, M. Nanno, K. Maki, K. Ikuta, H. Nariuchi, and H. Ishikawa. 1996. Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of c-kit+ IL-7R+ Thy1+ lympho-hemopoietic progenitors develop. *J Exp Med* 184:1449-1459.

[82] Pabst, O., H. Herbrand, T. Worbs, M. Friedrichsen, S. Yan, M.W. Hoffmann, H. Korner, G. Bernhardt, R. Pabst, and R. Forster. 2005. Cryptopatches and isolated lymphoid follicles: dynamic lymphoid tissues dispensable for the generation of intraepithelial lymphocytes. *Eur J Immunol* 35:98-107.

[83] Lorenz, R.G., D.D. Chaplin, K.G. McDonald, J.S. McDonough, and R.D. Newberry. 2003. Isolated lymphoid follicle formation is inducible and dependent upon

lymphotoxin-sufficient B lymphocytes, lymphotoxin beta receptor, and TNF receptor I function. *J Immunol* 170:5475-5482.

[84] Taylor, R.T., A. Lugering, K.A. Newell, and I.R. Williams. 2004. Intestinal cryptopatch formation in mice requires lymphotoxin alpha and the lymphotoxin beta receptor. *J Immunol* 173:7183-7189.

[85] Yamamoto, M., M.N. Kweon, P.D. Rennert, T. Hiroi, K. Fujihashi, J.R. McGhee, and H. Kiyono. 2004. Role of gut-associated lymphoreticular tissues in antigen-specific intestinal IgA immunity. *J Immunol* 173:762-769.

[86] Ivanov, II, G.E. Diehl, and D.R. Littman. 2006. Lymphoid tissue inducer cells in intestinal immunity. *Curr Top Microbiol Immunol* 308:59-82.

[87] McDonald, K.G., J.S. McDonough, and R.D. Newberry. 2005. Adaptive immune responses are dispensable for isolated lymphoid follicle formation: antigen-naive, lymphotoxin-sufficient B lymphocytes drive the formation of mature isolated lymphoid follicles. *J Immunol* 174:5720-5728.

[88] Kelly, K.A., and R. Scollay. 1992. Seeding of neonatal lymph nodes by T cells and identification of a novel population of CD3-CD4+ cells. *Eur J Immunol* 22:329-334.

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Chapter 10

A Pair-Wise Relationships Model of Hematopoietic Fate Determination*

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Abstract

For more than twenty years, most models of hematopoiesis have been branching trees that include an early lymphoid/myeloid dichotomy. However, the field now appears to be turning away from such deterministic viewpoints. Events such as the recent demise of the common lymphoid progenitor have spawned a plethora of new developmental trees, but without leading to consensus on any single depiction. Instead of precise trees, we offer a model that simply considers a series of pair-wise developmental relationships between the various hematopoietic lineages, with decisions also influenced by signals from cell-surface receptors (for interleukins, colony-stimulating factors, etc.). The

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evidence for pair-wise relationships comes from: *(i)* the sets of fates available to various cell lines and oligopotent progenitors; and *(ii)* the patterns of regulation of various hematopoietic fates by complex sets of transcription factors. Each transcription factor seems, by promoting or suppressing lineage options on either side of fate boundaries, to contribute to determining the boundaries of sets of contiguous and related fates and to drive final choices between adjacent fates.

Introduction

Since the 1960s the hematopoietic system has been a key model for determining how diverse types of cells are generated within an organ system, with the discovery of tissue-specific hematopoietic stem cells (HSC) a fundamental early step. HSC self-renew and give rise, via several types of intermediate progenitor cells, to all types of blood and immune cells – platelets, erythrocytes, neutrophils, basophils, eosinophils, macrophages, at least two types of dendritic cells, B and T lymphocytes (of several types), natural killer cells and maybe others. An understanding of how cell lineages are forged demanded ways of identifying progenitor cells that are committed to various lineage fates. Till and McCullochk [1] started this process when they observed that donor bone marrow cells injected into recipient mice whose own bone marrow had been eliminated by irradiation gave rise to clonal colonies in the spleen that contained various types of mature blood cells. Moreover, bone marrow cells that were dispersed in semi-solid medium *in vitro* gave rise to heterogeneous colonies of differentiating and mature cells, each made up of the progeny of a single progenitor cell [2]. For hematopoiesis to occur *in vivo*, the developing cells must interact with marrow stromal cells. Recognition of these interactions led to the development of adherent monolayer cultures of stromal cells that would predominantly support either myelopoiesis or lymphopoiesis by providing appropriate growth and/or survival factors [3, 4].

So what are the developmental pathways that the progeny of HSCs follow to develop into each type of differentiated cell? Information from diverse experimental systems, both *in vivo* and *in vitro*, has led to the proposal of a multiplicity of branching 'tree' maps of hematopoietic development – sometimes giving the impression that each major research group was nurturing its own bit of woodland. In recent years an added inconvenience has been the need for some of these trees to include more than one developmental route to some types of blood cell.

Rather than comparing the individual merits and deficiencies of hierarchical lineage trees, we will consider another way of representing the information that trees attempt to summarise. This alternative model views hematopoiesis as a process in which the sets of possibilities available to HSCs and the various progenitor populations they give rise to are contiguous segments of a single, continuous, and plastic spectrum of lineage options. It represents the tendency of maturing HSCs and haemopoietic progenitors to progress from having a complete spectrum of lineage options towards more restricted subsets of available lineage options, but considers all of the subsets of options available to haemopoietic cells as contiguous segments of this fixed spectrum. We shall compare this idea of a continuous spectrum of pair-wise inter-relationships between the various hematopoietic fates against the

bifurcating patterns of progressive loss of lineage options that characterize more traditional 'tree' representations.

The Sequential Determination Model

Starting in 1985, one of us developed the Sequential Determination (SD) model of hematopoiesis, an early version of which is in Figure 1 [5]. This envisaged that the various lineage options become available in HSCs in a preferred order and then become latent – the depicted sequence encompasses the full hematopoietic spectrum, from megakaryocyte development at one pole to T lymphocyte development at the other: see the legend for more details.

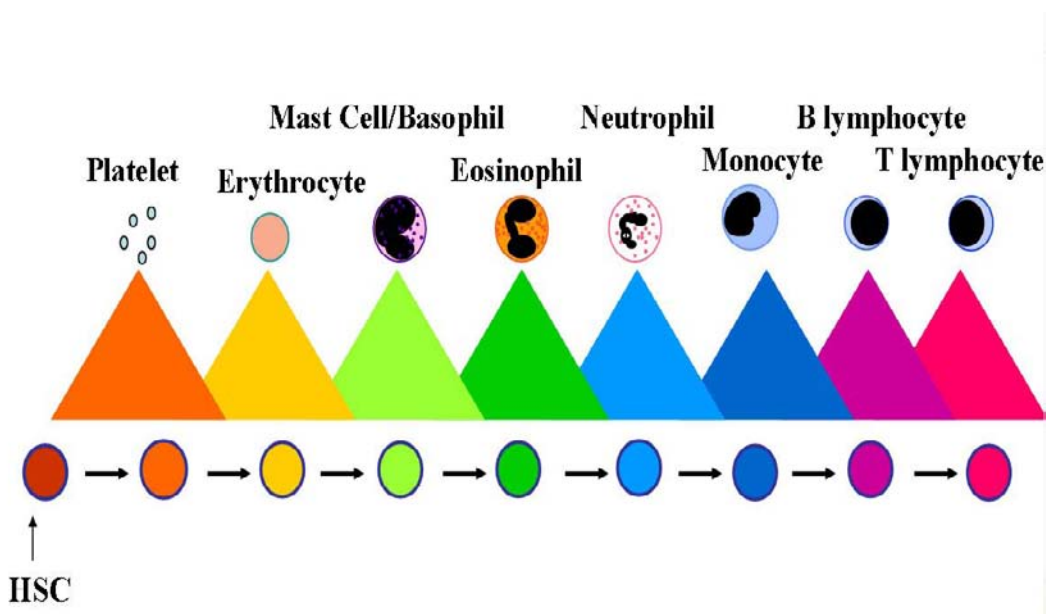

Figure 1. The Sequential Determination model of hematopoiesis.

The model, originally proposed in 1985 [5, 17], envisages a developmental program running in HSC that leads to a preferred sequence for the acquisition and loss of lineage potentials. A maximum of two options is available at any given time: as the probability of one decreases that of the next increases, so there is an overlap between each pair of options. The SD model arose from consideration of the combinations of lineage option pairings that were retained in various bipotent (and oligopotent) progenitor cells and in cell lines. Eight different fates were recognized in 1985, but only some of the 28 possible pairings of these – presumed to reflect close relationships – were observed experimentally, and these were combined into the sequence shown. Some of these relationships are exemplified by hematopoietic cell lines: HL60 cells can differentiate towards basophils, eosinophils, neutrophils or monocytes [18-21]); HT93 cells show eosinophil-neutrophil bipotency; and YJ cells show eosinophil-neutrophil-monocyte tripotency [22].

Lymphoid/Myeloid Progenitors in the Bone Marrow

A simple myeloid-lymphoid dichotomy was ruled out by the observations which revealed that the potentials for B lymphocyte and monocyte differentiation do not always segregate early. This conclusion has been reinforced by the detailed characterization of progenitor cell populations from mouse bone marrow that combine lymphoid and myeloid potentials [6]. These Early Progenitors with Lymphoid and Myeloid potential (EPLM) are B220^{+ve}, CD117^{low}, CD93^{+ve}, CD19^{-ve}, CD3^{-ve} and NK1.1^{-ve}, and they resemble pre-B cells that are found in knock-out mice lacking Pax-5, a B cell transcription factor [23]. *In vivo*, EPLM give rise to T and B lymphocytes, but they can be provoked to differentiate *in vitro* to B and T lymphocytes, natural killer (NK) cells, dendritic cells or macrophages. Differentiation to T lymphocytes requires a persistent Notch signal and IL-7; in the absence of Notch and the presence of IL-7, B lymphocytes are generated; NK cells develop following a transient Notch signal in the presence of IL-2 (or, probably, IL-15) [24]; and dendritic cell or macrophage development is Notch-independent but requires macrophage colony-stimulating factor (M-CSF) [25]. Despite the T lineage potential of EPLM, the thymus does not harbour EPLM-like cells, so EPLM probably represent the last stage in a developmental pathway that is predominantly B lymphocyte-directed, but in which a Notch signal can rescue T lymphopoiesis.

Endeavours to understand hematopoiesis in the mouse have focused on a bone marrow cell population that expresses high levels of stem cell antigen-1 (Sca-1) and *c*-kit (the receptor for stem cell factor, SCF) and lacks surface markers of differentiating or mature blood cells. These cells, termed LSK (Lin^{-} Sca-1^{+} Kit^{+}) cells, can reconstitute hematopoiesis in ablated mice. Also in the isolated LSK cell population are some multipotent progenitor cells (MPPs) that – unlike reconstituting HSCs – express Flt3 (LSKFlt3^{hi}). This LSKFlt3^{hi} subpopulation also includes lymphoid-primed multipotent progenitors (LMPPs) that have granulocyte, monocyte and lymphoid potentials and little or no aptitude for megakaryocytic or erythroid differentiation [26]. Megakaryocyte/erythroid potential is observed only in those LSKFlt3^{hi} cells that express the thrombopoietin receptor.

LSKFlt3^{hi} cells that lack the thrombopoietin receptor have the potentials for granulocyte, monocyte and lymphoid differentiation and their transcriptional patterns include elements typical of each of these lineage programmes. As they develop, a graded increase in transcriptional priming for lymphoid differentiation coincides with a graded reduction in the potentials for granulocyte and monocyte differentiation, suggesting that they progress in a gradual manner from including all potentials, through loss of megakaryocye/erythroid potentials but retention of propensity for differentiation towards granulocytes and monocytes, finally to become restricted to lymphoid pathways [15, 27] – this is the sequence of preferred options postulated by the SD model and also matches the branching patterns of many trees.

Lymphoid Progenitors in the Thymus Retain Myeloid Potential

Thymus-settling progenitors (TSP) are cells that have recently arrived in the thymus and are $CCR9^{+ve}$, $CD135^{+ve}$, $CD117^{high}$, $CD44^{+ve}$ and $CD25^{-ve}$ [28]. Upon loss of CCR9 and CD135, TSP are converted to early thymocyte progenitors (ETP, also called DN1 cells). In turn, ETP/DN1 gain CD25 to become double negative-2 (DN2) cells; and DN2 cells, upon loss of CD117 and CD44 and gain of cytoplasmic CD3, become DN3 cells. This DN2 to DN3 transition is particularly crucial, but still poorly defined [29]. In addition to maturing into T lymphocytes, TSPs have the potential to develop to myeloid cells, dendritic cells and B lymphocytes. B lineage potential is lost at the transition from TSP to ETP, prior to loss of CD135 expression, and myeloid-dendritic cell and NK cell potentials are lost as the cells convert from DN2 to DN3. DN3 cells contain abundant T cell receptor (TCR) β-chain V-DJ rearrangements and are fully T lymphocyte-committed. TCR β-chain D-J rearrangements are present in myeloid and NK cells generated from DN2 cells, so these rearrangements must occur before cells become committed to become T lymphocytes and they do not prevent differentiation towards other fates [25, 30].

Studies of clones of cells that are derived from ‘T lymphocyte progenitors’ under culture conditions that permit both lymphoid and myeloid cell-types to mature has demonstrated the existence of progenitors that combine lymphoid and myeloid potentials: ETP and DN2 cells both retain the potential for either T lymphocyte or myeloid differentiation, but have already lost the ability to develop into B lymphocytes [31, 32]. Their myeloid potential is predominantly monocytic, but granulocytes, dendritic cells and NK cells are also observed.

Multiple Routes towards a Particular Fate

As just illustrated, each of the diverse types of hematopoietic progenitor that has been identified maintains the ability to mature along a defined set of lineage options, and description of each ‘new’ type of progenitor has tended to demand some refinement of previous lineage maps. Redrawn versions of some of the recent tree models that are shown in Figure 2 illustrate two issues. Some elements, notably an early lymphoid/myeloid division, are common to all of the three maps, but the maps differ in their exact views of the relationships between myeloid and lymphoid cells. As a consequence, these trees suggest a variety, and sometimes a multiplicity, of routes from HSCs to myeloid fates.

That some hematopoietic fates might be reached by more than one route is an idea that to a degree echoes an early ‘stochastic’ model of hematopoiesis, which held that the progeny of HSCs choose in a fairly random manner from those lineage options that they still retain, with the various lineage potentials becoming assorted amongst committed colony-forming cells in an unpredictable pattern [35]. Later studies showed that the patterns of expression of the various transcription factors that govern lineage specification in progenitor cells are widely expressed at low levels in a variety of combinations, in a manner that seems compatible with this type of ‘stochastic’ model [36, 37], and it was suggested that these fluctuations then

influence the apparently random adoption of fates [38]. Although simple versions of such stochastic models of fate determination are now not widely favoured, such findings suggest that the properties of intermediate progenitor cells are more plastic than has often been acknowledged (see later).

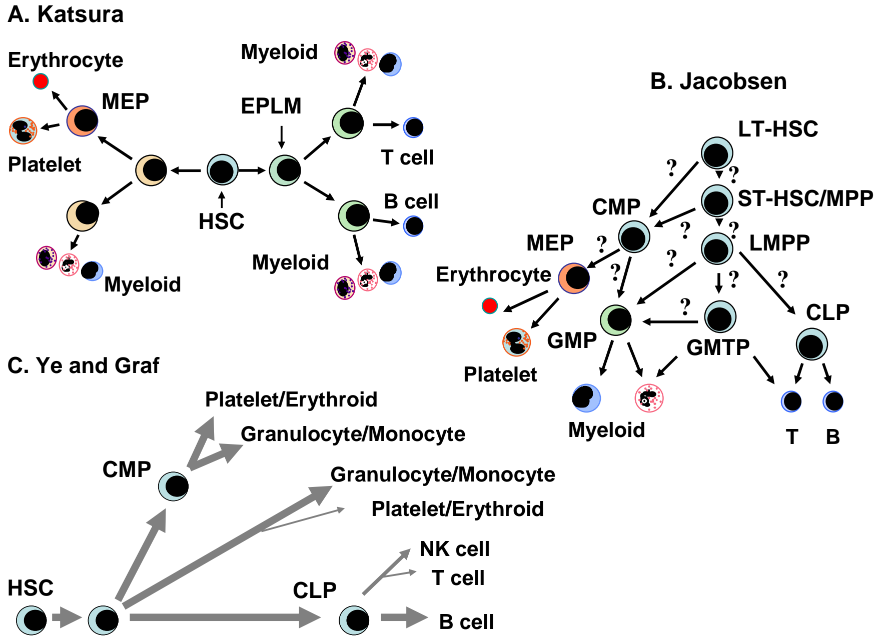

Figure 2. Branching models that suggest multiple routes to myeloid cells.

In the Katsura model a common lymphoid-myeloid progenitor gives rise to progenitors whereby there are close relationships between the options for myeloid and T lymphocyte development and for myeloid and B lymphocyte development [33]. In addition to HSCs veering in these directions, they also veer towards the potentials for erythroid and megakaryocyte development whilst retaining the potentials for myeloid development. In the model proposed by Jacobsen [15] there are multiple routes towards some end cell types. The question marks alongside the arrows indicate that the relationship between progenitor cell populations remains to be definitively established. The Ye and Graf model envisages flows along branches of a tree towards the options for megakaryocyte/erythroid/myeloid differentiation and those for lymphoid differentiation [34].

A Continuum Depiction of Lineage Inter-Relationships and their Links to Progressive Specification during Hematopoiesis

Given the limitations of the models that lie at two extremes – invariant lineage trees or stochastic lineage choice – what view of hematopoiesis would both recognise that intermediate progenitors have tendencies to give rise to particular combinations of fates (and to exclude others) and also reject strict determinacy of the type that lineage trees suggest. For

example, how do 'committed lymphoid progenitors' and 'committed myeloid progenitors' both give rise to apparently identical dendritic cells [39]?

