METHODS IN MOLECULAR BIOLOGY™

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Dendritic Cell Protocols

Second Edition

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Foreword

Research on dendritic cells (DC) has certainly come of age. The initial recognition of DC by Steinman and Cohn was followed by a long lag before the immunological community was convinced that these relatively rare cells were special. It is now accepted that these antigen-presenting cells are essential for the initiation of primary T-cell responses as well as for the maintenance of self-tolerance. When improved methods of DC isolation and analysis revealed multiple DC subsets, there was an understandable reluctance to accept this level of complexity. This has changed to an enthusiastic investigation of a network of DC subtypes differing in location and migration routes, in their patterns of microbial recognition receptors, in whether they process antigen for MHC class I or MHC class II presentation, and in the cytokines and chemokines they produce. The initially confusing impression that all haematological pathways might lead to DC is changing as intermediate precursors in steady-state DC development are being isolated. New culture systems for producing the different DC subtypes have been developed to supplement the earlier culture procedure for generating inflammatory-type DC from monocytes. This volume usefully details many of the basic techniques now used in this modern era of DC research.

However, these methods are not only a way of duplicating past experiments; they must help to lead us to the future. Although there is still much to be learnt from our steady-state laboratory mouse models, especially on the mucosal immune system, there are already some clear shifts of emphasis. DC are dynamic players in the response to infection, and their individual responses to particular microbial invasions need to be determined. Some approaches to this are presented. The wealth of knowledge obtained from studies on murine DC must be translated to the human DC system, despite some differences in surface markers and the problems of moving beyond blood as a human DC source. Some techniques used in the emerging work on human DC are presented here as well. Since DC are few in number but have a key role in regulating immune responses, they are an attractive target for immune modulation strategies. The initial approach of using antigen-loaded monocyte-derived DC as cellular adjuvants in tumour immunotherapy will evolve as methods, such as those presented in this volume are developed to enhance DC immunogenic potential or to render DC tolerogenic. Eventually, we must develop methods of targeting antigens or drugs to DC in situ. The experimental procedures presented in this book represent a valuable resource for expanding our understanding of DC biology and eventually translating this into clinical applications.

Ken Shortman
Melbourne, Australia
June, 2009

Preface

Exciting discoveries in a field of medical research can sometimes be tainted with frustration due to conflicting results within the field. Some of the ensuing controversies can be due to differing experimental conditions, by misinterpretation of the data, and, sometimes, simply the alignment of the planets. While mortals cannot remedy the latter, we can try and reach some consistency with the first – a tabulation of clear and informative protocols.

More often than not, it is the tidbits of information that one absorbs from a colleague while observing a protocol that are the crucial difference between empowering experimental success and demoralizing technical failure. I am sure everyone has his or her favourite example – 'make sure you angle the tube at 45°, facing west, while chanting "Kumbaya" before you resuspend the cells in degassed, isotonic, pH-balanced, sterile, 5.6°C buffer.' Sheesh. This book attempts, with a 'Notes' section, to highlight some of the seemingly mundane and trivial practical aspects to ensure a successful dendritic cell biology protocol.

In this edition of Methods in Dendritic Cell Research, I have tried to compile a list of protocols from experts in the field that cover some of the basics and some of the more complex forays into the exploration of DC development and function, both in mice and humans. This is a field that, like a vine, is ever-growing and by its nature therefore ever-entangling.

Many authors (named in parentheses below) have worked hard on the fantastic contributions presented in this book. To set the stage, we start of with an informative introduction of human DCs (Hart) and mouse DCs (Garbi), including the subtypes, their development, and their function.

The methods chapters are then split into human and mouse (rodent) protocols. Described in the human section are protocols for the isolation of blood DC subtypes (Radford), primary skin Langerhans cells (Geijtenbeek or Stoitzner), as well as the generation of gene-manipulated human DCs (Schotte) or DCs in humanized mice (Le Grand). From a more clinically relevant perspective, we then also discover methods to generate DCs that can induce different Th responses (Kalinski), tolerize T cells (Thomson), as well as a protocol hoped to be a 'standard' for comparison between different trials using patient-derived DCs (Erdmann).

In the mouse section, we see methods for the generation of DCs in vitro (Naik and O'Keeffe) with more recent protocols in the isolation (Onai) or generation (Naik) of DC precursors. Ex vivo isolation of DCs or their precursors is not forgotten. There is an extended protocol for the isolation of primary immature DCs from several organs by its inventor (Vremec) and other protocols for the isolation of DC precursors from the thymus (Wu), intestinal rat DCs (MacPherson), lung DCs (Stumbles), and cutaneous DCs (Merad or Stoitzner). Protocols of DC function also have a home here including T-cell activation assays with low numbers of DCs and high sensitivity (Belz), of DC migration and location properties based on labelling and depletion (Randolph) or parabiosis (Waskow), their phagosomal properties (Amigorena), regulatory T-cell (T reg) generation (Wang), and the use of the CD11c-DTR model of DC depletion (Jung).

Finally, we have several detailed models on the identification and functional testing of DCs in disease. These include asthma (Lambrecht), virus infection (Belz), and bacterial infection (Busch).

This methods book hopes to become a bench-side handbook for both beginners and experts in the field of DC research. It is hoped to be a long-term reference for some of the most popular methods in the field by those who lead the field. Hopefully, it will inspire new forays into the study of DC biology – independent of the alignment of the planets.