Starting from the 'sequential' model [5] we have further developed our depiction of the developmental relationships between the various hematopoietic cell types: see Fig 3, which is a modification of an earlier figure in [40]. Below we consider whether this might better express the diverse ways in which cells progress – from the initial 'stem-cell' state of HSCs, through various oligopotent ('progenitor cell') states, to one of the many final types of fully differentiated cells.

The mixed final cell populations that arise when the progeny of normal oligopotent progenitors are allowed to mature, regularly recapitulate the same sets of partnerships between pairs (and larger multiples) of final hematopoietic fates. The same applies to the many cell lines derived from hematopoietic cells. For example, neutrophil/monocyte and erythrocyte/megakaryocyte pairings are commonplace. By contrast, other lineage combinations – such as a progenitor or cell-line giving rise only to megakaryocytes and T lymphocytes – have not been seen and are presumed to be prohibited.

Figure 3 simply arranges the known pair-wise relationships between lineage fates around a broken circle. It makes no assumptions about any underlying branching pattern that might appear to dictate a preferred route to a particular fate. This pattern of pair-wise relationships appears to be governed by a cell-intrinsic program that operates in stem cells and in the various oligopotent progenitors derived from them. Each fate potential varies between being dominant (and likely to be adopted), being available (but less available than one of its neighbours), and already having been lost (though maybe not irretrievably). Like others, this model envisages that a cell progressively closes down other fates as it moves towards finally committing to a differentiated fate – with the last fate potentials to be lost being the two with which the chosen fate has immediate pair-wise relationships. Many different transcriptional programs may have been primed in the HSCs and multipotent progenitors, but all but one will have been extinguished – or at least suppressed to ineffectual levels – once final commitment occurs [41-44].

A problem with tree representations is that they are best suited to depicting only one route from an initiating stem cell to each final differentiated cell. By contrast, the depiction in Figure 3 simply considers a series of pair-wise relationships between adjacent fates available to HSCs. Each final differentiated fate can be represented by a small arc that takes in only that lineage, and each type of intermediate progenitor is represented by an arc that encompasses all of the contiguous fates that it can yield: arcs representing many of the commonly analyzed progenitor populations are displayed outside the central circle. If the fate repertoires of two or more types of progenitor include the same mature lineage then the arcs that represent these progenitors overlap appropriately (for example, EPLM and LMPP in Figure 3).

This depiction shares important features with the lineage trees discussed above, but presents relationships between the various lineages as a continuum – from megakaryocyte/erythroid potentials, through myeloid (neutrophil/monocyte) potentials to those for lymphoid development. Whilst there is still some uncertainty about exactly how to place each lineage option, the most profound separation seems to be between the megakaryocyte and erythroid pathways on the one hand and the various types of lymphoid cells on the other, with

granulocytic and phagocytic lineages intermediate. For example, the progressive lineage restriction of mouse LSK cells – from pluripotency, through the loss of erythrocyte/megakaryocyte and then granulocytic/monocytic potentials, finally to lymphoid restriction – corresponds to this pattern. Similarly, Lai and Kondo have suggested that there is a hierarchy to for the loss of differentiation potentials in which early loss of erythroid potential is followed by loss of both neutrophil and monocyte potentials and that a silencing of myeloid genes is prerequisite for commitment to lymphoid development [45].

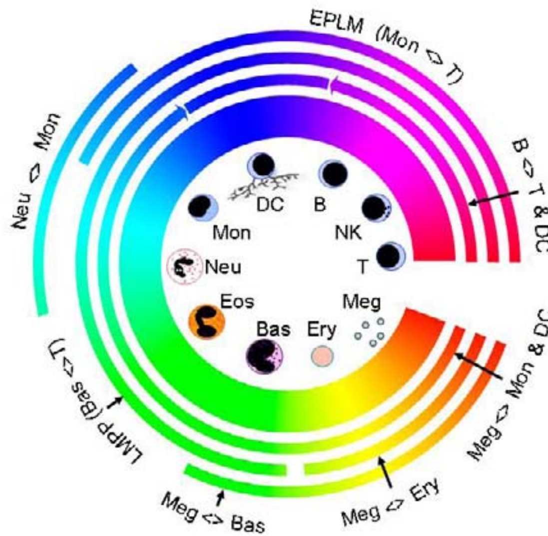

Figure 3. A simple depiction of the specification of hematopoietic lineages.

The figure shows a simple depiction of the pair-wise relationships between different hematopoietic lineages. Cell-intrinsic programs running in both HSCs and in the various oligopotent progenitors derived from them allows the initial wide spectrum of fates to become progressively restricted to parts of the spectrum (as shown by the arcs) and finally to a chosen lineage. The arrowheads on some arcs indicate that apparently identical dendritic cells can be derived from a progenitor with meg <> monocyte potentials and a progenitor with B <> T potentials. Hence these two arcs overlap as to dendritic cell potential.

The original SD model did not include dendritic cells and NK cells. A sub-population of CLPs expresses the tyrosine kinase receptor Flk2, and *in vitro* CLPs yield B and T lymphocytes, dendritic cells and NK cells, but not cells of myeloid lineages [46]. Hence, these are a set of related fates. The option for NK cell development is placed next to that for T lymphocytes: this reflects the order of loss of potentials by stem cells entering the thymus (see above) and the fact that these cells have related cytolytic functions. As already mentioned, apparently identical dendritic cells can be derived from cells purified either as CMP or CLP, even though these are clearly distinguishable progenitors [47], again suggesting where dendritic cells may be placed in the sequence: the option for dendritic cell

development may best be viewed as operating independently of myeloid and lymphoid pathways. Placing dendritic cells between monocytes and B lymphocyte is compatible with their generation from both ‘myeloid’ and ‘lymphoid’ progenitors. However, the fact that ETP and DN2 cells in the thymus have lost the potential for B lymphocyte development yet retained myeloid (including macrophage and dendritic cell) potential [25] appears at odds with this sequence. Perhaps the contiguity of fates is not inviolate, and can change under certain *in vivo* and *in vitro* circumstances by, for example, the strength of Notch signaling (see below). As mentioned earlier, adult mouse bone marrow and foetal liver yield a bipotent monocyte/B lymphocyte progenitor – it would be of interest to know whether this cell might be persuaded to exhibit dendritic cell (and T lymphocyte?) potential under appropriate conditions.

Evolutionary Emergence of the Mammalian Hematopoietic Repertoire

It is likely that the inter-relationships between cell-types in the complex adaptive hematopoietic system of mammals and other jawed vertebrates are evolutionarily derived from the simpler, mainly innate, immune systems in the common ancestors from which they evolved. New specialized lineages are likely to have been added, one by one, to various parts of the hematopoietic continuum: examples include the multiple types of lymphocytes and granulocytes. Primordial defense mechanisms such as non-specific recognition and phagocytosis of pathogens by macrophages (which are also needed to clear apoptotic cells during morphogenesis and tissue turnover) seem to have evolved earlier than lymphocyte-mediated specific and adaptive immunity [48, 49]. For example, *Drosophila melanogaster* has innate and non-specific immunity, mediated by macrophage-like plasmatocytes and neutrophil-like crystal cells, but lacks lymphocyte-mediated adaptive immunity. Coagulation of the type that is mediated by platelets, which probably evolved to restrict pathogen dispersal within an organism (for instance, in *Drosophila melanogaster*), is considered an even more primordial defense mechanism than phagocytosis.

Monocytes, B lymphocytes and dendritic cells share some common functions, including the processing of antigens and presentation of antigenic peptides at their cell surface in association with MHC Class II molecules. Some B lymphocytes from teleost fish and the amphibian *Xenopus laevis* are phagocytic, whereas the more specialized B lymphocytes of mammals have lost this capacity [50]. This suggests that B lymphocytes and monocytes might have evolved from a previous phagocytic cell type, with functional attributes segregated between the two cell-types and refined. Consideration of the notion that B and T lymphocytes evolved more recently than myeloid cells has led to an interesting interpretation of the different numbers of transcription factors that are required to specify these groups of lineages. Activation of two factors seems to be enough to specify each of the myeloid fates, but more complex combinations of at least four factors are needed to specify B and T lymphocyte development: it is suggested that “the basic wiring of myeloid cells may have served as a platform for the development of more complex cell phenotypes” [51].

Mapping Transcription Factor Requirements onto the Pair-Wise Relationships Model

What are the processes in HSCs and multipotent progenitors that cause the segregation and adoption of fates? The general answer is that changes in the concentrations of transcription factors (TFs) govern how a cell progressively loses its plasticity and finally adopts a particular lineage fate. A complex network of TFs selectively enhances or represses expression of individual genes, which in concert facilitate or repress each lineage option. Important variables include the achievement by TFs of various thresholds within a range of TF concentrations and the covalent regulation of TF activities, for example by phosphorylation or sumoylation. The relative concentrations (or activities) of two or more factors are important. This can be exemplified by the CCAAT enhancer-binding protein-α (C/EBPα) and GATA-2: granulocyte/monocyte-restricted progenitor cells express C/EBPα; elevated GATA-2 expression redirects these cells to the eosinophil lineage; but if C/EBPα expression is suppressed prior to GATA-2 expression the granulocyte/monocyte progenitors commit to mast cell development [52].

The TF circuitry is interactive, with TFs regulating one another's expression and activity. For example, EDGA expression is a target of GATA-1, and GATA-1 and PU-1 exemplify mutual cross-inhibition. Erythroid and megakaryocytic cells express GATA-1 and myelomonocytic lineages express PU-1; elevating GATA-1 promotes erythroid/megakaryocytic fates; elevating PU-1 shifts the balance towards myelomonocytic options; and over-expression of each can suppress expression of the other [53] (reviewed in [51]). Another example of interference circuitry is FOG-1 restriction of mast cell development by disrupting the requirement for GATA-1 to associate with PU-1 [54].

How do the requirements for activity of the various TFs map onto the pair-wise relationships spectrum of lineage fates? To what degree do the expression and activation states of various TFs correspond with the array of pair-wise relationships between lineages? WAnd what changes in TF activity characterise choices between adjacent fates, leading to the losses of fate potentials that occur as cells move outwards into the ever-smaller arcs of Fig 3? Fig 4 attempts to summarise available information on these points. It is apparent that the permissive and/or stimulatory effects of each TF tend to be exerted on a contiguous series of interrelated fates, and that a stimulatory TF often exerts inhibitory effects on the lineage options on one or both sides of that span.

Shared usage of TFs by contiguous fates is exemplified by the roles played by GATA-1 and its structurally similar binding partner Friend Of GATA-1 (FOG-1) in the megakaryocyte < > erythrocyte < > basophil/mast cell < > eosinophil span of the spectrum (see Figure 4). GATA-2 plays a role in early development along pathways and is later replaced by GATA-1 [55, 56]. GATA-1 concentration is important, as variations in its expression in Myb-Ets-transformed myeloblasts can reprogram these cells to become thromboblasts (high GATA-1) or eosinophils (lower GATA-1) [57]. GATA-1 restricts mast cell development: mice with an inactivating GATA-1 mutation have an excess of mast cell progenitors [58], and a megakaryocyte/erythrocyte/mast cell progenitor is expanded in mice carrying a GATA-1^{low} mutation [59]. Other TFs are also important – high GATA-1 plus FOG-1 specifies

megakaryocyte/erythroid-restricted progenitors, but moderate GATA-1 plus C/EBPα/β generates eosinophils [53]. Three findings emphasise the importance of GATA-1 to eosinophil development: *(i)* mice carrying inactivated GATA-1 do not make eosinophil progenitors; *(ii)* GATA-1 levels rise when murine embryonic stem cells undergo eosinophil development [60]; and *(iii)* over-expressing GATA-1 in granulocyte-macrophage-restricted cells forces eosinophil development [57, 61].

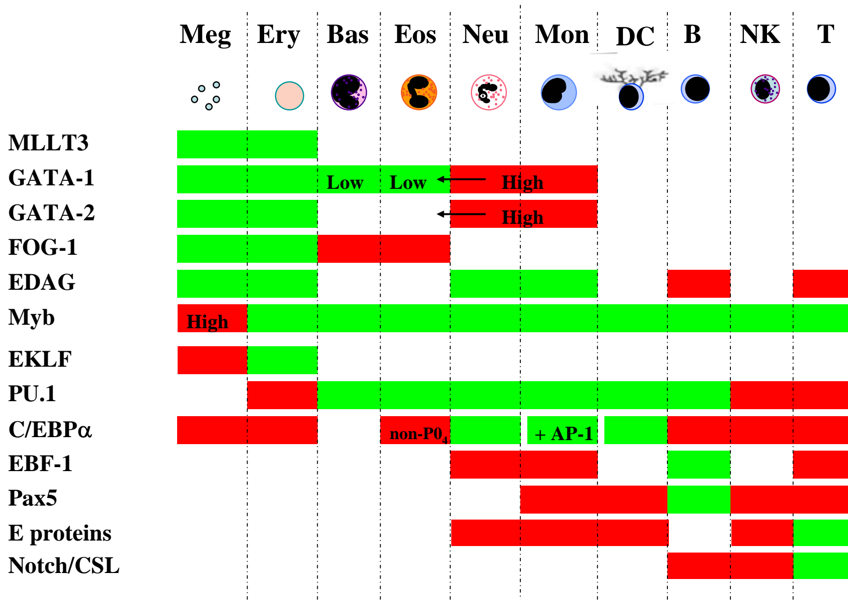

Figure 4. Mapping TF usage to the continuum of pair-wise relationships.

The green blocks indicate which TFs must be active for adoption of a particular fate, and the red blocks identify TFs that prevent cells from adopting that fate. Text on the blocks adds detail to information given by the colour (e.g. a TF must be at a high concentration or be phosphorylated to achieve the function depicted). The arrows indicate diversion to an adjacent fate. Meg, megakaryocyte; Ery, erythroid; Bas, basophil/mast cell; Eos, eosinophil; Neu, neutrophil; Mon, monocyte; DC, dendritic cell; B, B lymphocyte; NK, natural killer cell; T, T lymphocyte.

Like GATA-1, FOG-1 is essential for erythropoiesis and megakaryopoiesis and restricts mast cell development. Loss of FOG-1 arrests erythropoiesis and causes complete failure of megakaryopoiesis [62, 63]. Conditional FOG-1 expression in mouse ES cells followed by induction of hematopoietic cell differentiation reprograms hematopoietic progenitors from a mast cell to a neutrophil fate. FOG-1 expression in mature mast cells leads to their dedifferentiation [54], and ectopic expression of FOG-1 in mast cell progenitors redirects them to the erythroid, megakaryocytic and granulocytic lineages [64].

As in the above lineage grouping, there is a progenitor restricted to the adjacent megakaryocyte and erythroid lineages (reviewed in [65]) and MLLT3 interacts with GATA-1 to facilitate both of these fates [66]. By contrast, the mast cell and eosinophil pathways both

require down-regulation of FOG-1 alongside GATA-1 expression [64]: FOG-1 may skew cells from the mast cell fate by disrupting an association between GATA-1 and PU-1 [54].

C/EBPα is important for differentiation to lineages within the eosinophil/neutrophil/monocyte/dendritic cell span. Neonatal mice lacking C/EBPα lack granulocytes, monocytes and their progenitors [67, 68], and C/EBPα is also required for eosinophil and dendritic cell maturation [69]. The level of activity of C/EBPα influences options within this span of the sequence, with increased C/EBPα activity skewing cells towards a preference of monocyte over granulocyte development. For example, estradiol activation of an introduced C/EBPα-estrogen receptor construct in murine marrow mononuclear cells leads to a preponderance of monocytes and their progenitors over granulocytes and their progenitors [70], and C/EBPα:AP-1 heterodimers direct monocytic commitment more potently than C/EBPα homodimers [71]. In a further level of regulation, inhibitory phosphorylation of C/EBPα by glycogen synthase kinase-3 (GSK-3) can bias the neutrophil/eosinophil balance in hematopoietic progenitors towards eosinophils, and expression of a C/EBPα construct that cannot be phosphorylated at the inhibitory site directs differentiation towards neutrophils. At a yet higher level, inhibitory phosphorylation of GSK-3 by protein kinase B (PKB/c-akt, which is itself regulated downstream of phosphoinositide 3-kinase activation) prevents C/EBPα phosphorylation, promotes neutrophil and monocyte development and suppresses eosinophil differentiation [72].

PU.1 activity makes stem/progenitor cells veer away from the erythroid end of the spectrum towards myeloid fates [73-75], and is essential for murine embryonic stem cells to generate eosinophils [60], neutrophils and macrophages [76] and dendritic cells [77], and also for basophils/mast cells to produce IL-4 [78]. At the other end of the lineage sequence, PU.1 concentration must be low for B lymphocyte development [79].

The concentration of the Erythroid Differentiation-Associated Gene product (EDAG) must be low for successful differentiation to fates at the lymphoid end of the lineage spectrum. EDAG is expressed in HSCs, early progenitors and megakaryocytic and erythroid progenitors and is a target of GATA-1 [80]. In transgenic mice, increased EDAG expression causes an expansion of myeloid cells, blocks B lymphocyte development at the B1 to pre-BII stage and blocks T lymphocyte development at the most immature DN1 stage [81]. Multi-component TF complexes that include basic helix-loop-helix (HLH) E proteins (including SCL/TAL1, E2A, HEB and E2-2, and the inhibitor Id) play multiple roles both in the choice between T lymphocyte and non-T lymphocyte fates and in later developmental stages, for example, during erythrocyte development [82, 83]. When DN3 thymocytes are diverted towards myeloid or dendritic cell fates – by re-introducing C/EBPα or PU.1, respectively – one of the earliest effects is suppression of the inhibition of the net activity of E proteins [51, 83-85].