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June, 2009

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Section I

Reviews

Chapter 1

Review of Human DC Subtypes

Xinsheng Ju, Georgina Clark, and Derek N.J. Hart

Abstract

Dendritic cells (DC) are critical to the induction and regulation of the innate and adaptive immune responses. They have been implicated in the pathogenesis of many autoimmune and chronic inflammatory diseases as well as contributing to the development of tumours by their lack of appropriate function. As such, understanding human DC biology provides the insight needed to develop applications for their use in the treatment of diseases. Currently, studies on mouse DC outnumber those on human cells; however, the comparison between mouse and human models has been somewhat misleading due to the basic biological and practical differences between the two models. In this review, we summarise the current understanding of human DC subtypes by describing the phenotype of the populations and how this relates to function. We also hope to clarify the differences in nomenclature between the human and mouse models that have arisen by way of the different experimental models.

Key words: Dendritic cells, monocytes, langerhans cells, subsets, precursor, phenotype.

1. Introduction

Dendritic cells (DC) were first identified functionally as a haematopoietic-derived cell population able to initiate a primary T-lymphocyte response. They were identified initially in mouse lymphoid tissues (1) and then soon after as interdigitating cells in rat and human tissues (2–4). Studies in mice and humans have diverged dramatically over the years. This may reflect fundamental differences but in part relate to the difficulties involved in working on humans compared to inbred mouse strains. The tissue sources used are different due to ethical constraints, comparable reagents are unavailable and the motivations differ. Generally, the primary reason for human studies is to translate the research into clinical

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outcomes for patients, whereas mouse studies focus on proving or disproving a scientific question – a subtle but important difference. Research in the human system can be focused on diagnostically relevant or therapeutically feasible protocols. Many experiments performed in the mouse models are unfortunately very difficult or impossible to corroborate in humans. Studies in an inbred mouse strain may, for example, be very different in the outbred human. Consequently, some of the assumptions made about human DC are transposed from small animal experiments that have not been confirmed in man and may not necessarily apply.

Human DC subtypes have been primarily defined using peripheral blood, although a large body of data is available describing the immunohistological analysis of DC populations in human tissues. Unlike in the mouse, few studies have isolated DC from the tissues to analyse the functional capacity of these cells. This chapter will discuss the common subpopulations of human DC, whilst an accompanying chapter (**Chapter 3**) concentrates on the purification of the major human blood DC (BDC) populations. Broadly speaking human DC have been segregated on the basis of (1) location, e.g. peripheral tissues, lymphoid tissues, blood, (2) source, e.g. in vitro monocyte-derived, CD34-derived, or (3) phenotype, e.g. $Lin^{-}HLA\text{-}DR^{+}CD11c^{+/-}$. A functional classification (e.g. inflammatory, tolerogenic, migratory, tissue resident) is more popular in the mouse. In humans, DC were first identified as $HLA\text{-}DR^{+}$ interstitial cells in the kidney (2, 3). Following the identification of the tissue-resident $HLA\text{-}DR^{+}$ cells in the kidney, different populations of DC were described in the tonsil (5–7), thymus (8), liver (9), interstitium of non-lymphoid organs (interstitial DC) (10, 11), and bone marrow (12–14). However, it was recognised early that studies of human DC populations would require firstly lineage-specific surface markers and secondly, a convenient source and means of purifying this rare population. The ability to generate DC in vitro, with many of the properties of the DC manufactured in vivo, initiated an explosion of DC research as these cells were relatively easy to come by. However, again comparisons with the natural DC populations highlighted some obvious differences between ex vivo- and in vitro-obtained cells (15, 16). Recently, microarray analyses enabled an extensive comparative mRNA analysis of mouse and human DC populations and went some way towards reconciling the differences (16, 17).

Many groups, including this laboratory, have attempted to develop surface markers to expedite the study and isolation of human DC populations (**Table 1.1**). However, the generation of new antibodies has been slow and even today, there are very few antibodies that make a singular contribution to the identification of human DC populations (18). The use of informative panels

Table 1.1
Commonly used biomarkers of human DC populations

DC population	Antibody or antibody mixes	Antigen and function	References
All DC	HLA-DR	MHC-II molecule	(19)
	Lin mix (CD3, CD14, CD19, CD20, CD56, CD235)	T, B, NK, erythrocyte markers	(19)
$CD1c^+$-DC	CD11c	Binds CD54, fibrinogen	(19)
	CD1c (BDCA-1)	Antigen presentation	(19)
pDC	CD123	IL-3 receptor	(96)
	CD303 (BDCA-2)	Inhibit IFN-α production	(27)
	CD304 (BDCA-4)	Axonal guidance, angiogenesis, cell survival, migration	(97, 98)
$CD141^+$-DC	CD141 (BDCA-3)	Initiation of protein C anticoagulant signal	(19)
$CMRF\text{-}44^+$-DC	CMRF-44	DC differentiation or activation marker	(81, 82)
	CMRF-56	DC differentiation or activation marker	
$CD83^+$-DC	CD83	DC activation marker	
LC	CD1a	Antigen presentation	(63)
	CD207 (Langerin)	Integral component of Birbeck granules	(49)
DC in mucous and lymphoid tissue	CD209 (DC-SIGN)	C-type lectin with mannose-binding specificities	(99)
	CD103	Associates with integrin β7 to bind E-Cadherin	(57)

particularly lineage-negative (Lin^-; CD3, CD14, CD19, CD20, CD56) panels remains the standard. Despite these difficulties, five populations of allostimulatory leucocytes have been distinguished in the peripheral blood (19), tonsil (7), and spleen (20).