Progression of Lineage Restriction towards a Single Fate

Centrifugal progression from the central spectrum out to ever-smaller arcs representing decreasing numbers of options involves closure of fates. Changing concentrations of the relevant TFs tend to promote one fate or a group of contiguous fates whilst suppressing those to either side of the promoted sector.

c-Myb and the Erythroid Kruppel-like factor (EKLF) play a directive role in megakaryocytic versus erythroid fate choice. High c-Myb in megakaryocyte/erythroid-restricted progenitors promotes erythropoiesis and suppresses megakaryopoiesis, as revealed by a transgenic mouse in which an enhancer for *c-myb* gene expression was disabled by gene insertion: a marked decrease in c-Myb leads to an increase in megakaryocytes and a decrease in erythroid cells [86]. Various c-*myb*-dysfunctional mice have increased megakaryopoiesis and diminished erythropoiesis: these include a *c-myb* knockdown [87] and mice bearing mutations in the DNA-binding or leucine zipper domains of c-Myb [88] or in p300, which interacts with c-Myb [89].

EKLF plays an important role in erythroid differentiation. GATA-2 and Smad5 cooperate to induce expression of EKLF in a progenitor population, and EKLF is controlled by GATA-1 once cells have become erythroid-commited [90]. EKLF represses megakaryocyte differentiation [91], at least partly by repressing mRNA levels of Fli-1, a TF that drives expression of megakaryocytic-specific genes [91, 92]. Sumoylation of EKLF is important for inhibition of megakaryopoiesis: mutation of a single sumoylation site attenuates its repressive ability without affecting its ability to stimulate transcription. The sumoylated EKLF interacts with the Mi-2β ATPase-containing subunit of the NuRD repression complex rather than with activators such as p300, CBP and P/CAF [93].

As already noted, differences in TF concentrations can simultaneously drive cells towards a contiguous set of fates and suppress options to either side of this group. For example, PU.1 enforcement of myeloid and B lymphocyte identity (see above) appears to involve diversion of cells away from erythroid, NK cell and T lymphocyte options [94]. Genes activated by high PU.1 tend to be myeloid-specific, whereas erythroid-, NK cell- and T-lymphocyte-affiliated genes are repressed. Similarly, C/EBPα can divert megakaryocyte/erythroid progenitors towards macrophages [95], is important for myelopoiesis and suppresses lymphopoiesis: its enforced expression in CLPs, pro-T and pro-B cells promotes myeloid differentiation. C/EBPα inhibits B lymphoid development by cross-inhibiting the B lymphoid-promoting transcription factor Pax5 [96] and maybe other regulators: enforced C/EBPα expression completely suppresses Pax5 [97].

The Early B Cell Factor (EBF-1, also called EBF or Olf-1) provokes B cell development whilst antagonising myeloid and T lymphocyte options. It is expressed exclusively in B lineage cells and is essential for B lymphocyte commitment of multi-potent progenitors. Moreover, enforced EBF-1 expression in multi-potent progenitors directs B lymphocyte development at the expense of myelopoiesis, and sustained EBF-1 expression in HSCs or in foetal liver progenitor cells from $Pax5^{-/-}$ embryos suppresses both T cell and myeloid potentials [98]. There is no information on whether EBF-1 blocks the dendritic cell and NK cell fate options: dendritic cells and NK cells, as well as myeloid cells and T cells, are

generated when progenitor cells from EBF-$1^{-/-}$ mice are transferred into lethally irradiated host mice, so it is not required for development along these pathways.

Environmental Signals Influence the Progress of Cells towards TF-Prescribed Lineage Options

TFs determine the availability of lineage options to progenitor cells, environmental signals, such as cytokines, couple to the TF network to facilitate and/or force options.

Notch is the prototype of a family of transmembrane receptors that bind ligands of the Jagged and Delta/Serrate families. Ligand binding leads to proteolytic cleavage of an intracellular domain of Notch which translocates to the nucleus and converts its nuclear cofactor CSL into a transcriptional activator. Notch signaling is important for T lymphocyte development: entry into the earliest discernable T cell progenitor stage fails in mice lacking Notch [99]. Continued Notch signaling in the thymus is essential for the commitment of multipotent thymus settling progenitors (TSP) to the T lymphocyte lineage, and for progression of early thymocyte progenitors (ETP) towards T lymphocytes.

Notch signaling is also important to closing the B lymphocyte and NK cell options in TSP/ETP [100]. Expression of activated Notch-1 in fetal liver-derived progenitors drives T lymphocyte development and inhibits differentiation along the B lymphocyte and NK cell pathways [101], and co-culture of these progenitors on stromal cells that express Delta-like-1 has the same effect [102]. Both the strength and duration of Notch signaling regulate progenitor cell fates. A weak signal is enough to inhibit the B lymphocyte potential, a weak and transient signal is needed to initiate progenitor cell development into NK cells, and a strong signal blocks NK cell development [24, 103].

Wnt proteins are a family of secreted signals that regulate self-renewal of HSCs and the fates of these cells and progenitor cells in culture [104, 105] and can also modulate the primary effects of Notch signaling on lineage choice [106]. For example, Wnt modulates Notch's effects on the balance of T lymphocyte and NK cell development. Treatment of human $CD34^{+ve}$ cord blood cells with Wnt3a provokes an increase in Notch activation, and these cells can be driven along the NK and T lymphocyte pathways by the Notch ligand Delta-1. Co-addition of Wnt3a increases the proportion of T lymphocyte precursors, and inhibiting Wnt signaling – even when Delta 1 is present – favours NK cell development.

β-catenin is generally though to be essential for Wnt signaling, but the combined absence of β- and γ-catenins has no effect on hematopoiesis *in vivo* [107, 108]. This emphasizes that environmental cues mainly exert ancillary, rather than deterministic, influences on hematopoiesis – they particularly support survival, self-renewal and proliferation of cells at various developmental stages [109].

Cell Fate Decisions and Leukemia

In recent years, attention has focused on identifying rare leukemia stem cells and determining how these differ from their normal counterparts. The resulting information can be used to direct the targeting of anti-leukemia therapies [110, 111]. In many types of leukemia the cells undergo maturation arrest at an early stage of lineage development, and understanding this is important to understanding the genesis and progression of these diseases. Moreover, TFs that play a role in cell fate decisions are perturbed in leukemia: these include tandem duplication of Myb in T acute lymphoblastic leukemia and inactivation of Pax5 (essential for B lineage commitment) in B progenitor acute lymphoblastic leukemia (reviewed in [112]). AML-ETO can redirect erythroid-programmed progenitor cells to granulocytic fates, and the AML-1-ETO chromosomal translocation is commonly associated with acute myelogenous leukemia in which granulocyte precursors accumulate in the blood and marrow [113]. The AML-1-ETO fusion protein inhibits the transactivation activity of PU.1 [114], and suppression of PU.1 function has been proposed as a key component of the malignant transformation process in myeloid leukemia (reviewed in [115]).

In the model shown in Fig 3, choices between one fate and the adjacent fates are decisions between a series of fates arrayed along a continuum, and outward movement from the centre represents a drift of lineage potentiality away from pluripotency to more restricted sets of potentials. Under some special circumstances, cells may also move in the opposite direction – towards greater lineage flexibility. This level of plasticity within stem and progenitor cells, together with the modulating influences of cytokines such as erythropoietin, thrombopoietin, GM-CSF and others, probably permits the balance of various types of blood cell to be matched to the prevailing needs of the organism. In other words, HSC and progenitor cells are behaving autonomously but also in a 'social' manner. But, what happens to a cell that gets 'stuck' in a particular fate window and so loses the means to move sideways within the spectrum of options or outwards towards more restricted and mature states? A possibility is that these abnormal stem cells accumulate and preferentially give rise to cells of the lineage option at which the HSC or progenitor cell is 'stuck': for example, giving rise to erythroleukemias or acute monocytic leukemias. The bulk of the cells in these and other leukemias remain substantially immature – their maturation is incomplete. In most cases, whether this is coupled to perturbations of the processes that drive HSC fate decisions remains to be seenincompletely understood. But, TFs function repeatedly to trigger and sustain the process of commitment and progression along differentiation pathways [83], so any transcription factor defect that perturbs fate determination might also affect progression along a maturation pathway.

Conclusion

Our preferred depiction of the development of blood cells, which is an updated version of our earlier Sequential Determination model, is that HSCs make a series of decisions between invariant pair-wise relationships between possible lineage fates. Unlike the classical 'tree' depictions of hematopoiesis, our model makes no assumptions about underlying branching

patterns that might appear to dictate a preferred route to a particular fate. Cells may find their way to a final outcome via more than one type of intermediate progenitor. In other words, there is greater plasticity in the sequences in which the various steps of lineage restriction occur than is acknowledged by most models.

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References

[1] Till, J. E. and McCulloch, C. E., A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* 1961. 14: 213-222.

[2] Bradley, T. R. and Metcalf, D., The growth of mouse bone marrow cells in vitro. *Aust J Exp Biol Med Sci* 1966. 44: 287-299.

[3] Dexter, T. M., Haemopoiesis in long-term bone marrow cultures. A review. *Acta Haematol* 1979. 62: 299-305.

[4] Whitlock, C. A. and Witte, O. N., Long-term culture of B lymphocytes and their precursors from murine bone marrow. *Proc Natl Acad Sci U S A* 1982. 79: 3608-3612.

[5] Brown, G., Bunce, C. M. and Guy, G. R., Sequential determination of lineage potentials during haemopoiesis. *Br J Cancer* 1985. 52: 681-686.

[6] Graf, T., Immunology: blood lines redrawn. *Nature* 2008. 452: 702-703.

[7] Kawamoto, H., A close developmental relationship between the lymphoid and myeloid lineages. *Trends Immunol* 2006. 27: 169-175.

[8] Weissman, I. L., Anderson, D. J. and Gage, F., Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. *Annu Rev Cell Dev Biol* 2001. 17: 387-403.

[9] Borzillo, G. V., Ashmun, R. A. and Sherr, C. J., Macrophage lineage switching of murine early pre-B lymphoid cells expressing transduced fms genes. *Mol Cell Biol* 1990. 10: 2703-2714.

[10] Boyd, A. W. and Schrader, J. W., Derivation of macrophage-like lines from the pre-B lymphoma ABLS 8.1 using 5-azacytidine. *Nature* 1982. 297: 691-693.

[11] Davidson, W. F., Pierce, J. H., Rudikoff, S. and Morse, H. C., 3rd, Relationships between B cell and myeloid differentiation. Studies with a B lymphocyte progenitor line, HAFTL-1. *J Exp Med* 1988. 168: 389-407.

[12] Tanaka, T., Wu, G. E. and Paige, C. J., Characterization of the B cell-macrophage lineage transition in 70Z/3 cells. *Eur J Immunol* 1994. 24: 1544-1548.

[13] Cumano, A., Paige, C. J., Iscove, N. N. and Brady, G., Bipotential precursors of B cells and macrophages in murine fetal liver. *Nature* 1992. 356: 612-615.

[14] Montecino-Rodriguez, E., Leathers, H. and Dorshkind, K., Bipotential B-macrophage progenitors are present in adult bone marrow. *Nat Immunol* 2001. 2: 83-88.

[15] Luc, S., Buza-Vidas, N. and Jacobsen, S. E., Delineating the cellular pathways of hematopoietic lineage commitment. *Semin Immunol* 2008. 20: 213-220.

[16] Fisher, A. G., Burdet, C., Bunce, C., Merkenschlager, M. and Ceredig, R., Lymphoproliferative disorders in IL-7 transgenic mice: expansion of immature B cells which retain macrophage potential. *Int Immunol* 1995. 7: 415-423.

[17] Brown, G., Bunce, C. M., Lord, J. M. and McConnell, F. M., The development of cell lineages: a sequential model. *Differentiation* 1988. 39: 83-89.

[18] Collins, S. J., The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. *Blood* 1987. 70: 1233-1244.

[19] Denburg, J. A., Basophil and mast cell lineages in vitro and in vivo. *Blood* 1992. 79: 846-860.

[20] Fabian, I., Lass, M., Kletter, Y. and Golde, D. W., Differentiation and functional activity of human eosinophilic cells from an eosinophil HL-60 subline: response to recombinant hematopoietic growth factors. *Blood* 1992. 80: 788-794.

[21] Fischkoff, S. A. and Condon, M. E., Switch in differentiative response to maturation inducers of human promyelocytic leukemia cells by prior exposure to alkaline conditions. *Cancer Res* 1985. 45: 2065-2069.

[22] Yamaguchi, Y., Nishio, H., Kasahara, T., Ackerman, S. J., Koyanagi, H. and Suda, T., Models of lineage switching in hematopoietic development: a new myeloid-committed eosinophil cell line (YJ) demonstrates trilineage potential. *Leukemia* 1998. 12: 1430-1439.

[23] Balciunaite, G., Ceredig, R., Massa, S. and Rolink, A. G., A B220+ CD117+ CD19- hematopoietic progenitor with potent lymphoid and myeloid developmental potential. *Eur J Immunol* 2005. 35: 2019-2030.

[24] Rolink, A. G., Balciunaite, G., Demoliere, C. and Ceredig, R., The potential involvement of Notch signaling in NK cell development. *Immunol Lett* 2006. 107: 50-57.

[25] Rolink, A. G., Massa, S., Balciunaite, G. and Ceredig, R., Early lymphocyte development in bone marrow and thymus. *Swiss Med Wkly* 2006. 136: 679-683.

[26] Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C. T., Bryder, D., Yang, L., Borge, O. J., Thoren, L. A., Anderson, K., Sitnicka, E., Sasaki, Y., Sigvardsson, M. and Jacobsen, S. E., Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell* 2005. 121: 295-306.

[27] Luc, S., Anderson, K., Kharazi, S., Buza-Vidas, N., Boiers, C., Jensen, C. T., Ma, Z., Wittmann, L. and Jacobsen, S. E., Down-regulation of Mpl marks the transition to lymphoid-primed multipotent progenitors with gradual loss of granulocyte-monocyte potential. *Blood* 2008. 111: 3424-3434.

[28] Zediak, V. P., Maillard, I. and Bhandoola, A., Closer to the source: notch and the nature of thymus-settling cells. *Immunity* 2005. 23: 245-248.

[29] Ceredig, R., Bosco, N. and Rolink, T., Problems defining DN2 thymocytes. *Immunol Cell Biol* 2008. 86: 545-547.

[30] Balciunaite, G., Ceredig, R. and Rolink, A. G., The earliest subpopulation of mouse thymocytes contains potent T, significant macrophage, and natural killer cell but no B-lymphocyte potential. *Blood* 2005. 105: 1930-1936.

[31] Bell, J. J. and Bhandoola, A., The earliest thymic progenitors for T cells possess myeloid lineage potential. *Nature* 2008. 452: 764-767.

[32] Wada, H., Masuda, K., Satoh, R., Kakugawa, K., Ikawa, T., Katsura, Y. and Kawamoto, H., Adult T-cell progenitors retain myeloid potential. *Nature* 2008. 452: 768-772.

[33] Katsura, Y., Redefinition of lymphoid progenitors. *Nat Rev Immunol* 2002. 2: 127-132.

[34] Ye, M. and Graf, T., Early decisions in lymphoid development. *Curr Opin Immunol* 2007. 19: 123-128.

[35] Suda, T., Suda, J. and Ogawa, M., Disparate differentiation in mouse hemopoietic colonies derived from paired progenitors. *Proc Natl Acad Sci U S A* 1984. 81: 2520-2524.

[36] Cross, M. A. and Enver, T., The lineage commitment of haemopoietic progenitor cells. *Curr Opin Genet Dev* 1997. 7: 609-613.

[37] Enver, T. and Greaves, M., Loops, lineage, and leukemia. *Cell* 1998. 94: 9-12.

[38] McAdams, H. H. and Arkin, A., Stochastic mechanisms in gene expression. *Proc Natl Acad Sci U S A* 1997. 94: 814-819.

[39] Ishikawa, F., Niiro, H., Iino, T., Yoshida, S., Saito, N., Onohara, S., Miyamoto, T., Minagawa, H., Fujii, S. I., Shultz, L. D., Harada, M. and Akashi, K., The developmental program of human dendritic cells is operated independently of conventional myeloid and lymphoid pathways. *Blood* 2007.

[40] Brown, G., Hughes, P. J., Michell, R. H., Rolink, A. G. and Ceredig, R., The sequential determination model of hematopoiesis. *Trends Immunol* 2007. 28: 442-448.

[41] Hu, M., Krause, D., Greaves, M., Sharkis, S., Dexter, M., Heyworth, C. and Enver, T., Multilineage gene expression precedes commitment in the hemopoietic system. *Genes Dev* 1997. 11: 774-785.

[42] Miyamoto, T., Iwasaki, H., Reizis, B., Ye, M., Graf, T., Weissman, I. L. and Akashi, K., Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. *Dev Cell* 2002. 3: 137-147.

[43] Ye, M., Iwasaki, H., Laiosa, C. V., Stadtfeld, M., Xie, H., Heck, S., Clausen, B., Akashi, K. and Graf, T., Hematopoietic stem cells expressing the myeloid lysozyme gene retain long-term, multilineage repopulation potential. *Immunity* 2003. 19: 689-699.

[44] Delassus, S., Titley, I. and Enver, T., Functional and molecular analysis of hematopoietic progenitors derived from the aorta-gonad-mesonephros region of the mouse embryo. *Blood* 1999. 94: 1495-1503.

[45] Lai, A. Y. and Kondo, M., Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *J Exp Med* 2006. 203: 1867-1873.

[46] Karsunky, H., Inlay, M. A., Serwold, T., Bhattacharya, D. and Weissman, I. L., Flk2+ common lymphoid progenitors possess equivalent differentiation potential for the B and T lineages. *Blood* 2008. 111: 5562-5570.

[47] Ishikawa, F., Niiro, H., Iino, T., Yoshida, S., Saito, N., Onohara, S., Miyamoto, T., Minagawa, H., Fujii, S., Shultz, L. D., Harada, M. and Akashi, K., The developmental program of human dendritic cells is operated independently of conventional myeloid and lymphoid pathways. *Blood* 2007. 110: 3591-3660.

[48] Fisher, H., *Macrophages*. Springer, Berlin, Heidelberg, New York.: 1976.

[49] Manning, M. and Turner, R., *Comparative Immunobiology*. Blackie, Glasgow, London.: 1976.

[50] Li, J., Barreda, D. R., Zhang, Y. A., Boshra, H., Gelman, A. E., Lapatra, S., Tort, L. and Sunyer, J. O., B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nat Immunol* 2006. 7: 1116-1124.