DC subtypes in the different human tissues are inevitably defined first according to their phenotype, indicated by specific markers (**Table 1.2**). Using the markers for nomenclature is a simple and, provided commonly available markers are employed, consistent manner to describe a population compared to alternative descriptive or functional names which may hide the specific function of such cell type. Phenotypes should not change but our current knowledge as to DC biology may change

Table 1.2
Human DC populations defined by phenotype ex vivo

Phenotype	Location	Function	Other markers in common use	References
Lin$^-$HLA-DR$^+$ CD11c$^+$CD1c$^+$ (CD1c$^+$-DC)	Blood	Antigen uptaken, migration and activation of T cells		(100)
Lin$^-$HLA-DR$^+$ CD303$^+$ (pDC)	Blood	Type-I IFN production	CD304, CD123	(27)
Lin$^-$HLA-DR$^+$ CD141$^+$ (CD141$^+$-DC)	Blood	IL-10 and IFN-β production	Clec-9	(28, 29)
Lin$^-$HLA-DR$^+$ CD34$^+$ (CD34$^+$-DC)	Blood, BM	Low allostimulatory capacity		(19)
Lin$^-$HLA-DR$^+$ CMRF-44$^+$ (CMRF-44$^+$-DC)	Blood, GVHD	Allogeneic T-cell stimulation	CD83, CMRF-56	(81, 82, 101)
Lin$^-$CD1a$^+$ Langerin$^+$ (LC)	Skin epidermis	Antigen uptaken and migration; induce T-cell tolerance; cross-presentation?	E-Cadherin, Birbeck granules	(51)
CD103$^+$-DC	Mesenteric LN	Induce regulatory T cells in gut and gut homing of T cells		(57)

dramatically. Human DC subtypes fit broadly into the subset divisions outlined in (**Table 1.3**). The common experimental practice that uses monocytes as DC precursors has continued the debate as to the relationship between monocytes, DC, and the myeloid lineage. Within each of these subpopulations are further segregation of DC populations that have particular phenotypic or functional markers and these are described below in more detail.

2. Subdivision by Cell Surface Phenotype of DC from Different Tissue Sources

2.1. Bone Marrow

CD34$^+$ precursors: CD34 is one of the markers for haematopoietic stem cells in blood, bone marrow (BM), and cord blood. CD34$^+$ cells are capable of differentiating into the full range of haematopoietic cell lineages. Transplantation of ex vivo expanded human umbilical cord blood CD34$^+$ cells into Rag2$^{-/-}\gamma^{-/-}$

Table 1.3
Human monocyte and DC subsets

Cellular description	Cell surface phenotype	References
Monocytes	$CD14^{++}CD16^{-}$ $CD14^{+}CD16^{+}$	(44, 102)
Blood dendritic cells		
Precursors	$CD34^{+}$	(19)
	$CD123^{+}CD303^{+}$	(30)
$CD11c^{+}$-DC	$CD1c^{+}$	(103)
	$CD141^{+}$	(19)
	$CD16^{+}$	(41, 42)
Tissue derived		
Skin	Langerhans cells	(51)
	Dermal DC	(54)
Tonsil	HLA-DR^{++} $CD11c^{+}CD83^{+}$ CMRF-56^{+}	(7)
	HLA-DR^{+} $CD11c^{+}$ $CD13^{+}$	(7)
	HLA-DR^{+} $CD11c^{+}$ $CD13^{-}$	(7)
	HLA-DR^{+} $CD11c^{-}$ $CD123^{+}$	(7)
	HLA-DR^{+} $CD11c^{-}$ $CD123^{-}$	(7)
In vitro derived		
	$CD34^{+}$ derived	(63)
	$CD14^{+}$ derived	(45, 46)

mice reconstitutes lymphocyte and dendritic cells (21). Previously, we demonstrated DC in human bone marrow that had specific antigen-presenting capacity and T-cell stimulatory function (22). Within blood, there are $CD34^{+}$HLA-$DR^{+}Lin^{-}CD11c^{-}$ with low, but measurable allostimulatory capacity (21). These cells do not express CD80/CD86, but are $CD40^{+}$ (21). Whilst they may contribute to antigen-presenting capacity (particularly given the in vitro studies using cytokine-derived $CD34^{+}$-DC), these are not generally included in DC populations. Nonetheless, it has emphasised the point that CD34 mAb should be used experimentally to exclude these cells from Lin^{-}HLA-DR^{+} populations.

2.2. Blood DC

Blood provides a delivery system for distributing BM-derived DC to lymphoid and other tissues. It may also contain at least in some circumstances, DC derived from other tissues, e.g. vascularised organs or grafts (22).

Freshly isolated human peripheral blood HLA-DR$^+$Lin$^-$CD11c$^+$ DC are able to stimulate allo- and antigen-specific T-cell responses. With careful isolation, these cells are CD80$^-$ but do express CD86. Minimal culture (37°C, human AB serum) of DC results in increased expression of HLA-DR and upregulation of activation markers such as CMRF-44 and CMRF-56. Human pDC from the peripheral blood are in an immature state and require activation with TLR ligands to induce maturation associated with the ability to present antigen. However, these BDC do not divide in order to become functionally mature. On the other hand, mouse peripheral blood DC are unable to stimulate antigen-specific responses without prior activation (23). This apparent difference has led to the general assumption that peripheral blood DC are precursor cells (24). Whilst this may be the case for mouse peripheral blood DC, this is probably not the case in humans. Whether this process is differentiation of a precursor population or an appropriate response to stimuli depends on one's definition of "precursor". The same thought process must also be applied to other monocyte populations circulating in blood. DC can be derived from common myeloid or lymphoid progenitors. In vivo evidence for a human DC lineage differentiation pathway from haematopoietic stem cells has followed clinical transplant studies in xenogenic models (25).

A classification of human blood DC includes the cells outlined in Table **1.3** and separates into five subpopulations (**Fig. 1.1**). The HLA-DR$^+$Lin$^-$CD11c$^+$ BDC populations divide into three allostimulatory cell populations: CD1c$^+$, CD141$^+$, and CD16$^+$ cells. Blood CD1c$^+$ myeloid DC originate from bone marrow, they circulate in the blood and migrate constantly to the second lymphoid organs and peripheral tissues as resting interstitial DC. These cells express myeloid markers such as CD13, CD33, CD11c, CD1c, but are Lin$^-$. The majority of myeloid DC are CD14$^-$ but a minor population expresses low levels of CD14 and are considered to be a myeloid DC precursor (12). It is conceivable that CD1c$^+$ myeloid DC may develop from CD14$^+$ monocytes in vivo. CD1c$^+$ DC comprise about 0.6% of peripheral blood mononuclear cells or approximately 19% of the HLA-DR$^+$Lin$^-$ population.

CD141$^+$-DC are a very rare type of DC and only in 0.04% of PBMC (or 3% of the DC population), they are CD11c^{dim}CD1c$^-$CD4$^+$CD123$^-$ and Lin$^-$ (27). Clec9 is a new marker strongly expressed on both human and mouse CD141$^+$ DC (28, 29). Isolating these cells from human peripheral blood is discussed in an accompanying paper (**Chapter 3**). Human CD141$^+$ DC expressed high levels of toll-like receptor (TLR) 3 but lacked TLRs 4, 5, and 7 whilst CD1c$^+$ DC expressed the TLRs1–8 and TLR10.

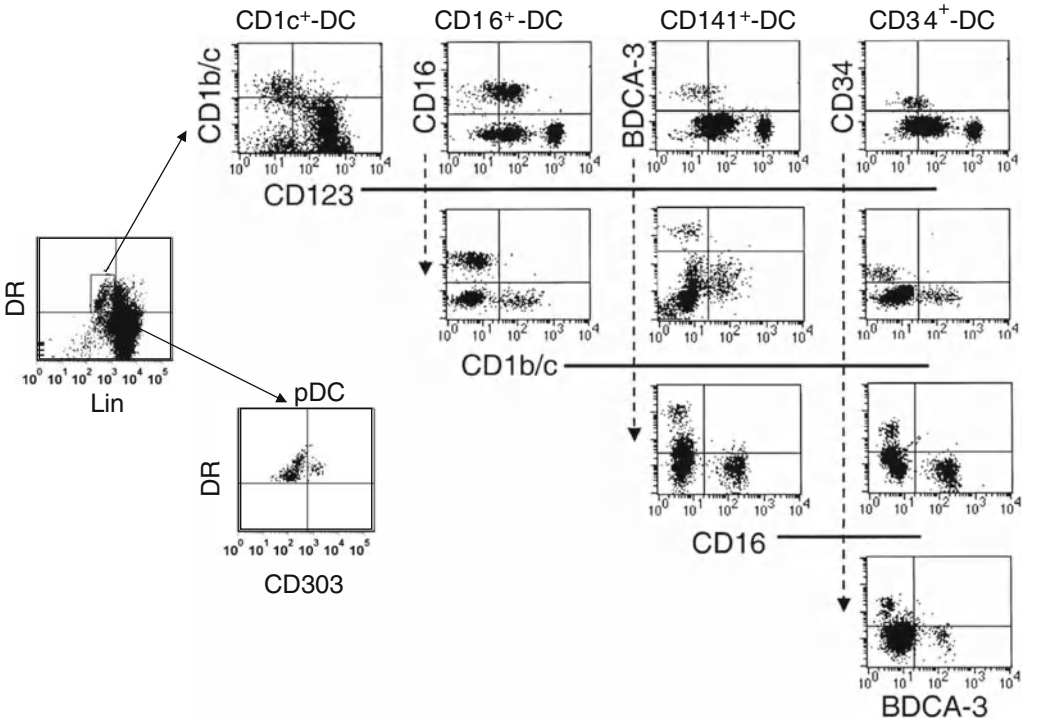

Fig. 1.1. Phenotype of the five human peripheral blood DC subsets. To identify the five DC subsets, the gating strategy is firstly to gate on the Lin^- DR^+ population in the living cell gate, secondly gate according to different phenotypic markers. Methods for purification of each population are described in the accompanying chapter (**Chapter 3**).

Plasmacytoid DC (pDC) are a specific type of DC that are found in an immature form in the peripheral blood but in higher numbers in inflamed tissue and tonsil (30). They are the major IFN-α/β producers upon stimulation. pDC show plasmacytoid morphology, express pre-T-cell receptor α-chain, and are $CD123^{++}CD4^+CD11c^-CD303^+CD304^+$. CD303, whilst a useful marker on fresh blood pDC, is downregulated upon culture in vitro and the antibody blocks secretion of IFN-α (27). ILT-7 was recently identified as a human pDC-specific marker, which is negative in other DC subtypes (31–33). pDC express TLR7 and TLR9 and differentiate into mature antigen-presenting cells in response to TLR7 and TLR9 ligands. Blood pDC also express L-selectin and migrate to second lymphoid organs through the high endothelial venules around which they congregate (34). pDC and myeloid DC exhibit some important differences in terms of antigen uptake, presentation, and T-cell stimulatory activity. In human pDC activated with IL-3 are unable to uptake FITC-dextran, in contrast to monocyte-derived DC (35). pDC have limited ability to prime naïve $CD4^+$ T cells but are able to induce IL-10-producing regulatory T cells upon activation (36, 37). pDC are also found to be capable of cross-presentation antigens to $CD8^+$ T cells (38, 39).