[51] Laiosa, C. V., Stadtfeld, M. and Graf, T., Determinants of lymphoid-myeloid lineage diversification. *Annu Rev Immunol* 2006. 24: 705-738.

[52] Iwasaki, H., Mizuno, S., Arinobu, Y., Ozawa, H., Mori, Y., Shigematsu, H., Takatsu, K., Tenen, D. G. and Akashi, K., The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. *Genes Dev* 2006. 20: 3010-3021.

[53] Graf, T., Differentiation plasticity of hematopoietic cells. *Blood* 2002. 99: 3089-3101.

[54] Sugiyama, D., Tanaka, M., Kitajima, K., Zheng, J., Yen, H., Murotani, T., Yamatodani, A. and Nakano, T., Differential context-dependent effects of friend of GATA-1 (FOG-1) on mast-cell development and differentiation. *Blood* 2008. 111: 1924-1932.

[55] McCormack, M. P., Hall, M. A., Schoenwaelder, S. M., Zhao, Q., Ellis, S., Prentice, J. A., Clarke, A. J., Slater, N. J., Salmon, J. M., Jackson, S. P., Jane, S. M. and Curtis, D. J., A critical role for the transcription factor Scl in platelet production during stress thrombopoiesis. *Blood* 2006. 108: 2248-2256.

[56] Anguita, E., Hughes, J., Heyworth, C., Blobel, G. A., Wood, W. G. and Higgs, D. R., Globin gene activation during haemopoiesis is driven by protein complexes nucleated by GATA-1 and GATA-2. *Embo J* 2004. 23: 2841-2852.

[57] Kulessa, H., Frampton, J. and Graf, T., GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts, and erythroblasts. *Genes Dev* 1995. 9: 1250-1262.

[58] Metcalf, D., Majewski, I., Mifsud, S., Di Rago, L. and Alexander, W. S., Clonogenic mast cell progenitors and their excess numbers in chimeric BALB/c mice with inactivated GATA-1. *Proc Natl Acad Sci U S A* 2007. 104: 18642-18647.

[59] Ghinassi, B., Sanchez, M., Martelli, F., Amabile, G., Vannucchi, A. M., Migliaccio, G., Orkin, S. H. and Migliaccio, A. R., The hypomorphic Gata1low mutation alters the proliferation/differentiation potential of the common megakaryocytic-erythroid progenitor. *Blood* 2007. 109: 1460-1471.

[60] Hamaguchi-Tsuru, E., Nobumoto, A., Hirose, N., Kataoka, S., Fujikawa-Adachi, K., Furuya, M. and Tominaga, A., Development and functional analysis of eosinophils from murine embryonic stem cells. *Br J Haematol* 2004. 124: 819-827.

[61] Hirasawa, R., Shimizu, R., Takahashi, S., Osawa, M., Takayanagi, S., Kato, Y., Onodera, M., Minegishi, N., Yamamoto, M., Fukao, K., Taniguchi, H., Nakauchi, H. and Iwama, A., Essential and instructive roles of GATA factors in eosinophil development. *J Exp Med* 2002. 195: 1379-1386.

[62] Tsang, A. P., Visvader, J. E., Turner, C. A., Fujiwara, Y., Yu, C., Weiss, M. J., Crossley, M. and Orkin, S. H., FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation. *Cell* 1997. 90: 109-119.

[63] Tsang, A. P., Fujiwara, Y., Hom, D. B. and Orkin, S. H., Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG. *Genes Dev* 1998. 12: 1176-1188.

[64] Cantor, A. B., Iwasaki, H., Arinobu, Y., Moran, T. B., Shigematsu, H., Sullivan, M. R., Akashi, K. and Orkin, S. H., Antagonism of FOG-1 and GATA factors in fate choice for the mast cell lineage. *J Exp Med* 2008. 205: 611-624.

[65] Pang, L., Weiss, M. J. and Poncz, M., Megakaryocyte biology and related disorders. *J Clin Invest* 2005. 115: 3332-3338.

[66] Pina, C., May, G., Soneji, S., Hong, D. and Enver, T., MLLT3 regulates early human erythroid and megakaryocytic cell fate. *Cell Stem Cell* 2008. 2: 264-273.

[67] Zhang, D. E., Zhang, P., Wang, N. D., Hetherington, C. J., Darlington, G. J. and Tenen, D. G., Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proc Natl Acad Sci U S A* 1997. 94: 569-574.

[68] Heath, V., Suh, H. C., Holman, M., Renn, K., Gooya, J. M., Parkin, S., Klarmann, K. D., Ortiz, M., Johnson, P. and Keller, J., C/EBPalpha deficiency results in hyperproliferation of hematopoietic progenitor cells and disrupts macrophage development in vitro and in vivo. *Blood* 2004. 104: 1639-1647.

[69] Richards, J., Le Naour, F., Hanash, S. and Beretta, L., Integrated genomic and proteomic analysis of signaling pathways in dendritic cell differentiation and maturation. *Ann N Y Acad Sci* 2002. 975: 91-100.

[70] Wang, D., D'Costa, J., Civin, C. I. and Friedman, A. D., C/EBPalpha directs monocytic commitment of primary myeloid progenitors. *Blood* 2006. 108: 1223-1229.

[71] Cai, D. H., Wang, D., Keefer, J., Yeamans, C., Hensley, K. and Friedman, A. D., C/EBP alpha:AP-1 leucine zipper heterodimers bind novel DNA elements, activate the PU.1 promoter and direct monocyte lineage commitment more potently than C/EBP alpha homodimers or AP-1. *Oncogene* 2008. 27: 2772-2779.

[72] Buitenhuis, M., Verhagen, L. P., van Deutekom, H. W., Castor, A., Verploegen, S., Koenderman, L., Jacobsen, S. E. and Coffer, P. J., Protein kinase B (C-AKT) regulates hematopoietic lineage choice decisions during myelopoiesis. *Blood* 2007.

[73] Rhodes, J., Hagen, A., Hsu, K., Deng, M., Liu, T. X., Look, A. T. and Kanki, J. P., Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *Dev Cell* 2005. 8: 97-108.

[74] Galloway, J. L., Wingert, R. A., Thisse, C., Thisse, B. and Zon, L. I., Loss of gata1 but not gata2 converts erythropoiesis to myelopoiesis in zebrafish embryos. *Dev Cell* 2005. 8: 109-116.

[75] Stopka, T., Amanatullah, D. F., Papetti, M. and Skoultchi, A. I., PU.1 inhibits the erythroid program by binding to GATA-1 on DNA and creating a repressive chromatin structure. *Embo J* 2005. 24: 3712-3723.

[76] Dahl, R., Walsh, J. C., Lancki, D., Laslo, P., Iyer, S. R., Singh, H. and Simon, M. C., Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. *Nat Immunol* 2003. 4: 1029-1036.
[77] Bharadwaj, A. S. and Agrawal, D. K., Transcription factors in the control of dendritic cell life cycle. *Immunol Res* 2007. 37: 79-96.
[78] Henkel, G. and Brown, M. A., PU.1 and GATA: components of a mast cell-specific interleukin 4 intronic enhancer. *Proc Natl Acad Sci U S A* 1994. 91: 7737-7741.
[79] DeKoter, R. P. and Singh, H., Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science* 2000. 288: 1439-1441.
[80] Yang, L., Wang, L., Kalfa, T. A., Cancelas, J. A., Shang, X., Pushkaran, S., Mo, J., Williams, D. A. and Zheng, Y., Cdc42 critically regulates the balance between myelopoiesis and erythropoiesis. *Blood* 2007. 110: 3853-3861.
[81] Li, C. Y., Zhan, Y. Q., Li, W., Xu, C. W., Xu, W. X., Yu, D. H., Peng, R. Y., Cui, Y. F., Yang, X., Hou, N., Li, Y. H., Dong, B., Sun, H. B. and Yang, X. M., Overexpression of a hematopoietic transcriptional regulator EDAG induces myelopoiesis and suppresses lymphopoiesis in transgenic mice. *Leukemia* 2007. 21: 2277-2286.
[82] Rothenberg, E. V., Regulatory factors for initial T lymphocyte lineage specification. *Curr Opin Hematol* 2007. 14: 322-329.
[83] Rothenberg, E. V., Moore, J. E. and Yui, M. A., Launching the T-cell-lineage developmental programme. *Nat Rev Immunol* 2008. 8: 9-21.
[84] Franco, C. B., Scripture-Adams, D. D., Proekt, I., Taghon, T., Weiss, A. H., Yui, M. A., Adams, S. L., Diamond, R. A. and Rothenberg, E. V., Notch/Delta signaling constrains reengineering of pro-T cells by PU.1. *Proc Natl Acad Sci U S A* 2006. 103: 11993-11998.
[85] Dionne, C. J., Tse, K. Y., Weiss, A. H., Franco, C. B., Wiest, D. L., Anderson, M. K. and Rothenberg, E. V., Subversion of T lineage commitment by PU.1 in a clonal cell line system. *Dev Biol* 2005. 280: 448-466.
[86] Mukai, H. Y., Motohashi, H., Ohneda, O., Suzuki, N., Nagano, M. and Yamamoto, M., Transgene insertion in proximity to the c-myb gene disrupts erythroid-megakaryocytic lineage bifurcation. *Mol Cell Biol* 2006. 26: 7953-7965.
[87] Emambokus, N., Vegiopoulos, A., Harman, B., Jenkinson, E., Anderson, G. and Frampton, J., Progression through key stages of haemopoiesis is dependent on distinct threshold levels of c-Myb. *Embo J* 2003. 22: 4478-4488.
[88] Sandberg, M. L., Sutton, S. E., Pletcher, M. T., Wiltshire, T., Tarantino, L. M., Hogenesch, J. B. and Cooke, M. P., c-Myb and p300 regulate hematopoietic stem cell proliferation and differentiation. *Dev Cell* 2005. 8: 153-166.
[89] Kasper, L. H., Boussouar, F., Ney, P. A., Jackson, C. W., Rehg, J., van Deursen, J. M. and Brindle, P. K., A transcription-factor-binding surface of coactivator p300 is required for haematopoiesis. *Nature* 2002. 419: 738-743.
[90] Lohmann, F. and Bieker, J. J., Activation of Eklf expression during hematopoiesis by Gata2 and Smad5 prior to erythroid commitment. *Development* 2008. 135: 2071-2082.

[91] Frontelo, P., Manwani, D., Galdass, M., Karsunky, H., Lohmann, F., Gallagher, P. G. and Bieker, J. J., Novel role for EKLF in megakaryocyte lineage commitment. *Blood* 2007. 110: 3871-3880.

[92] Eisbacher, M., Holmes, M. L., Newton, A., Hogg, P. J., Khachigian, L. M., Crossley, M. and Chong, B. H., Protein-protein interaction between Fli-1 and GATA-1 mediates synergistic expression of megakaryocyte-specific genes through cooperative DNA binding. *Mol Cell Biol* 2003. 23: 3427-3441.

[93] Siatecka, M., Xue, L. and Bieker, J. J., Sumoylation of EKLF promotes transcriptional repression and is involved in inhibition of megakaryopoiesis. *Mol Cell Biol* 2007. 27: 8547-8560.

[94] Kamath, M. B., Houston, I. B., Janovski, A. J., Zhu, X., Gowrisankar, S., Jegga, A. G. and DeKoter, R. P., Dose-dependent repression of T-cell and natural killer cell genes by PU.1 enforces myeloid and B-cell identity. *Leukemia* 2008. 22: 1214-1225.

[95] Fukuchi, Y., Shibata, F., Ito, M., Goto-Koshino, Y., Sotomaru, Y., Ito, M., Kitamura, T. and Nakajima, H., Comprehensive analysis of myeloid lineage conversion using mice expressing an inducible form of C/EBP alpha. *Embo J* 2006. 25: 3398-3410.

[96] Friedman, A. D., Transcriptional control of granulocyte and monocyte development. *Oncogene* 2007. 26: 6816-6828.

[97] Hsu, M. C., Toellner, K. M., Vinuesa, C. G. and Maclennan, I. C., B cell clones that sustain long-term plasmablast growth in T-independent extrafollicular antibody responses. *Proc Natl Acad Sci U S A* 2006. 103: 5905-5910.

[98] Pongubala, J. M., Northrup, D. L., Lancki, D. W., Medina, K. L., Treiber, T., Bertolino, E., Thomas, M., Grosschedl, R., Allman, D. and Singh, H., Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5. *Nat Immunol* 2008. 9: 203-215.

[99] Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H. R. and Aguet, M., Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* 1999. 10: 547-558.

[100] Lehar, S. M., Dooley, J., Farr, A. G. and Bevan, M. J., Notch ligands Delta 1 and Jagged1 transmit distinct signals to T-cell precursors. *Blood* 2005. 105: 1440-1447.

[101] Schmitt, T. M., Ciofani, M., Petrie, H. T. and Zuniga-Pflucker, J. C., Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J Exp Med* 2004. 200: 469-479.

[102] Schmitt, T. M. and Zuniga-Pflucker, J. C., Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* 2002. 17: 749-756.

[103] Carotta, S., Brady, J., Wu, L. and Nutt, S. L., Transient Notch signaling induces NK cell potential in Pax5-deficient pro-B cells. *Eur J Immunol* 2006. 36: 3294-3304.

[104] Ross, J. and Li, L., Recent advances in understanding extrinsic control of hematopoietic stem cell fate. *Curr Opin Hematol* 2006. 13: 237-242.

[105] Brandon, C., Eisenberg, L. M. and Eisenberg, C. A., WNT signaling modulates the diversification of hematopoietic cells. *Blood* 2000. 96: 4132-4141.

[106] Aoyama, K., Delaney, C., Varnum-Finney, B., Kohn, A. D., Moon, R. T. and Bernstein, I. D., The interaction of the Wnt and Notch pathways modulates natural killer versus T cell differentiation. *Stem Cells* 2007. 25: 2488-2497.

[107] Koch, U., Wilson, A., Cobas, M., Kemler, R., Macdonald, H. R. and Radtke, F., Simultaneous loss of beta- and gamma-catenin does not perturb hematopoiesis or lymphopoiesis. *Blood* 2008. 111: 160-164.

[108] Jeannet, G., Scheller, M., Scarpellino, L., Duboux, S., Gardiol, N., Back, J., Kuttler, F., Malanchi, I., Birchmeier, W., Leutz, A., Huelsken, J. and Held, W., Long-term, multilineage hematopoiesis occurs in the combined absence of beta-catenin and gamma-catenin. *Blood* 2008. 111: 142-149.

[109] Robb, L., Cytokine receptors and hematopoietic differentiation. *Oncogene* 2007. 26: 6715-6723.

[110] Buzzai, M. and Licht, J. D., New molecular concepts and targets in acute myeloid leukemia. *Curr Opin Hematol* 2008. 15: 82-87.

[111] Krause, D. S. and Van Etten, R. A., Right on target: eradicating leukemic stem cells. *Trends Mol Med* 2007. 13: 470-481.

[112] O'Neil, J. and Look, A. T., Mechanisms of transcription factor deregulation in lymphoid cell transformation. *Oncogene* 2007. 26: 6838-6849.

[113] Yeh, J. R., Munson, K. M., Chao, Y. L., Peterson, Q. P., Macrae, C. A. and Peterson, R. T., AML1-ETO reprograms hematopoietic cell fate by downregulating scl expression. *Development* 2008. 135: 401-410.

[114] Vangala, R. K., Heiss-Neumann, M. S., Rangatia, J. S., Singh, S. M., Schoch, C., Tenen, D. G., Hiddemann, W. and Behre, G., The myeloid master regulator transcription factor PU.1 is inactivated by AML1-ETO in t(8;21) myeloid leukemia. *Blood* 2003. 101: 270-277.

[115] Dakic, A., Wu, L. and Nutt, S. L., Is PU.1 a dosage-sensitive regulator of haemopoietic lineage commitment and leukaemogenesis? *Trends Immunol* 2007. 28: 108-114.

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Chapter 11

The Epigenetics of Haematopoiesis*

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Abstract

Epigenetics is the generation of heritable changes in gene expression without alteration of the DNA sequence. During hematopoiesis, pluripotent hematopoietic stem cells (HSCs) differentiate to produce all the blood cell types. A major goal in cell biology is to understand how the gene expression potential remains extensive but controlled in HSCs and their progeny, namely progenitor cells, yet becomes directionally restricted as differentiation occurs to produce separate blood cell lineages, defined by their individual patterns of gene expression and silencing. This chapter examines the principles underlying the epigenetic regulation of gene expression status during hematopoiesis, in the context of the three-dimensional nuclear landscape.

Introduction

The recent development of high-throughput and genome-wide methods to analyze chromatin interactions has provided us with abundant new information about the impact on gene expression wrought by changes in the spatial organization of the genome during development. The DNA of the genome is packaged as chromatin. Local changes in chromatin

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structure facilitate the control of gene expression by permitting or restricting access of transcriptional modulators to gene control elements. It is becoming increasingly apparent that topological changes in chromatin that allow distant control elements to interact are more common than previously supposed. At the local level, chromatin loop formation can allow contact between enhancers and promoters to facilitate high levels of transcription. Conversely, alternative loop structures can form that prevent such contacts, leading to transcriptional down-regulation. Loops can be remodelled during differentiation. Large-scale chromatin loop formation allows groups of genes to explore the nuclear space beyond the limits of their home chromosome territory and permits regulatory contact between different chromosomes.

Recent improvements in live cell microscopy, together with the ability to tag gene loci and proteins, have revealed the nucleus to be a highly dynamic environment. The nuclear compartmentalization of both gene loci and proteins has been found to have a significant impact on the initiation and maintenance of gene expression status. How compartmentalization is achieved and maintained within the context of high locus mobility and transitory regulator protein-chromatin interactions is a major question.

The relatively static, two-dimensional model of gene regulation is being replaced by a more dynamic model encompassing the effects on gene regulation of the three-dimensional organization of the epigenome. We are now in a position to begin to understand gene regulation in a way that moves beyond the study of individual genes to encompass a global understanding of the whole genome in its endogenous environment of nuclear space and time.

Mammalian Haematopoiesis

Mammalian haematopoiesis is the process by which HSCs give rise to all blood lineages, including T and B lymphocytes (lymphoid lineage), erythrocytes, neutrophils, basophils, eosinophils, macrophages, monocytes, megakaryocytes and platelets (myeloid lineage) [1] (Fig 1). Each cell type is characterized by a specific pattern of actively transcribed and silenced genes that is established during cellular differentiation. HSCs differentiate according to the functional needs of the organism through their response to extracellular signals, mediated by the actions of regulatory proteins that modulate gene expression by association with, and spatial organization of, genes and their control elements.

Epigenetic regulation refers to heritable changes in gene expression that occur without alteration in DNA sequence. The mechanisms of epigenetic regulation include DNA methylation [2] as well as control of the accessibility of the DNA sequence through modification of chromatin, in which genomic DNA is packaged. 'Euchromatin' refers to a less condensed, more accessible and gene-rich form of chromatin, whereas 'heterochromatin' refers to the more condensed, less accessible and gene-poor form of chromatin, present at centromeres and telomeres. The building blocks of chromatin are the nucleosomes, in turn composed of histones that can be modified. Complex, transcription status-dependent, patterns of histone modification arise by methylation, acetylation, phosphorylation, SUMOylation and ubiquitinylation of the histone tails. This ‘histone code’ can be interpreted by factors that are

able to recognize specific chromatin motifs [3] and alter gene expression profiles accordingly.

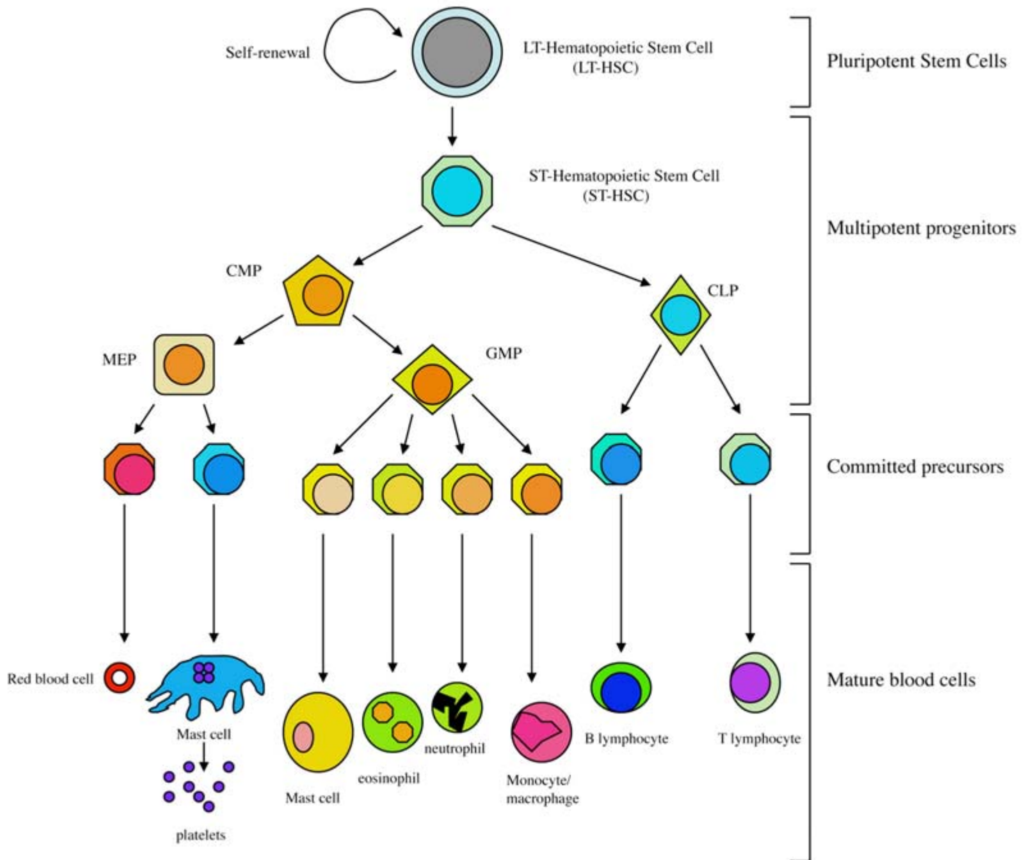


Figure 1. Schematic representation of lineage restriction during hematopoiesis.

Characteristic combinations of chromatin marks have been correlated with transcriptionally active, inactive or 'poised' chromatin states. Increasing the degree of histone acetylation of chromatin at a genetic locus, for example, influences gene expression both by increasing the accessibility of the DNA at gene control elements to *trans*-acting transcription factors [5], and by affecting the physical properties of chromatin: acetylation decreases the compactness of chromatin and increases its flexibility [6]. The relative abundance of the different combinations of chromatin marks at regulated loci can be modified during development, and correlates with changes in gene expression.

How does the gene expression potential remain extensive but controlled in HSCs and multi-potent progenitors, yet become directionally reduced as specific cell types form (i.e. alternative lineage possibilities close off) during development? Once cells are committed to terminal differentiation, further stabilization or spreading of local chromatin modifications can confer a heritable (less easily reversible) gene expression status. For example, when the mouse terminal transferase gene *Dntt* is heritably silenced during thymocyte maturation,

histone modifications characteristic of silent chromatin are nucleated at the promoter. Repositioning of the *Dntt* locus to nuclear compartments centred on pericentromeric heterochromatin correlates with the heritable silencing of the *Dntt* gene [7]. When the *Dntt* locus is repositioned to pericentromeric heterochromatin in activated thymocytes, the histone modifications characteristic of the silent state spread across the coding region of the gene. In contrast, in a transformed thymocyte cell line (VL3-3M2) where *Dntt* silencing can be initiated yet remains reversible, the locus is not repositioned within the nucleus, and although similar histone modification changes are observed at the promoter, they do not spread [8].

Committed cell types retain plasticity of gene expression potential, however, despite reaching an advanced stage of differentiation. For example, the commitment of hematopoietic progenitors to the B cell lineage and their development to mature B lymphocytes was found to depend on the transcription factor *Pax5*. Deletion of *Pax5* in mice allowed their mature B lymphocytes to dedifferentiate into early, uncommitted progenitors that are able to rescue T lymphopoiesis in T lymphocyte-deficient mice [9]. Such findings suggest that 'heritable' gene expression patterns may be actively maintained, even during the latter stages of differentiation.

Recently, the importance of higher order folding of the chromatin into activation- or repression-promoting loops, the formation of larger loops allowing loci to explore the nuclear space, together with nuclear compartmentalization of the genome and the regulatory contact between the chromatin of different chromosomes within the nucleus are becoming recognized as mechanisms of epigenetic control [10]. The linear model of gene regulation has been extended to take account of the three-dimensional organization of the epigenome. The restriction of a locus to a nuclear compartment rich in either transcriptional activators or repressors could favour the stabilization of an active or silent state. The local balance of opposing activities that promote either heterochromatin or euchromatin formation determines the epigenetic state of a genomic region [11].

Gene expression can also be controlled at the post-transcriptional level, e.g. by antisense microRNA (miRNA) silencing (targeted destruction of the RNA message). It has become apparent recently that miRNAs, small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs) are able to regulate gene expression at the transcriptional, post-transcriptional and epigenetic level [12].

This chapter focuses on the way in which regulated changes of chromatin structure and conformation at specific gene loci, together with changes in locus position within the nuclear space, contribute to the control and heritability of gene expression patterns during lineage specification, differentiation and terminal maturation of the functional cell types of the blood.

Regulation of the Globin Loci during Erythropoiesis

Erythropoiesis is the process by which red blood cells are produced. In mammals, the yolk sac and then the foetal liver are the first sites of primitive then definitive erythropoiesis [13]. In mice, erythropoiesis starts around embryonic day 8.25 (E8.25) in the yolk sac and E10 in the foetal liver. In the adult, the spleen is the major erythropoietic organ. During

definitive red blood cell maturation, cells progress through a series of stages defined on the basis of changing cellular morphology. Committed immature erythroid progenitors called 'pronormoblasts' are the first morphologically distinct erythroid precursor cells, which mature into 'basophilic', 'chromatophilic' and then 'orthochromatophilic' normoblasts. Haemoglobin accumulates in the cytoplasm as maturation progresses, the nuclear chromatin compacts and cell diameter decreases. The nucleus is eventually extruded, leaving a reticulocyte, which matures into a biconcave erythrocyte, packed full of haemoglobin (Fig 2).

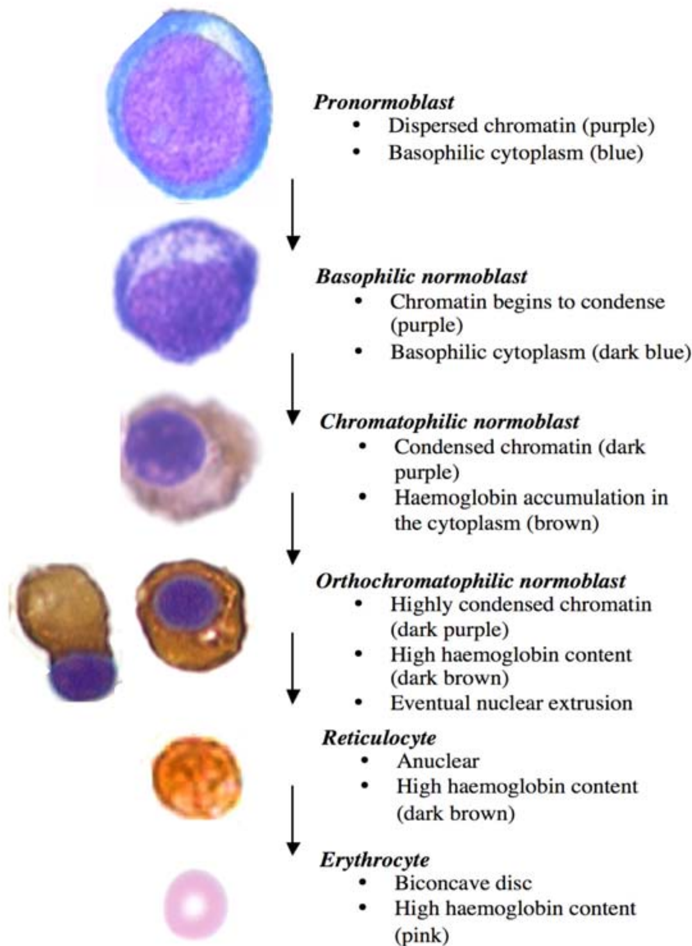


Figure 2. The progression of definitive erythroid maturation.

The successive stages of definitive erythroid maturation can be distinguished on the basis of relative nuclear size and cytoplasmic haemoglobin content. Cytospin preparations of foetal liver cultures were stained with 'Diff Quick', containing a basophilic stain to stain the cytoplasm (blue) and nuclear chromatin (purple), together with a benzidine solution, which stains haemoglobin (brown).

Despite the increasingly compact chromatin structure and down-regulation of the vast majority of genes [14], some genes are up-regulated during terminal erythroid maturation. For example the α- and β-globin genes as well as *Bcl-x*$_L$ (an anti-apoptotic protein that maintains the viability of mature definitive erythroid cells) are up-regulated after the induction of maturation [15]. The co-ordinated expression of the erythroid-specific globin genes ensures that the α- and β-globin proteins are produced in balanced amounts at the right developmental stage. The analysis of α- and β-globin gene expression in transgenic mice, primary erythroid cells and cell lines, and cells derived from thalassemia patients has established many of the principles underlying the regulation of mammalian gene expression [16-18].

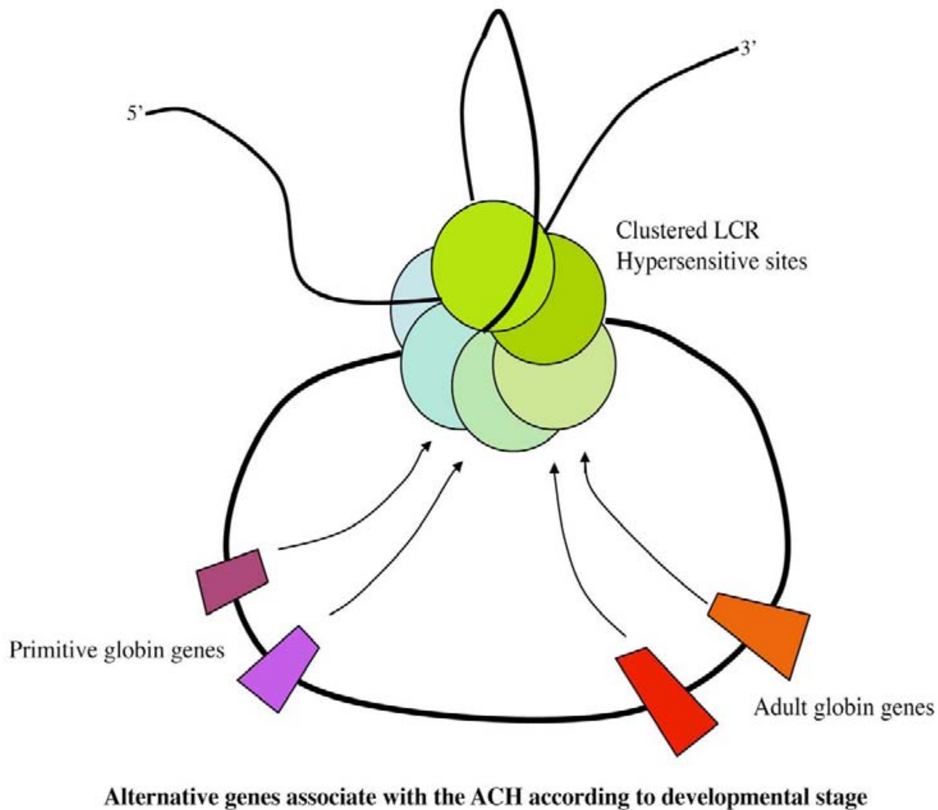
In both humans and mice, the β-globin locus consists of a set of β-like globin genes arranged in the order of their expression during development, together with a locus control region (LCR) containing five DNAse 1 hypersensitive sites spread over 20-30kb, located 10-60 kb upstream of the genes. The LCR contains binding sites for ubiquitous and tissue-specific transcription factors and is required for the correct level of gene expression, but not for chromatin opening activity [19]. The tissue-specific expression of embryonic, foetal and adult globin genes is developmentally regulated and there are two stage-specific switches of expression: firstly, from embryonic globin to foetal globin expression and secondly, from foetal to adult globin expression. The LCR is acetylated to the same extent in both foetal and adult erythroid cells, though the degree of acetylation of the coding regions correlates with expression status as it changes during development [20].

Several studies have shown a correlation between the silent state of a gene and its proximity to pericentromeric heterochromatin [21, 22]. Suppression of β-globin transgene silencing and maintenance of its open chromatin conformation were found to require a functional enhancer and separation from the pericentromeric heterochromatin [23]. It is likely that a multi-step process is involved in the initiation of β-globin expression: localization away from pericentromeric DNA to achieve general locus acetylation and open chromatin structure, followed by LCR and promoter hyperacetylation [24] to permit association of transcription factors. It has been established that β-globin expression requires chromatin looping between the β-globin LCR and the promoter of the particular globin gene expressed at a given time. The structure formed has been termed the Active Chromatin Hub (ACH) [25]. An analogous structure forms at the α-globin locus, involving the α-globin genes and the LCR-like (HS-containing) upstream regulatory region, despite the very different local chromatin environments of the α- and β-globin loci [26]. Recently, the formation of such structures has been found to underpin the developmental control of gene expression at many loci, and can be considered to represent a paradigm of epigenetic control.

Chromatin Loops Allow Interactions between Widely Spaced Regulatory Elements

The clustering of interacting regulatory elements separated by large genomic distances creates chromatin loops [10]. Looping allows positive or negative regulatory communication between widely spaced sites in the genome, by bringing co-operating elements into

proximity, or by topologically separating them. The ability of distant enhancer elements to interact with promoter regions has been recognized for some time [27]. The development of techniques able to probe chromatin interaction across the genome, such as Chromosome Conformation Capture (3C) [28], RNA Trap [25] and 4C [29], has provided new tools with which to probe interactions between chromatin regions [30].



After Palstra et al., 2003.

Figure 3. Schematic representation of the Active Chromatin Hub (ACH).

Erythroid-specific clustering of the hypersensitive sites of the β-globin LCR and the concomitant clustering of binding sites for transcription factors and associated chromatin modifiers provides a microenvironment favouring efficient globin transcription. Active globin genes associate with the ACH when transcribed according to developmental stage (indicated by arrows), with one gene being transcribed at any given time whilst the others are topologically excluded.

Loop formation can augment transcription by establishing contact between enhancers and promoters, possibly increasing local transcription factor concentration. Loops form between the upstream enhancers and the active promoter within a locus, such that non-transcribed genes within the locus are topologically excluded from interaction with the enhancer [28]. Loci retain a linear conformation in cells that do not express any gene of the locus, such that no chromatin contacts can be detected between the upstream regulatory regions and downstream promoters. Enhancer-promoter contacts facilitate efficient loading of polymerase

onto the promoter within a looped structure, to allow the transition from basal to high-level expression of the activated β-globin gene, for example [31].