The $CD16^+$ BDC population represents the largest $HLA\text{-}DR^+Lin^-$ population in the blood comprising up to 50% of the $CD11c^+$ DC. The literature sometimes confuses these cells as a monocyte subpopulation or DC precursor (28). Care must be taken to ensure that the $CD14^+$ monocytes are removed from the gating strategies during analysis or from the Lin^- preps during preparation. The $CD16^+CD14^-$ cell population expresses 6-Sulpho LacNAc, an O-linked carbohydrate modification of PSGL-1 recognised by binding to the MDC-8 mAb (16). These cells are $CD1c^-CD11c^+C5aR^+CD45RA^+HLA\text{-}DR^+$. They are the principal source of TNF-α and IL-12p70 when blood leucocytes are stimulated with TLR4 ligand LPS or CD40 ligands (43, 44). Most importantly, these cells express receptors for the inflammatory mediators C3a and C5a but not the skin homing molecule, CLA. In xenogenic models, these cells specifically migrate to C5a (42). They are able to produce allogeneic proliferative responses of naïve $CD4^+$ T lymphocytes but produce poor autologous T-cell responses compared to $MDC\text{-}8^-$ cells. The $CD16^+$ DC are a proinflammatory cell type possibly indicating similarities with monocyte-derived DC (MoDC). From the gene microarray data, $CD16^+$-DC cluster with neutrophils and monocytes but not with $CD1c^+$-DC and pDC.

2.3. Monocytes

The lineage marker most commonly used for monocytes is CD14. Peripheral blood monocytes express high levels of CD14 and these $CD14^+$ cells can be subdivided by their expression of CD16. The $CD14^{++}CD16^-$ population of monocytes is the predominant population of monocytes in human peripheral blood. These cells are thought to correspond to the tissue-resident cells that are the precursors to tissue macrophages, Kupffer cells, osteoclasts, microglia, etc. (43). The $CD14^+CD16^+$ population forms less than 10% of the normal $CD14^+$ monocyte population in healthy individuals. However, these cells have the properties of proinflammatory cells that, on activation, may correspond to a similar cell type as the monocyte-derived DC (44). Careful attention to detail is required when analysing human peripheral blood monocyte subpopulations (**Table 1.3**). $CD14^{++}$ monocytes have been routinely used as a DC precursor following the demonstration that they can differentiate into DC-like cells in vitro (45, 46). Consequently $CD14^{++}$ cells have sometimes been referred to in the literature as DC1 precursors (47). However, the evidence indicates that monocytes are most likely precursors of a DC that differentiates in inflammatory situation and that they are not the major precursors of steady-state DC.

2.4. Non-lymphoid Tissue DC

DC have been identified in most human non-lymphoid tissue (9, 48–51). Their presence in the skin has major historical importance as the original description of the Langerhans cell (LC).

There are two major DC subpopulations in the skin: LC and Dermal DC. LC reside mainly in the epidermis where they constantly monitor for the presence of foreign antigens. LC express Langerin (CD207), CD1a, and E-Cadherin and contain specific intracellular or membrane-bound structures, referred to as Birbeck granules, that are not found in other DC subsets (49). Murine LC are capable of cross-presentation of antigen to $CD8^+$ T cells but is yet to be described for human cells (50). Under steady-state conditions, immature LC migrate continuously into lymph nodes without further maturation and induce T-cell tolerance (51). They are not continuously replenished by migratory blood precursors, but rather renew through local proliferation under steady condition (52). LC might not be essential for priming T cells to foreign antigens that enter the skin, but instead, may induce immune tolerance. Dermal DC are distinguished from LC by their complement of C-type lectins ($CD209^+CD206^+CD207^-$) and a more activated phenotype (53). The difference in the receptor expression indicates that the two skin DC populations may recognise and present different microbial antigens (54, 55).

2.5. Lymphoid Tissue DC

Tonsil: Tonsil is the most available lymphoid tissue although it must be remembered firstly that they are gut-associated lymphoid tissue and not lymph nodes and secondly that most tonsils available ethically, are those removed due to prolonged inflammation. The Lin^-HLA-DR^+ DC population of tonsil can be subdivided into five populations of which four are interdigitating DC. Isolation of the tonsil DC populations needs to minimise further cellular activation during the procedure from this generally already inflamed tissue. The phenotypes of the populations were HLA-$DR^{++}CD11c^+CD83^+$ CMRF-56^+, HLA-DR^+ $CD11c^+$ $CD13^+$, HLA-DR^+ $CD11c^+$ $CD13^-$, HLA-DR^+ $CD11c^-$ $CD123^+$, and HLA-DR^+ $CD11c^-$ $CD123^-$ (7). The relationship to each of these populations requires functional studies. Activation markers such as NKp46 further divide the Lin^-HLA-DR^+CD123^+ population of tonsil pDC (5, 7, 56).

Lymph node: DC expressing CD103, the integrin α chain, reside in the human intestinal lymph node (LN). $CD103^+$-DC efficiently induce gut homing receptor CCR9 on responding T cells with regulatory function (iTreg) via a retinoic acid receptor-dependent mechanism (57). The ability of $CD103^+$-DC to induce CCR9 expression is maintained in patients with Crohn's disease indicating a role for $CD103^+$-DC in the intestinal homeostasis and inflammation. It might represent a potential targeting of human intestinal inflammatory disease.