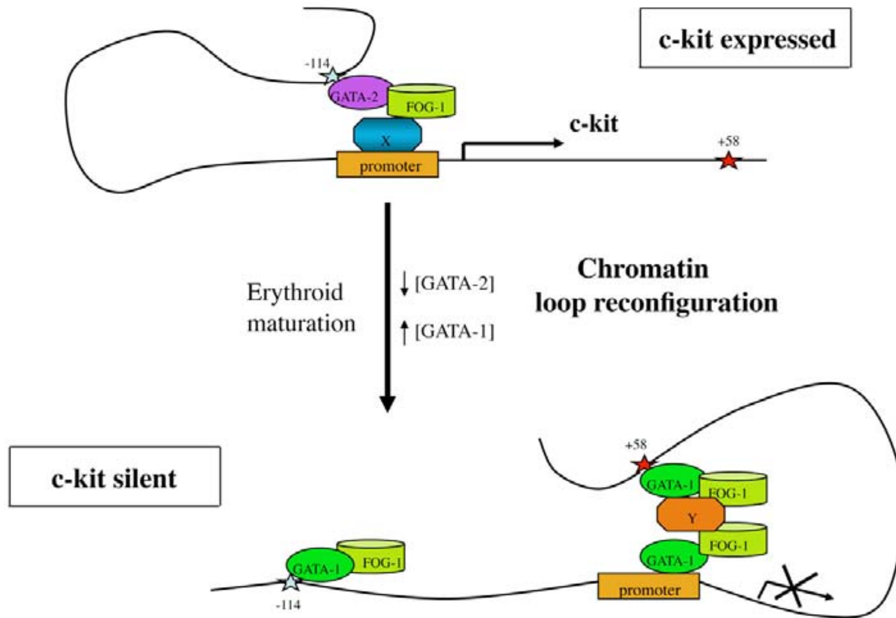
Looped chromatin structures can be stabilized by the recruitment of protein complexes. GATA-1 is a hematopoietic transcription factor expressed in erythroid, megakaryocyte, eosinophil and mast cell precursors. It is required for erythroid differentiation, the expression of erythroid-specific genes, and establishes an open chromatin structure at the β-globin locus. Contact between distant regulatory elements of the β-globin locus requires GATA-1 and its associated factor Friend of GATA-1 (FOG-1), for example [32]. The introduction of GATA-1 into cells that do not normally express it resulted in chromatin remodelling at the β-globin locus, along with transcription of erythroid-specific genes [33].

Chromatin loops may be additionally stabilized through attachment to structural components of the nucleus. Loop ends may be tethered to the nuclear matrix [34] or perhaps to transcription and replication sites [35, 36], an energetically favourable situation [37]. The way in which particular looped chromatin conformations are formed, maintained or reconfigured during cellular differentiation is currently an active area of research.

Loop Reconfiguration during Development Correlates with Altered Gene Expression Status

Chromatin interactions are highly dynamic, and loops can be reconfigured to permit changes in gene expression during development. Research is underway to elucidate the molecular mechanisms by which hematopoietic transcriptional regulators function co-operatively or antagonistically according to cellular context and the temporal progression of development. Many transcription factors have been found to share a limited number of broadly expressed co-regulators, yet show the ability to distinguish between and selectively bind to a small sub-set of consensus sequences amongst the many scattered throughout the genome in a cell-type and developmental stage-specific way [38].

The concentration of regulator proteins can change during development, to favour their inclusion in or exclusion from regulatory complexes. For example, when the *Kit* gene is expressed early in erythroid development, a distal enhancer bound by the transcription factor GATA-2 is in physical proximity to the active *Kit* promoter. When *Kit* expression is down regulated upon erythroid maturation, GATA-1 concentration increases and GATA-1 displaces GATA-2 to trigger the loss of the enhancer-promoter interaction, with a reciprocal increase in spatial proximity of distinct downstream GATA elements. This indicates that the GATA family is involved in the formation of chromatin loops during both gene activation and repression [39]. Loop reconfiguration was not found to be accompanied by changes in overall chromatin acetylation, excluding the role of changed chromatin flexibility in this case [40].



After Jing et al., 2008.

Figure 4. The reconfiguration of chromatin looping at the Kit locus during erythroid maturation.

In immature erythroid cells, GATA-2 binds to the enhancer at -114 to form an activating chromatin loop, and c-kit is transcribed. Upon maturation, the concentration of GATA-1 increases and it replaces GATA-2 at the enhancer, increasing the interaction of downstream elements at the expense of the enhancer-promoter interaction, perhaps contributing to the loss of c-kit expression.

Some functions of the GATA family members in the control of hematopoiesis are unique and others are redundant. The differential stability of the alternative members of the GATA family may be important in determining relative chromatin site occupancy, and therefore the establishment of the networks controlling hematopoiesis [41]. The co-factor FOG-1 is required for repressive chromatin loop formation, indicating that the GATA-induced changes in chromatin conformation are not simply the result of transcriptional inhibition during erythroid maturation [39].

Regulatory complex composition can change in accordance with changes in the chromatin status of the regulated locus during gene activation. For example, MafK-NF-E2p18 has a tandem binding site in the 5'HS2 of the β-globin LCR. MafK-NF-E2p18 switches from a repressive to an activating mode during erythroid maturation. When it exchanges the dimerization partner Bach1 for NF-E2p45, β-globin expression is activated in Murine ErythroLeukemia (MEL) cells. This shift occurs when the β-globin locus is poised for expression (i.e. when the chromatin structure of the locus is 'open') and is associated with changes in MafK's association with other co-activators [42].

It is likely that other, as yet unidentified, factors are involved in balancing the probability of forming particular activation or repression-promoting loops at particular loci or nuclear locations, to allow dynamic regulation of changes in gene expression. Such factors may also

be required for the recruitment of activator or repressor complexes and to co-operatively maintain a transcribed or silent state. Erythroid Krüppel-Like Factor (EKLF) associates with GATA-1 and FOG-1 at the active β-globin locus on formation of the β-globin Active Chromatin Hub (ACH), indicating that several interacting factors are necessary, though perhaps not sufficient for loop formation[43].

The way in which chromatin context at particular enhancers confers regulatory protein complex selectivity to promote the formation of a particular loop conformation or to inhibit the formation of an alternative chromatin arrangement remains an open question. The higher-order organization of chromatin loops with respect to each other, and the way in which loops may be recruited to and anchored at particular nuclear compartments in a way that might promote efficiency of co-regulation of tissue-specific genes is another current area of interest.

Association of co-expressed loci with the same transcription factory [44] might be a way in which control elements can be brought into the presence of high concentrations of loop-stabilizing factors confined to transcription-promoting nuclear compartments. In foetal liver cells, for example, where there is α1- and α2- but not ξ-globin expression, only the active genes co-localize with the expressed neighbouring housekeeping genes, and their promoters are enriched with Pol II. In contrast, in cells that do not express α-globins, the α-globin genes do not associate with the expressed housekeeping genes [26]. Much remains to be discovered about the parameters governing chromatin loop formation. Since entire chromatin domains cannot be reconstituted *in vitro*, it is vital to dissect the underlying mechanisms at endogenous loci using the range of new molecular and genetic tools that are becoming available.

Insulation Loops Cluster and Inhibit Gene Expression

Insulation is a major mechanism for the epigenetic control of gene expression. Insulators are DNA elements that prevent inappropriate interactions between adjacent chromatin domains. In an analogous way to the formation of transcription-enhancing chromatin loops, insulator elements are able to cluster and inhibit gene expression by isolating enhancer and promoter elements in spatially distinct chromatin domains or regions of the nucleus. 'Enhancer blockers', active only when located between an enhancer and the promoter it affects, isolate enhancers from promoters by establishing separate chromatin domains. 'Barriers' prevent the spread of heterochromatin, permitting active genes to be surrounded by constitutively silent chromatin structures [45]. Enhancer-blocking insulator sequences can interact with each other or tether the chromatin fibre to structural domains within the nucleus, isolating the enhancer and promoter sequences on separate chromatin loops. Barrier insulators may generate comparable structures.

The cHS4 element [46] is located at the 5' end of the chicken β-globin locus, and combines enhancer-blocking and barrier activities. The enhancer-blocking activity of this element correlates with binding of the ubiquitously expressed zinc finger protein CTCF. The ubiquitously expressed CTCF has multiple context-dependent functions including acting as a transcriptional activator, repressor and an enhancer blocker. It possesses 11 zinc-fingers,

permitting dimerization and interaction with DNA and a range of other proteins. CTCF molecules can cluster together to generate closed loop domains [47], implying that CTCF may function through topological organization of the genome [48]. It is also involved in the generation of chromatin loops at the β-globin locus [49].

It has been proposed that CTCF could tether the chromatin fibre to the nucleolar surface by interacting with nucleophosmin, such that the CTCF molecules don't interact with each other but instead create open loop domains to separate enhancers and promoters from each other. The finding that cHS4 transgenes localize to the nucleolar surface in a CTCF-dependent manner provides support for this model [47].

Barrier activity may be linked to the ability of barrier elements to target a region of chromatin to a specific nuclear compartment that is refractory to the spreading of heterochromatin [50]. Barrier activity has been linked to tethering to the nuclear pore, an area unfavourable to heterochromatin-mediated silencing in yeast since it is enriched in transcriptional activators that favour euchromatin formation [51]. The mechanisms by which enhancer-blocking and barriers act are thought likely to be evolutionarily conserved [46].

The barrier insulation activity of cHS4 is not CTCF-dependent [52], but is rather thought to depend on the ability of upstream transcription factor 1 and 2 (USF1 and USF2) sequence-specific DNA binding proteins [53]. Mutations in cHS4 which abolish USF1 and USF2 binding eliminate both HAT recruitment and barrier activity, suggesting that cHS4-mediated acetylation and H3K4 methylation of the local nucleosomes makes them resistant to H3K9 trimethylation, H3K9 trimethyl-dependent HP1 binding and hence the spread of heterochromatin.

Imprinting Loops are Able to Control Allelic Exclusion

The versatile CTCF protein has a key role in imprinting (i.e. the expression of a single allele from either the maternal or paternal chromosome) in B lymphocytes. Alternate interactions between the H19 imprinting control region (ICR) and one of the two Igf2 differentially methylated regions (DMR) has been proposed as an 'imprint switch' model of regulation of the reciprocal imprinting of Igf2 and H19. A scanning chromosome conformation capture (3C) method was used to reveal the existence of a complex 'knotted loop' on the maternal chromosome, where Igf2 is silent, keeping the Igf2 enhancers away from the Igf2 promoters but allowing them to interact with the H19 promoter, permitting H19 expression from the maternal chromosome. On the paternal chromosome, from which Igf2 is expressed, this loop is absent and so the enhancers and promoters are able to interact. The looping structure at the maternal allele is formed by interactions involving DMR1, the ICR and enhancers. Binding of the transcription factor CTCF to the maternal (unmethylated) ICR in conjunction with the presence of multi-complex components, including inter-chromosomal interactions, create a barrier blocking access of all enhancers to Igf2, thereby silencing the maternal Igf2 allele. This configuration exists in newborn liver, murine embryonic fibroblasts (MEF) and embryonic stem (ES) cells, persists through mitosis and, therefore, confers epigenetic memory [54].

It is of interest that cohesins are found at most CTCF sites. Cohesins appear to regulate gene expression and promoter-enhancer interactions, and may be recruited by CTCF to a subset of DNAse 1 hypersensitive sites. CTCF appears to be required to enrich cohesins at particular binding sites, where their role is separate from the well-characterized function of cohesion [55]. Since CTCF binding is sensitive to DNA methylation status, cohesin positioning may integrate DNA sequence and epigenetic state [56].

CTCF binding sites are often found adjacent to the binding sites of other regulatory factors, and so CTCF action may be highly context-dependent. Its function can be affected by modifications such as ADP-ribosylation. The diversity of CTCF function and mechanisms of action are only beginning to be appreciated.

Large Chromatin Loops

Chromosomes occupy reasonably distinct territories within the nucleus [57]. Whole gene clusters, several mega bases (Mb) in size, have been found to loop out of their home chromosome territories on activation [58]. The correlation between chromatin decondensation and relocation outside a chromosome territory is not absolute. The *Hoxd* locus, for example, is active and visibly decondensed whilst remaining within its chromosome territory in embryonic nuclei [59]. Although not all transcribed genes form part of giant chromatin loops, they may be more common that previously anticipated. Chromosome territory intermingling has been found to be more extensive than expected [60], and the formation of giant chromatin loops may contribute to this.

The Nuclear Compartmentalization of the Genome

The regulated localization of genes within the nuclear space is increasingly thought to be a key contributor to the epigenetic regulation of genome function. The functional consequence of relocating genes within the nucleus relative to nuclear landmarks is under investigation. Nuclear compartmentalization of activating or silencing factors at specific ‘nuclear addresses’ may facilitate programmed epigenetic changes at loci following their recruitment to such compartments, through the spatio-temporal concentration of activating or silencing factors (or complex components) in the functionally distinct compartments, driving the equilibrium of factor association with the recruited locus in favour of either transcription or repression.

Under the microscope, the nucleus appears to be partitioned into areas of heterochromatin and euchromatin, nucleoli and concentrated areas (or speckles) rich in particular proteins. Transcription 'factories', for example, are rich in RNA polymerases and other proteins required for transcription [61]. Estimation of the number of nascent transcripts, active polymerases and sites of transcription within a typical nucleus implies that each transcription ‘factory’ may contain an average of 30 active polymerases with their associated transcripts [62]. Different genes have been found to share the same transcription factory [63]. Genes have been shown to congregate at shared transcription factories (or transcriptional

'industrial estates'), which may contribute to the co-ordinated regulation of tissue-specific genes in *cis* [44] or *trans* [64, 65]. The murine α-globin genes α-1 and α-2, for example, participate in an active chromatin loop involving their upstream regulatory elements in erythroid cells (where they are expressed) and this loop shares a transcription factory with four neighbouring housekeeping genes. The highly expressed housekeeping genes remain clustered in brain, but the silent α-globin genes are excluded from the transcription factory [26].

Gene loci can move within the nucleus, and the principles governing their movements and inter-chromosomal interactions are currently under intensive study [66]. Although genes may relocate according to their own activation status, adjacent genes may also be relocated with them, perhaps resulting in selective pressure to maintain clusters of broadly expressed genes in proximity on particular chromosomes. The way in which spatially separate genes travel to 'network' at the same nuclear compartments is an area of ongoing investigation.

Genome-wide bioinformatic analysis of mammalian gene expression profiles (transcriptosome mapping) together with mapping of gene position on the chromosomes have confirmed that genes are non-randomly distributed in the genome. Highly expressed housekeeping genes, for example, are often clustered within specific chromosomal regions (RIDGES) [67]. When the linear arrangement of genes together with chromosome organization were analyzed during erythroid development, co-regulated genes were found to be proximal and lineage-specific gene domains were found to be juxtaposed spatially [68].

Activated genes may travel within the nuclear environment in a stochastic way, with increased mobility when the chromatin is in an open, flexible state [69]. Alternatively, directed motion may occur through interaction with motor proteins and actin filaments [70]. It has been proposed that the spatial associations of active genes would nevertheless be rare events, such that cells in which they have been observed have in fact been selected for preferential survival [71].

Intra-chromosomal interactions between loci have been found, and may possess important biological functions. Control regions on one chromosome can influence gene expression of a locus on a different chromosome [72, 73]. The interaction of co-regulated genes from different chromosomes with the same (possibly specialized) transcription factory may increase the efficiency of co-ordinated regulation of gene expression [65]. These findings have contributed to the replacement of the traditional linear model of gene control with a three dimensional alternative model.

Nuclear Localization: A Cause or Consequence of Transcriptional Status?

Until recently, it has remained unclear whether genes move to functional nuclear compartments as a cause or a consequence of their state of activation [74]. It is, therefore, essential to show that co-localization of factors in nuclear compartments has significant functional consequences. Recent support for this notion is provided by studies showing that inducible tethering of tagged loci to the periphery of mammalian nuclei correlates with decreased (though not entirely extinguished) gene expression [75], and that passage though

mitosis is required for re-localization to occur [76]. Repression of the tagged loci was found to spread to genes tens of Mb away, indicating that the periphery is an area of decreased transcriptional efficiency. The down-regulation was found to be reversible on release of the tagged locus from the periphery, indicating that heritable changes in gene expression (and, therefore, heritable modifications in the chromatin at the locus) had not occurred [77]. The nuclear periphery is not considered to be a homogenous silent 'compartment' since active and silent regions are known to co-exist there [78]. For example, expression of the β-globin locus is initiated at the nuclear periphery prior to its movement to a more central nuclear position during erythroid maturation, a process that requires the LCR [79].

Repositioning a locus may exclude it from undergoing a process confined to a specific nuclear location or compartment. During B lymphocyte development, for example, the 3Mb Igκ locus undergoes monoallelic recombination. Prior to induction, both Igκ alleles are in a structurally contracted state, Vκ genes are transcribed at the basal level and hence both alleles appear poised for recombination. Upon induction, only one Igκ allele is relocated to a nuclear compartment centred on pericentromeric heterochromatin, and recombination within the non-centromeric allele follows [80].

Regulatory complexes with common components have activating roles at some genes, whilst having repressive roles at others in the same nucleus at the same developmental stage. Compartmentalization of the different factors and/or their target loci may represent a way in which factor composition at a locus is controlled during development. The structurally similar GATA family members are important regulators of hematopoiesis. GATA-1 promotes erythroid, megakaryocyte and mast cell development, GATA-2 is required for the function of multipotent hematopoietic precursors and GATA-3 regulates lymphopoiesis. GATA proteins are able to bind to a range of different regulatory proteins and form complexes able to recruit other activators, repressors and chromatin-modifying factors to their target loci. GATA-1 can recruit FOG-1 and NuRD to repress some target loci, or can interact with the activating transcription factors Sp1, EKLF or PU.1, perhaps reflecting unique requirements for the regulation of distinct loci. GATA factor levels change dynamically during erythropoiesis [81]. GATA-1 targets differ in their sensitivity to changes in GATA-1 levels and/or activity [82]. In addition, GATA-1 can activate or repress target genes in a FOG-1 dependent or independent manner [83]. The development of ChIP-on-chip has recently increased the factor binding data available for the elucidation of the rules governing protein complex binding [84]. The control of target site sensitivity to the presence of altered transcription factor concentrations is an active area of investigation.

Chromatin Mobility within the Nucleus

Advances in live cell microscopy, such as tracking of fluorescently-tagged proteins at unique chromatin sites [85], have allowed chromatin motion to be tracked *in vivo*. Chromatin in mammalian cells has been revealed to be highly mobile at the local level, but chromosomes appear to be relatively static at the global level during the cell cycle. Both degrees of motion may be functionally significant. As a gene becomes active, its chromatin is remodelled to be more accessible to transcription factors as well as more flexible. These

changes may also have the effect of making the locus more mobile and able to move in a stochastic manner, allowing it to randomly find a nuclear compartment that corresponds to and supports its transcriptional status, such as a transcription factory, where its rate of transcription can be enhanced [69].

Photo bleaching experiments involving fluorescently-tagged proteins have revealed that proteins are highly mobile within the nucleus [86]. The heterochromatin-associated protein HP1 is a key component of condensed chromatin and is involved in creating a compact heterochromatic structure at many inactive genes [87]. Fluorescence recovery after photobleaching (FRAP) revealed HP1 to be much more mobile in both the euchromatin and heterochromatin of resting murine T lymphocytes than previously imagined [88]. Transcription factors are also highly mobile, with typical binding times on chromatin of only a few seconds [89]. The binding of the key hematopoietic transcription factor NF-κB to its cognate sites is highly transient, for example [90]. The free proteins are highly mobile within the nuclear space, and can access nuclear compartments with ease [91].

The retention of a specific gene locus (with consensus binding sites within its promoter for both activating and repressing factors) within a particular nuclear compartment may depend on competition between activating and repressing factors, whose concentrations change during development. The promoter of the lymphocyte specific gene terminal deoxytransferase (TdT), for example, contains overlapping binding sites for Ikaros (a repressor of TdT expression, enriched at pericentromeric heterochromatin) and an Ets transcriptional activator. Binding and competition experiments show that Ikaros and Ets compete for TdT promoter occupancy. When TdT is down-regulated, it is associated with heterochromatic foci, where Ikaros is abundant, favouring binding of Ikaros to the promoter to maintain the silent state. Increasing the concentration of Ets activator could shift the equilibrium, displacing Ikaros in favour of Ets and perhaps relocating TdT away from the heterochromatic domain to a nuclear location favouring TdT transcription [92].

A number of nuclear bodies rich in different factors have been identified [93] and many of these are highly dynamic, changing nuclear location and their extent of interaction with chromatin throughout the cell cycle [94]. RUNX1 is an essential factor for tissue-specific gene expression during hematopoiesis. It is located at punctate foci in the nucleus that are involved in transcriptional control and association with the nuclear scaffold. Removal of the C-terminal localization signal results in mis-localization of RUNX proteins and defective hematopoiesis, implying that the dynamic association of RUNX proteins at fixed nuclear foci provides a mechanism for formation of localized regulatory complexes essential for RUNX-dependent differentiation [95].

The post-translational modification of mRNAs and proteins within specific nuclear compartments can change their localization and hence modify the structure and function of the compartments [96]. Some nuclear bodies may represent storage areas for inactive factors, though others are thought to be functionally active. The impact of their large-scale movement on gene regulation within the nuclear environment continues to be investigated.

Chromosome territories have been found to maintain a distribution within the nucleus according to chromosome size and/or gene density [97]. Different relative distributions of chromosome territories have been reported to be lineage- or differentiation stage-specific. The mouse chromosome 6 territory (home of the differentially expressed T lymphocyte

markers CD4 and CD8) in immature $CD4^{+ve}CD8^{+ve}$ thymocytes was found to undergo differential changes in nuclear position depending on whether the cells differentiated into $CD4^{+ve}$ or $CD8^{+ve}$ single positive cells [98]. Since the size and gene-richness of the territory is similar before and after differentiation, such reproducible differences in the distribution of chromosome territories may have functional significance.

Recent technical developments include high-throughput techniques for the analysis of contacts between distinct regions of the genome, more sensitive proteomics (mass spectrometry) together with the development of sensitive microscopy in the 2-200 nm range and improvements in modelling and bioinformatics. These techniques are generating vast mounts of data that should greatly increase our understanding of the dynamics of genome organization [99].

Non-Coding RNAs are Epigenetic Regulators of Gene Expression

Most transcription occurs from intergenic regions of the genome in mammals. Transcriptosome studies have revealed a plethora of non-coding RNAs in mammals, some of which are involved in pathways that ultimately act on genome architecture and gene expression. Long non-coding RNAs (nc-RNAs) are able to repress transcription in *cis* using mechanisms that may involve sequestration into nuclear sub-compartments and/or through association with or recruitment of repressive complexes to chromatin [100]. The ability of anti-sense nc-RNA to mediate transcriptional silencing by targeting a gene to the perinucleolar region, for example, shows that anti-sense RNA may mediate epigenetic control through restricting genes to regions of the nucleus rich in heterochromatin-forming machinery [101].

The Three-Dimensional Organization of the Genome

If the epigenetic regulation of gene expression and silencing is dependent on the topological organization of the genome, organizational bridging factors must exist. Some may be expressed ubiquitously, whilst others are likely to be tissue-specific. There are a number of candidate molecules. The ubiquitously expressed factor CTCF is able to dimerize, and has 14,000-15,000 binding sites in the human genome. Knockdown of CTCF doesn't result in global changes in gene expression [102], however, and so other candidates must be considered. SATB1, for example, has been implicated in the regulation of thymocyte architecture, since it is able to direct long-range interactions within the T helper 2 cytokine locus [103, 104]. Other candidates include cohesins, which co-localize with CTCF [55, 56], the methyltransferase MeCP2 [105] and MENT [106]. Disruption of the function of key bridging factors is likely to affect visible nuclear architecture together with regulated gene expression and silencing and hence, the progression of differentiation. Identification of such factors is a new goal in cell biology.

Epigenetics and Disease: Epigenetic Drugs

Malignancy is often characterized by translocations resulting in fusion proteins that can lead to the deployment of transcription factors in a spatio-temporally inappropriate manner. This in turn can lead to the aberrant recruitment of chromatin modifying proteins (HATs as well as modifiers) and resultant inappropriate epigenetic effects on gene expression [107]. One effect of the resultant global shift in gene expression pattern is increased self-renewal of the malignant cells rather than their differentiation.

The essential role of chromatin modifying enzymes during blood development is highlighted by the finding that members from all families of chromatin regulators are deregulated in many haematological malignancies, leading to both local and global modification of the histone code in malignant cells. Leukemias are often characterised by expansion of progenitor cells that are unable to mature correctly, which may involve epigenetic faults. Acute myeloid leukaemia (AML) provides a classic example. Here, the most common genetic fault involves a translocation creating a fusion protein AML1/ETO, which participates in a protein complex with the retinoic acid receptor α (RARα) at regulatory regions of RARβ2, a key all-*trans* retinoic acid (RA) target gene. At these sites, AML1/ETO recruits a histone deacetylase (HDAC), DNA methyltransferase (DNMT) and DNA methyl binding activities that promote a repressed chromatin conformation. siRNA against the fusion protein or administration of the DNA methylation inhibitor 5-azacytidine (5-Aza-C) can revert these epigenetic alterations and restore the RA differentiation response in AML1/ETO blasts [108]. The discovery that the aberrant histone marks can be reversed and transcriptional regulation altered by administration of drugs that target histone-modifying factors has revealed potential for the development of epigenetic medicine or 'epi-drugs' [107].

The ability to reactivate fetal γ-globin in patients with sickle cell disease using the HDAC inhibitor sodium phenylbutyrate [109] and the DNMT inhibitor 5-aza-2'-deoxycytidine (5-Aza-dC) [110] highlights the therapeutic potential of reversing epigenetic marks. Gene re-activation is thought to occur by depletion of functional DNMTs, which bind 5-Aza-dC-incorporated DNA, and the drug may also selectively degrade DNMT1 [111]. The use of combined DNMT inhibitors and HDAC inhibitors has been employed to reactivate hypermethylated tumour suppressor genes in human cancer cell lines. Clinical trials in patients with AML involving administration of 5-Aza-C followed by sodium phenylbutyrate have demonstrated an enhanced clinical response rate associated with demethylation of the cyclin-dependent kinase inhibitor $p15^{INK4b}$ and acetylation of histones H3 and H4 [112].

Modification of the global acetylation state of a cell may have many effects, since the chromatin at many genomic sites, in addition to the intended target site, may be affected by the administration of relatively non-specific modulators of HDACs, such as trichostatin A (TSA). In addition, the activity of many transcriptional regulators can be altered by their degree of acetylation, leading to non-specific side effects and unexpected toxicity. The development of more specifically targeted chromatin modifying factor inhibitors together with cell-type specific delivery systems may ameliorate these effects to some extent, but currently the use of epigenetic medicines is limited by these concerns [113].

Conclusion

The extensively characterized regulation of the erythroid-specific globin genes, once thought specialized owing to the apparently complex nature of the locus organization, has increasingly become recognized as a paradigm of controlled gene expression following increasing realization that many gene loci are controlled by distant elements brought into proximity of the regulated alleles by higher-order chromatin structural transitions, resulting in chromatin loop formation. In addition, the discovery that *trans* interactions also occur within the nuclear space, which may be involved in the co-regulation of genes housed on separate chromosomes, has widened our appreciation of the role of dynamic changes in gene location within the nucleus as a mechanistic cornerstone of epigenetics. The involvement of widely expressed transcriptional regulator proteins (such as CTCF, the GATA family and EKLF, for example) acting in a context and developmental stage-specific way, in part influenced by the local chromatin microenvironment, shows that study of hematopoiesis is extremely valuable in informing us of the fundamental principles which convey genomic information.

The recent development of high throughput, genome-wide methods for analysis of the gene expression landscape has provided us with an abundance of information, and it is becoming apparent that genetic diversity between individuals in many gene loci is higher than previously imagined, making regulation of the epigenome increasingly significant for understanding the control of gene expression and its mis-regulation in disease. We are now in a position to begin to understand gene regulation in a way that moves beyond the study of individual genes to encompass a global understanding of the whole epigenome in its endogenous environment of nuclear space and time.

References

[1] Zhu, J. and Emerson, S. G., Hematopoietic cytokines, transcription factors and lineage commitment. *Oncogene* 2002. 21: 3295-3313.

[2] Bird, A., DNA methylation patterns and epigenetic memory. *Genes Dev* 2002. 16: 6-21.

[3] Strahl, B. D. and Allis, C. D., The language of covalent histone modifications. *Nature* 2000. 403: 41-45.

[4] Gangenahalli, G. U., Gupta, P., Saluja, D., Verma, Y. K., Kishore, V., Chandra, R., Sharma, R. K. and Ravindranath, T., Stem cell fate specification: role of master regulatory switch transcription factor PU.1 in differential hematopoiesis. *Stem Cells Dev* 2005. 14: 140-152.

[5] de la Cruz, X., Lois, S., Sanchez-Molina, S. and Martinez-Balbas, M. A., Do protein motifs read the histone code? *Bioessays* 2005. 27: 164-175.

[6] Hansen, J. C., Conformational dynamics of the chromatin fiber in solution: determinants, mechanisms, and functions. *Annu Rev Biophys Biomol Struct* 2002. 31: 361-392.

[7] Brown, K. E., Baxter, J., Graf, D., Merkenschlager, M. and Fisher, A. G., Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. *Mol Cell* 1999. 3: 207-217.

[8] Su, R. C., Brown, K. E., Saaber, S., Fisher, A. G., Merkenschlager, M. and Smale, S. T., Dynamic assembly of silent chromatin during thymocyte maturation. *Nat Genet* 2004. 36: 502-506.

[9] Cobaleda, C., Jochum, W. and Busslinger, M., Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors. *Nature* 2007. 449: 473-477.

[10] Fraser, P., Transcriptional control thrown for a loop. *Curr Opin Genet Dev* 2006. 16: 490-495.

[11] Kimura, A. and Horikoshi, M., Partition of distinct chromosomal regions: negotiable border and fixed border. *Genes Cells* 2004. 9: 499-508.

[12] Ambros, V. and Chen, X., The regulation of genes and genomes by small RNAs. *Development* 2007. 134: 1635-1641.

[13] McGrath, K. and Palis, J., Ontogeny of erythropoiesis in the mammalian embryo. *Curr Top Dev Biol* 2008. 82: 1-22.

[14] Bruno, L., Hoffmann, R., McBlane, F., Brown, J., Gupta, R., Joshi, C., Pearson, S., Seidl, T., Heyworth, C. and Enver, T., Molecular signatures of self-renewal, differentiation, and lineage choice in multipotential hemopoietic progenitor cells in vitro. *Mol Cell Biol* 2004. 24: 741-756.

[15] Gregory, T., Yu, C., Ma, A., Orkin, S. H., Blobel, G. A. and Weiss, M. J., GATA-1 and erythropoietin cooperate to promote erythroid cell survival by regulating bcl-xL expression. *Blood* 1999. 94: 87-96.

[16] Grosveld, F., van Assendelft, G. B., Greaves, D. R. and Kollias, G., Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell* 1987. 51: 975-985.

[17] Grosveld, F., Activation by locus control regions? *Curr Opin Genet Dev* 1999. 9: 152-157.

[18] Higgs, D. R. and Wood, W. G., Long-range regulation of alpha globin gene expression during erythropoiesis. *Curr Opin Hematol* 2008. 15: 176-183.

[19] Bender, M. A., Bulger, M., Close, J. and Groudine, M., Beta-globin gene switching and DNase I sensitivity of the endogenous beta-globin locus in mice do not require the locus control region. *Mol Cell* 2000. 5: 387-393.

[20] Yin, W., Barkess, G., Fang, X., Xiang, P., Cao, H., Stamatoyannopoulos, G. and Li, Q., Histone acetylation at the human beta-globin locus changes with developmental age. *Blood* 2007. 110: 4101-4107.

[21] Csink, A. K. and Henikoff, S., Genetic modification of heterochromatic association and nuclear organization in Drosophila. *Nature* 1996. 381: 529-531.

[22] Brown, K. E., Guest, S. S., Smale, S. T., Hahm, K., Merkenschlager, M. and Fisher, A. G., Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell* 1997. 91: 845-854.

[23] Francastel, C., Walters, M. C., Groudine, M. and Martin, D. I., A functional enhancer suppresses silencing of a transgene and prevents its localization close to centrometric heterochromatin. *Cell* 1999. 99: 259-269.

[24] Schübeler, D., Francastel, C., Cimbora, D. M., Reik, A., Martin, D. I. and Groudine, M., Nuclear localization and histone acetylation: a pathway for chromatin opening and

transcriptional activation of the human beta-globin locus. *Genes Dev* 2000. 14: 940-950.

[25] Carter, D., Chakalova, L., Osborne, C. S., Dai, Y. F. and Fraser, P., Long-range chromatin regulatory interactions in vivo. *Nat Genet* 2002. 32: 623-626.

[26] Zhou, G. L., Xin, L., Song, W., Di, L. J., Liu, G., Wu, X. S., Liu, D. P. and Liang, C. C., Active chromatin hub of the mouse alpha-globin locus forms in a transcription factory of clustered housekeeping genes. *Mol Cell Biol* 2006. 26: 5096-5105.

[27] Cullen, K. E., Kladde, M. P. and Seyfred, M. A., Interaction between transcription regulatory regions of prolactin chromatin. *Science* 1993. 261: 203-206.

[28] Tolhuis, B., Palstra, R. J., Splinter, E., Grosveld, F. and de Laat, W., Looping and interaction between hypersensitive sites in the active beta-globin locus. *Mol Cell* 2002. 10: 1453-1465.

[29] Simonis, M., Klous, P., Splinter, E., Moshkin, Y., Willemsen, R., de Wit, E., van Steensel, B. and de Laat, W., Nuclear organization of active and inactive chromatin domains uncovered by chromosome conformation capture-on-chip (4C). *Nat Genet* 2006. 38: 1348-1354.

[30] Brown, K. E., Chromatin folding and gene expression: new tools to reveal the spatial organization of genes. *Chromosome Res* 2003. 11: 423-433.

[31] Kooren, J., Palstra, R. J., Klous, P., Splinter, E., von Lindern, M., Grosveld, F. and de Laat, W., Beta-globin active chromatin Hub formation in differentiating erythroid cells and in p45 NF-E2 knock-out mice. *J Biol Chem* 2007. 282: 16544-16552.

[32] Vakoc, C. R., Letting, D. L., Gheldof, N., Sawado, T., Bender, M. A., Groudine, M., Weiss, M. J., Dekker, J. and Blobel, G. A., Proximity among distant regulatory elements at the beta-globin locus requires GATA-1 and FOG-1. *Mol Cell* 2005. 17: 453-462.

[33] Layon, M. E., Ackley, C. J., West, R. J. and Lowrey, C. H., Expression of GATA-1 in a non-hematopoietic cell line induces beta-globin locus control region chromatin structure remodeling and an erythroid pattern of gene expression. *J Mol Biol* 2007. 366: 737-744.

[34] Davie, J. R., The nuclear matrix and the regulation of chromatin organization and function. *Int Rev Cytol* 1995. 162A: 191-250.

[35] Cook, P. R., Predicting three-dimensional genome structure from transcriptional activity. *Nat Genet* 2002. 32: 347-352.

[36] Chakalova, L., Debrand, E., Mitchell, J. A., Osborne, C. S. and Fraser, P., Replication and transcription: shaping the landscape of the genome. *Nat Rev Genet* 2005. 6: 669-677.

[37] Marenduzzo, D., Micheletti, C. and Cook, P. R., Entropy-driven genome organization. *Biophys J* 2006. 90: 3712-3721.

[38] Kim, S. I. and Bresnick, E. H., Transcriptional control of erythropoiesis: emerging mechanisms and principles. *Oncogene* 2007. 26: 6777-6794.

[39] Jing, H., Vakoc, C. R., Ying, L., Mandat, S., Wang, H., Zheng, X. and Blobel, G. A., Exchange of GATA factors mediates transitions in looped chromatin organization at a developmentally regulated gene locus. *Mol Cell* 2008. 29: 232-242.

[40] Apostolou, E. and Thanos, D., Linking differential chromatin loops to transcriptional decisions. *Mol Cell* 2008. 29: 154-156.

[41] Lurie, L. J., Boyer, M. E., Grass, J. A. and Bresnick, E. H., Differential GATA factor stabilities: implications for chromatin occupancy by structurally similar transcription factors. *Biochemistry* 2008. 47: 859-869.