Thymus: Human thymus DC are $CD1a^-CD3^-CD4^+CD8^-$ cells and express high levels of CD123 on the membrane and

are able to develop into mature DC upon culture with IL-3 and CD40 ligation (58, 59).

Spleen: In human spleen, four DC subsets have been described. Most DC are $CD11c^+HLA\text{-}DR^+Lin^-$ cells that express CD54 and low levels of CD40 and CD86 but not CD80 or CD83. There is a smaller population of $CD11c^-$ DC. This suggests that the majority of human splenic DC have an immature phenotype similar to $CD11c^+$ BDC (20). There did not appear to be $CD16^+$ DC and CD141 was not tested. The $CD11c^+$ DC were subdivided based on their distribution: marginal zone DC, B-cell zone DC, and T-cell zone DC. Splenic DC expressing high levels of co-stimulatory molecules could be found in some donors but the authors argued that these donors may have had bacterial infection.

2.6. In Vitro Derived

The most common source of DC for human studies has been the cells derived in vitro from monocytes or $CD34^+$ haematopoietic stem cells. These derived cells are not identical to cells in vivo or purified ex vivo (15, 16, 60), nor are the products of cultures derived from cells purified by different technologies or in the presence of different media supplements. Claims attributing specific functions to specific subsets should be treated with suitable reservation as to direct correlation with in vivo functions.

$CD14^+$ derived: We have shown that there are spontaneous generation of DC from monocyte precursors (61). It has also been demonstrated that blood monocytes can give rise to mucosal DC in vivo (62). In vitro monocytes are cultured with IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) or monocyte-conditioned medium to induce DC differentiation (45, 46). Immature MoDC can be matured in the presence of LPS or TNF-α or the cocktail containing IL-6, IL-1β, TNF-α, and PGE_2.

$CD34^+$ derived: $CD34^+$ haematopoietic stem cells from cord blood, bone marrow, or mobilised peripheral blood are induced to differentiation into DC with GM-CSF, TNF-α (63). $CD34^+$-derived DC can differentiate into a number of phenotypically distinct populations including $CD1a^-$ interstitial DC and $CD1a^+$ LC subpopulation (64).

3. Functional Subsets

In the mouse, DC are also commonly classified according to their distinct function, i.e. migratory DC, tissue-resident DC, inflammatory DC. The application of this type of classification to humans will generally be based on assumptions made from

murine models. However, within one functional subdivision used in the mouse, human DC will often have differing phenotypes.

3.1. Migratory DC

Migratory DC in the human include tissue-derived DC, BDC, and LC. The most applicable model for studying human DC migration relies on therapeutic haematopoietic stem cell transplantation where donor and host-derived cells are able to be monitored (65).

3.2. Tissue-Resident DC

Human tissue-resident DC have been studied in the transplant arena where HLA-DR$^+$Lin$^-$ cells have been identified in the liver (9, 66), heart, cornea (65), and pancreas (67, 68) and this was extended to HLA-DR$^+$BDCA-1$^+$ or HLA-DR$^+$BDCA-2$^+$ cells in the kidney (48).

3.3. Inflammatory DC

When the host is healthy, it is difficult to identify inflammatory DC. Inflammatory DC are a heterogeneous population that includes tumour necrosis factor and inducible nitric oxide synthase (iNOS)-producing DC (Tip-DC), IL-20-producing DC, and IL-23-producing DC, etc. Tip-DC were originally found in the spleen of *Listeria monocytogenes*-infected mice, but are now found also in human psoriasis plaque with the expression of CD11c$^+$DR$^+$CD40$^+$CD86$^+$CD83$^+$DC-LAMP$^+$ DC-SIGN$^+$ but Langerin$^-$ CD14^{dim}CD1c$^-$ (69, 70). pDC are also rarely found in healthy skin, but increased in allergic disease of the airway (71) and diseased skin, e.g. atopic excema, psoriasis, and cutaneous lupus erythematodes (72–75).

3.4. Tolerogenic DC

Immature DC and pDC are capable of inducing tolerance through regulatory T-cell differentiation from naïve CD4$^+$ T cells. Tolerogenic properties can be enhanced by a number of mediators and some DC populations (CD1c$^+$) are able to secrete regulatory cytokines such as IL-10 following certain stimuli. In abnormal situations DC with altered phenotypes exist, i.e. in transplant recipients that have been weened from immunosuppressive drugs. These cells can be distinguished phenotypically as expressing ILT-3 and ILT-4 (76) or indoleamine 2,3-dioxygenase (IDO) enzyme. The ability to specifically induce these cells or monitor their generation in transplantation is a clinical relevance to allow clinicians to remove patient's from long-term immunosuppressive drugs whilst monitoring rejection episodes (77).

4. Clinically Relevant Subsets

4.1. Diagnostic

4.1.1. Bone Marrow

Mobilisation of haematopoietic stem cells (HSC) with G-CSF induces the mobilisation of pDC into the peripheral blood. An increased number of pDC has been associated with not only

enhanced engraftment of HSC transplants but also an increase in chronic graft versus host disease (GVHD).

4.1.2. Blood

Despite their rarity, human BDC are the most commonly studied DC populations. Recently developed protocols to enumerate BDC populations enabled the monitoring of human BDC subsets in the healthy and disease populations. This technology relies on flow cytometric-based TruCOUNT assays (78, 79) of whole blood. The four $CD14^-$ subsets of human BDC can be assessed from whole blood and the different populations show numerical differences through disease progression (79, 80). The CMRF-44 (IgM) and CMRF-56 (IgG) monoclonal antibodies bind in vitro matured blood DC with 54 and 66% of matured blood DC binding CMRF-44 and CMRF-56 mAb, respectively (81, 82). However, it has been observed that there is an increased number of activated DC in the peripheral blood during GVHD. The ability to monitor the presence of activated DC populations to predict GVHD episodes has proven effective in small studies (83). These studies have the potential to contribute directly to patient management in a variety of clinical scenarios.

4.1.3. Skin

BDCA-1^--DC were found in human psoriatic dermis, which accounts for 90% of $CD11c^+$ dermal-resident DC and belong to inflammatory DC and induce Th1/Th17 polarisation. Etanercept treatment reduces BDCA-1^--DC number but the BDCA-1^+-DC remained stable (84). Patients with psoriasis have pDC that are particularly sensitive to the keratinocyte peptide LL37 and responsive with increased IFN-α induction (85).

4.1.4. Cancer Biopsies

Tumours contain an abundance of immature DC and fewer mature DC compared with healthy tissues. A number of cytokines released by tumours recruit these immature DC from peripheral blood and the hypoxia in the tumour microenvironment might support the immature phenotype of DC (86). Tumour DC have been shown to deliver immune suppressive function, meanwhile, they promote tumour angiogenesis by secretion of proangiogenic cytokines.

4.1.5. Transplanted Organs

Human DC are currently exploited for monitoring the outcome of organ and bone marrow transplantation (GVHD and allograft tolerance).

4.2. Therapeutic

Lastly, most, but not all, clinical trials addressing DC tumour vaccination have used in vitro-derived DC preparations generated from either monocytes or $CD34^+$ cord blood cells. (For a detailed summary of DC-based international clinical trials *see* www.mmri.mater.org.au.) However, these trials have not been overly successful and it is commonly agreed that the in

vitro-derived cells have failings – the inability to migrate appropriately. The ability to purify BDC from aphaeresis products has provided the potential to initiate clinical trials using these cells (87–92). This description of CMRF-56$^+$ DC populations has enabled a method to be developed suitable for large-scale isolation of DC for clinical application (81, 82).

4.2.1. DC Malignancy

Whilst not a true malignancy, Langerhans Cell Histiocytosis is a rare disease with the abnormal proliferation of Langerhans cells in bone, skin, lung, and stomach. Most cases are found in children (93). Recently, a rare haematopoietic tumour sharing the phenotypic and functional features of pDC was identified and named pDC leukaemia (pDCL), but leukaemic pDC express CD56$^+$ and adaptor protein CD2AP$^+$; it comprises less than 1% of acute leukaemia cases. Some leukaemic pDC produce IFN-α, but lower than their normal counterparts. Leukaemic pDC are able to present viral antigen to CD4$^+$ and CD8$^+$ T cells and prime naïve CD4$^+$ T cells towards Th2 or Th1 pathway. Leukaemic pDC become fully competent antigen-presenting cells after culture with IL-3/CD40L (94, 95). Currently, only allogeneic haematopoietic stem cell transplantation might lead to complete remission.

5. Conclusion

Human DC subpopulations have been described based on phenotypic assessment. Careful flow cytometric analysis ensures that discrete populations are purified or studied. DC populations isolated from peripheral blood or human tissue differ from DC derived in vitro. The DC precursor cells are still not clearly identified in human and appear to differ from mouse cells. Direct comparisons, such as between the DC precursor population in mouse and humans, can be misleading due to differences in the systems. There is now good data indicating the ability to purify five DC populations based on phenotype and function from human peripheral blood. Understanding the functional difference among different DC subsets represents one of the remaining challenges in DC biology.

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Chapter 2

Review of Murine Dendritic Cells: Types, Location, and Development

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Abstract

Dendritic cells (DCs) are key coordinators of the immune response, governing the choice between tolerance and immunity. DCs are professional antigen-presenting cells capable of presenting antigen on MHC molecules and priming CD4 and CD8 T-cell responses. They form a heterogeneous group of cells based on phenotype, location, and function. In this review, murine DCs will be discussed regarding their function with special emphasis on their tissue distribution. Recent findings on DC homeostasis during cancer progression will be presented. Finally, the developmental pathways leading to DC differentiation from their precursors will be summarized.

Key words: Dendritic cells, dendritic cell subpopulations, dendritic cell precursors, cancer, inflammation.

1. Introduction

Dendritic cells (DCs) are a group of rare hematopoietic cells scattered in lymphoid and non-lymphoid organs throughout the body where they serve as pivotal coordinators of innate and adaptive immune responses (1, 2). DCs in lymphoid organs were first identified on a morphological basis by Steinman and Cohn in 1973 (3) and 5 years later they were realized to have a key immune function by potently stimulating naïve T cells (4). However, the first identification of DCs took place about a century earlier when Paul Langerhans discovered interdigitating cells in the skin in 1868, which he called Langerhans cells (5). Despite this historical milestone, he suggested a neural origin and

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function for Langerhans cells. It was over a century later that these cells were proven to fulfill immunological functions (6, 7) and not to have neural origin (8) but to develop from a mobile pool of hematopoietic cells (9, 10).