[42] Brand, M., Ranish, J. A., Kummer, N. T., Hamilton, J., Igarashi, K., Francastel, C., Chi, T. H., Crabtree, G. R., Aebersold, R. and Groudine, M., Dynamic changes in transcription factor complexes during erythroid differentiation revealed by quantitative proteomics. *Nat Struct Mol Biol* 2004. 11: 73-80.

[43] Vernimmen, D., De Gobbi, M., Sloane-Stanley, J. A., Wood, W. G. and Higgs, D. R., Long-range chromosomal interactions regulate the timing of the transition between poised and active gene expression. *Embo J* 2007. 26: 2041-2051.

[44] Osborne, C. S., Chakalova, L., Brown, K. E., Carter, D., Horton, A., Debrand, E., Goyenechea, B., Mitchell, J. A., Lopes, S., Reik, W. and Fraser, P., Active genes dynamically colocalize to shared sites of ongoing transcription. *Nat Genet* 2004. 36: 1065-1071.

[45] Gaszner, M. and Felsenfeld, G., Insulators: exploiting transcriptional and epigenetic mechanisms. *Nat Rev Genet* 2006. 7: 703-713.

[46] Chung, J. H., Whiteley, M. and Felsenfeld, G., A 5' element of the chicken beta-globin domain serves as an insulator in human erythroid cells and protects against position effect in Drosophila. *Cell* 1993. 74: 505-514.

[47] Yusufzai, T. M., Tagami, H., Nakatani, Y. and Felsenfeld, G., CTCF tethers an insulator to subnuclear sites, suggesting shared insulator mechanisms across species. *Mol Cell* 2004. 13: 291-298.

[48] Williams, A. and Flavell, R. A., The role of CTCF in regulating nuclear organization. *J Exp Med* 2008. 205: 747-750.

[49] Splinter, E., Heath, H., Kooren, J., Palstra, R. J., Klous, P., Grosveld, F., Galjart, N. and de Laat, W., CTCF mediates long-range chromatin looping and local histone modification in the beta-globin locus. *Genes Dev* 2006. 20: 2349-2354.

[50] Casolari, J. M., Brown, C. R., Komili, S., West, J., Hieronymus, H. and Silver, P. A., Genome-wide localization of the nuclear transport machinery couples transcriptional status and nuclear organization. *Cell* 2004. 117: 427-439.

[51] Ishii, K., Arib, G., Lin, C., Van Houwe, G. and Laemmli, U. K., Chromatin boundaries in budding yeast: the nuclear pore connection. *Cell* 2002. 109: 551-562.

[52] Recillas-Targa, F., Pikaart, M. J., Burgess-Beusse, B., Bell, A. C., Litt, M. D., West, A. G., Gaszner, M. and Felsenfeld, G., Position-effect protection and enhancer blocking by the chicken beta-globin insulator are separable activities. *Proc Natl Acad Sci U S A* 2002. 99: 6883-6888.

[53] West, A. G., Huang, S., Gaszner, M., Litt, M. D. and Felsenfeld, G., Recruitment of histone modifications by USF proteins at a vertebrate barrier element. *Mol Cell* 2004. 16: 453-463.

[54] Qiu, X., Vu, T. H., Lu, Q., Ling, J. Q., Li, T., Hou, A., Wang, S. K., Chen, H. L., Hu, J. F. and Hoffman, A. R., A complex deoxyribonucleic acid looping configuration

associated with the silencing of the maternal Igf2 allele. *Mol Endocrinol* 2008. 22: 1476-1488.

[55] Wendt, K. S., Yoshida, K., Itoh, T., Bando, M., Koch, B., Schirghuber, E., Tsutsumi, S., Nagae, G., Ishihara, K., Mishiro, T., Yahata, K., Imamoto, F., Aburatani, H., Nakao, M., Imamoto, N., Maeshima, K., Shirahige, K. and Peters, J. M., Cohesin mediates transcriptional insulation by CCCTC-binding factor. *Nature* 2008. 451: 796-801.

[56] Parelho, V., Hadjur, S., Spivakov, M., Leleu, M., Sauer, S., Gregson, H. C., Jarmuz, A., Canzonetta, C., Webster, Z., Nesterova, T., Cobb, B. S., Yokomori, K., Dillon, N., Aragon, L., Fisher, A. G. and Merkenschlager, M., Cohesins functionally associate with CTCF on mammalian chromosome arms. *Cell* 2008. 132: 422-433.

[57] Cremer, T., Cremer, M., Dietzel, S., Muller, S., Solovei, I. and Fakan, S., Chromosome territories--a functional nuclear landscape. *Curr Opin Cell Biol* 2006. 18: 307-316.

[58] Volpi, E. V., Chevret, E., Jones, T., Vatcheva, R., Williamson, J., Beck, S., Campbell, R. D., Goldsworthy, M., Powis, S. H., Ragoussis, J., Trowsdale, J. and Sheer, D., Large-scale chromatin organization of the major histocompatibility complex and other regions of human chromosome 6 and its response to interferon in interphase nuclei. *J Cell Sci* 2000. 113 (Pt 9): 1565-1576.

[59] Morey, C., Da Silva, N. R., Perry, P. and Bickmore, W. A., Nuclear reorganisation and chromatin decondensation are conserved, but distinct, mechanisms linked to Hox gene activation. *Development* 2007. 134: 909-919.

[60] Branco, M. R. and Pombo, A., Intermingling of chromosome territories in interphase suggests role in translocations and transcription-dependent associations. *PLoS Biol* 2006. 4: e138.

[61] Carter, D. R., Eskiw, C. and Cook, P. R., Transcription factories. *Biochem Soc Trans* 2008. 36: 585-589.

[62] Jackson, D. A., Iborra, F. J., Manders, E. M. and Cook, P. R., Numbers and organization of RNA polymerases, nascent transcripts, and transcription units in HeLa nuclei. *Mol Biol Cell* 1998. 9: 1523-1536.

[63] Osborne, C. S., Chakalova, L., Mitchell, J. A., Horton, A., Wood, A. L., Bolland, D. J., Corcoran, A. E. and Fraser, P., Myc dynamically and preferentially relocates to a transcription factory occupied by Igh. *PLoS Biol* 2007. 5: e192.

[64] Brown, J. M., Leach, J., Reittie, J. E., Atzberger, A., Lee-Prudhoe, J., Wood, W. G., Higgs, D. R., Iborra, F. J. and Buckle, V. J., Coregulated human globin genes are frequently in spatial proximity when active. *J Cell Biol* 2006. 172: 177-187.

[65] Spilianakis, C. G., Lalioti, M. D., Town, T., Lee, G. R. and Flavell, R. A., Interchromosomal associations between alternatively expressed loci. *Nature* 2005. 435: 637-645.

[66] Fraser, P. and Bickmore, W., Nuclear organization of the genome and the potential for gene regulation. *Nature* 2007. 447: 413-417.

[67] Caron, H., van Schaik, B., van der Mee, M., Baas, F., Riggins, G., van Sluis, P., Hermus, M. C., van Asperen, R., Boon, K., Voute, P. A., Heisterkamp, S., van Kampen, A. and Versteeg, R., The human transcriptome map: clustering of highly expressed genes in chromosomal domains. *Science* 2001. 291: 1289-1292.

[68] Kosak, S. T., Scalzo, D., Alworth, S. V., Li, F., Palmer, S., Enver, T., Lee, J. S. and Groudine, M., Coordinate gene regulation during hematopoiesis is related to genomic organization. *PLoS Biol* 2007. 5: e309.

[69] Soutoglou, E. and Misteli, T., Mobility and immobility of chromatin in transcription and genome stability. *Curr Opin Genet Dev* 2007. 17: 435-442.

[70] Nunez, E., Kwon, Y. S., Hutt, K. R., Hu, Q., Cardamone, M. D., Ohgi, K. A., Garcia-Bassets, I., Rose, D. W., Glass, C. K., Rosenfeld, M. G. and Fu, X. D., Nuclear receptor-enhanced transcription requires motor- and LSD1-dependent gene networking in interchromatin granules. *Cell* 2008. 132: 996-1010.

[71] de Laat, W. and Grosveld, F., Inter-chromosomal gene regulation in the mammalian cell nucleus. *Curr Opin Genet Dev* 2007. 17: 456-464.

[72] Spilianakis, C. G. and Flavell, R. A., Long-range intrachromosomal interactions in the T helper type 2 cytokine locus. *Nat Immunol* 2004. 5: 1017-1027.

[73] Ling, J. Q., Li, T., Hu, J. F., Vu, T. H., Chen, H. L., Qiu, X. W., Cherry, A. M. and Hoffman, A. R., CTCF mediates interchromosomal colocalization between Igf2/H19 and Wsb1/Nf1. *Science* 2006. 312: 269-272.

[74] Chakalova, L. and Fraser, P., Brushed aside and silenced. *Dev Cell* 2008. 14: 461-462.

[75] Reddy, K. L., Zullo, J. M., Bertolino, E. and Singh, H., Transcriptional repression mediated by repositioning of genes to the nuclear lamina. *Nature* 2008. 452: 243-247.

[76] Kumaran, R. I. and Spector, D. L., A genetic locus targeted to the nuclear periphery in living cells maintains its transcriptional competence. *J Cell Biol* 2008. 180: 51-65.

[77] Finlan, L. E., Sproul, D., Thomson, I., Boyle, S., Kerr, E., Perry, P., Ylstra, B., Chubb, J. R. and Bickmore, W. A., Recruitment to the nuclear periphery can alter expression of genes in human cells. *PLoS Genet* 2008. 4: e1000039.

[78] Akhtar, A. and Gasser, S. M., The nuclear envelope and transcriptional control. *Nat Rev Genet* 2007. 8: 507-517.

[79] Ragoczy, T., Bender, M. A., Telling, A., Byron, R. and Groudine, M., The locus control region is required for association of the murine beta-globin locus with engaged transcription factories during erythroid maturation. *Genes Dev* 2006. 20: 1447-1457.

[80] Fitzsimmons, S. P., Bernstein, R. M., Max, E. E., Skok, J. A. and Shapiro, M. A., Dynamic changes in accessibility, nuclear positioning, recombination, and transcription at the Ig kappa locus. *J Immunol* 2007. 179: 5264-5273.

[81] Weiss, M. J., Keller, G. and Orkin, S. H., Novel insights into erythroid development revealed through in vitro differentiation of GATA-1 embryonic stem cells. *Genes Dev* 1994. 8: 1184-1197.

[82] Kim, S. I., Bultman, S. J., Jing, H., Blobel, G. A. and Bresnick, E. H., Dissecting molecular steps in chromatin domain activation during hematopoietic differentiation. *Mol Cell Biol* 2007. 27: 4551-4565.

[83] Crispino, J. D., Lodish, M. B., MacKay, J. P. and Orkin, S. H., Use of altered specificity mutants to probe a specific protein-protein interaction in differentiation: the GATA-1:FOG complex. *Mol Cell* 1999. 3: 219-228.

[84] Huebert, D. J., Kamal, M., O'Donovan, A. and Bernstein, B. E., Genome-wide analysis of histone modifications by ChIP-on-chip. *Methods* 2006. 40: 365-369.

[85] Belmont, A., Dynamics of chromatin, proteins, and bodies within the cell nucleus. *Curr Opin Cell Biol* 2003. 15: 304-310.

[86] Misteli, T., Concepts in nuclear architecture. *Bioessays* 2005. 27: 477-487.

[87] Kwon, S. H. and Workman, J. L., The Heterochromatin Protein 1 (HP1) Family: Put Away a bias toward HP1. *Mol Cells* 2008. 26.

[88] Festenstein, R., Pagakis, S. N., Hiragami, K., Lyon, D., Verreault, A., Sekkali, B. and Kioussis, D., Modulation of heterochromatin protein 1 dynamics in primary Mammalian cells. *Science* 2003. 299: 719-721.

[89] Phair, R. D., Scaffidi, P., Elbi, C., Vecerova, J., Dey, A., Ozato, K., Brown, D. T., Hager, G., Bustin, M. and Misteli, T., Global nature of dynamic protein-chromatin interactions in vivo: three-dimensional genome scanning and dynamic interaction networks of chromatin proteins. *Mol Cell Biol* 2004. 24: 6393-6402.

[90] Bosisio, D., Marazzi, I., Agresti, A., Shimizu, N., Bianchi, M. E. and Natoli, G., A hyper-dynamic equilibrium between promoter-bound and nucleoplasmic dimers controls NF-kappaB-dependent gene activity. *Embo J* 2006. 25: 798-810.

[91] Hager, G. L., Elbi, C. and Becker, M., Protein dynamics in the nuclear compartment. *Curr Opin Genet Dev* 2002. 12: 137-141.

[92] Trinh, L. A., Ferrini, R., Cobb, B. S., Weinmann, A. S., Hahm, K., Ernst, P., Garraway, I. P., Merkenschlager, M. and Smale, S. T., Down-regulation of TDT transcription in CD4(+)CD8(+) thymocytes by Ikaros proteins in direct competition with an Ets activator. *Genes Dev* 2001. 15: 1817-1832.

[93] Spector, D. L., SnapShot: Cellular bodies. *Cell* 2006. 127: 1071.

[94] Chen, Y. C., Kappel, C., Beaudouin, J., Eils, R. and Spector, D. L., Live Cell Dynamics of Promyelocytic Leukemia Nuclear Bodies upon Entry into and Exit from Mitosis. *Mol Biol Cell* 2008. 19: 3147-3162.

[95] Harrington, K. S., Javed, A., Drissi, H., McNeil, S., Lian, J. B., Stein, J. L., Van Wijnen, A. J., Wang, Y. L. and Stein, G. S., Transcription factors RUNX1/AML1 and RUNX2/Cbfa1 dynamically associate with stationary subnuclear domains. *J Cell Sci* 2002. 115: 4167-4176.

[96] Handwerger, K. E. and Gall, J. G., Subnuclear organelles: new insights into form and function. *Trends Cell Biol* 2006. 16: 19-26.

[97] Croft, J. A., Bridger, J. M., Boyle, S., Perry, P., Teague, P. and Bickmore, W. A., Differences in the localization and morphology of chromosomes in the human nucleus. *J Cell Biol* 1999. 145: 1119-1131.

[98] Kim, S. H., McQueen, P. G., Lichtman, M. K., Shevach, E. M., Parada, L. A. and Misteli, T., Spatial genome organization during T-cell differentiation. *Cytogenet Genome Res* 2004. 105: 292-301.

[99] Grosveld, F. G. and Duboule, D., Differentiation and gene regulation. *Curr Opin Genet Dev* 2007. 17: 369-372.

[100] Umlauf, D., Fraser, P. and Nagano, T., The role of long non-coding RNAs in chromatin structure and gene regulation: variations on a theme. *Biol Chem* 2008. 389: 323-331.

[101] Mohammad, F., Pandey, R. R., Nagano, T., Chakalova, L., Mondal, T., Fraser, P. and Kanduri, C., Kcnq1ot1/Lit1 noncoding RNA mediates transcriptional silencing by targeting to the perinucleolar region. *Mol Cell Biol* 2008. 28: 3713-3728.

[102] Majumder, P., Gomez, J. A., Chadwick, B. P. and Boss, J. M., The insulator factor CTCF controls MHC class II gene expression and is required for the formation of long-distance chromatin interactions. *J Exp Med* 2008. 205: 785-798.

[103] Cai, S., Han, H. J. and Kohwi-Shigematsu, T., Tissue-specific nuclear architecture and gene expression regulated by SATB1. *Nat Genet* 2003. 34: 42-51.

[104] Cai, S., Lee, C. C. and Kohwi-Shigematsu, T., SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. *Nat Genet* 2006. 38: 1278-1288.

[105] Georgel, P. T., Horowitz-Scherer, R. A., Adkins, N., Woodcock, C. L., Wade, P. A. and Hansen, J. C., Chromatin compaction by human MeCP2. Assembly of novel secondary chromatin structures in the absence of DNA methylation. *J Biol Chem* 2003. 278: 32181-32188.

[106] Springhetti, E. M., Istomina, N. E., Whisstock, J. C., Nikitina, T., Woodcock, C. L. and Grigoryev, S. A., Role of the M-loop and reactive center loop domains in the folding and bridging of nucleosome arrays by MENT. *J Biol Chem* 2003. 278: 43384-43393.

[107] Rice, K. L., Hormaeche, I. and Licht, J. D., Epigenetic regulation of normal and malignant hematopoiesis. *Oncogene* 2007. 26: 6697-6714.

[108] Fazi, F., Zardo, G., Gelmetti, V., Travaglini, L., Ciolfi, A., Di Croce, L., Rosa, A., Bozzoni, I., Grignani, F., Lo-Coco, F., Pelicci, P. G. and Nervi, C., Heterochromatic gene repression of the retinoic acid pathway in acute myeloid leukemia. *Blood* 2007. 109: 4432-4440.

[109] Dover, G. J., Brusilow, S. and Samid, D., Increased fetal hemoglobin in patients receiving sodium 4-phenylbutyrate. *N Engl J Med* 1992. 327: 569-570.

[110] Saunthararajah, Y. and DeSimone, J., Clinical studies with fetal hemoglobin-enhancing agents in sickle cell disease. *Semin Hematol* 2004. 41: 11-16.

[111] Ghoshal, K., Datta, J., Majumder, S., Bai, S., Kutay, H., Motiwala, T. and Jacob, S. T., 5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. *Mol Cell Biol* 2005. 25: 4727-4741.

[112] Gore, S. D., Baylin, S., Sugar, E., Carraway, H., Miller, C. B., Carducci, M., Grever, M., Galm, O., Dauses, T., Karp, J. E., Rudek, M. A., Zhao, M., Smith, B. D., Manning, J., Jiemjit, A., Dover, G., Mays, A., Zwiebel, J., Murgo, A., Weng, L. J. and Herman, J. G., Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. *Cancer Res* 2006. 66: 6361-6369.

[113] Bruserud, O., Stapnes, C., Ersvaer, E., Gjertsen, B. T. and Ryningen, A., Histone deacetylase inhibitors in cancer treatment: a review of the clinical toxicity and the modulation of gene expression in cancer cell. *Curr Pharm Biotechnol* 2007. 8: 388-400.

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