Based on their ability to capture and present antigen on major histocompatibility I and II molecules (MHC-I and MHC-II), DCs have been classified as components of the antigen-presenting cell family, to which B cells and macrophages also belong. DCs display a myriad of immunological functions crucial for immune regulation. Of these, some demonstrated in vivo are (i) induction of T-cell immunity or tolerance depending on their activation status (11); (ii) generation of regulatory T cells (12); (iii) activation of NK cells (13); and (iv) promotion of homeostatic expansion of T cells (14) and NK cells (13) during lymphopenia. Regarding non-immune functions, DCs have also shown to be required for embryo implantation during pregnancy, possibly by promoting angiogenesis (15).

DCs form a very heterogeneous group of different cell populations based on phenotype, location, and function. It is, therefore, not surprising that the definition of what constitutes a DC is not as straightforward as one may anticipate. High expression levels of CD11c and MHC-II phenotypically mark DCs in most cases. However, in strong contrast to lymphocytes, there is no specific molecular definition of a DC (lymphocytes are clonally derived and characterized by expression of either the T-cell receptor or the B-cell receptor). Rather, DCs are defined based on functional properties (e.g., potent activation of T-cell responses) and a combination of cellular markers (e.g., $CD11c^{hi}$ and $MHC\text{-}II^{+}$). Although the identification of cellular markers for DCs has been very useful for research and clinical purposes, they are not fully specific to the DC compartment and not all DCs share the same phenotypical and functional determinants. This has led to some skepticism on the separation of DCs into a different category than monocytes and macrophages (16). Nevertheless, it is generally accepted that DCs form a heterogeneous cellular group with key functions in the regulation of immunity. To fulfill the many functions ascribed to DCs, they are present in different locations of the body and come in different flavors regarding phenotype and migration capabilities.

The aim of this review is to present recent data regarding the different types and subtypes of murine DCs regarding their phenotype and general function and to describe the locations where they are found. Providing detailed insight into the specific functions of each DC subtype is not the intention of this review, but excellent reviews exist on this subject (e.g., see Refs. (17–25)). A brief description of the role of DCs in cancer disease will also be presented. Finally, we will discuss the main known pathways for DC development. Procedures to phenotypically characterize

and purify murine DCs and their precursors by flow cytometry are described in Chapters 10–22.

2. Basic Dendritic Cell Types in the Mouse

Arguably, a first level of classifying different DC types useful for understanding their basic properties is whether they reside in lymphoid organs or migrate from non-lymphoid organs, whether they are conventional DCs or plasmacytoid DCs, and finally, whether they are present in the steady state or induced in certain inflammatory scenarios.

2.1. Lymphoid-Organ Resident DCs

They reside in lymphoid organs such as spleen, lymph nodes, bone marrow, and thymus. They are characterized by being $CD11c^{hi}$ $B220^{-}MHC\text{-}II^{+}$ and spend their lifetime in one organ. A main function of these DCs is to present lymph-borne or blood-borne antigen to T cells for either immunity or tolerance induction. Most of these DCs are also named classical or conventional DCs (cDCs), which are further divided depending on their phenotype and function. Typical examples constitute $CD8^{+}CD4^{-}$ cDCs, $CD8^{-}CD4^{+}$ cDCs, and $CD8^{-}CD4^{-}$ cDCs in spleen (26).

2.2. Migratory DCs

They are classically found in non-lymphoid organs and in the lymphoid organs to which they migrate. These DCs come in different flavors regarding organ, location within the organ, phenotype, and function. A common characteristic is their high expression level of CD11c and MHC-II and their ability to take up antigen and migrate to lymphoid organs (mainly the draining lymph node) where they shape T-cell responses. All organs examined so far contain these DCs; examples are epidermal Langerhans cells and dermal dendritic cells in the skin and DCs in other organs such as lung, kidney (27), liver, brain, eye (28), and digestive tract, among others. Within lymphoid organs, they have been found in the lymph nodes draining that particular organ (e.g., see Refs. (29, 30)) and thymus (31). It is likely that spleen and BM also contain migratory DCs that are blood-borne, because experimentally expanded DCs can enter those organs upon intravenous injection (32, 33). However, it is not clear whether endogenous, circulating differentiated DCs enter spleen and BM.

2.3. Plasmacytoid DCs (pDCs)

pDCs constitute a minority of DCs in the whole body. They are called "plasmacytoid" because of their "plasma cell-like" morphological appearance. pDCs are present in lymphoid and non-lymphoid organs and are characterized by being $CD11c^{int}$ $B220^{+}$ PDCA-1^{+} $MHC\text{-}II^{-/int}$ and by producing large quantities of

IFN-α following viral infections (34). In the steady state, pDCs present antigen on MHC poorly; however, following activation with microbial stimuli, they are converted into potent APCs (35).

2.4. Inflammatory DCs

These are not present in the steady state but generated under the influence of certain inflammatory regimes. Probably, they are the DC type less well characterized in terms of phenotype and function. An example of inflammatory DCs is the TNFα/iNOS-producing (Tip)-DC subset derived from monocytes and present in the spleen of mice infected with *Listeria monocytogenes* (36) and in the uroepithelium of *Escherichia coli*-infected mice (37). Tip-DCs are not required to induce T-cell responses (36), although they are important to clear the bacterial infection in some cases but not in others (37). Inflammatory DCs are also generated experimentally from transferred monocytes following methylated BSA- or thioglycollate-mediated inflammation in the peritoneum but not irradiation (38, 39) (K. Hochweller and N. Garbi, unpublished), indicating that not all inflammatory regimes are sufficient to generate inflammatory DCs.

Although this introduction of the different DC types is useful as a basis to DC heterogeneity, it says little about the complex network of DCs operating in the body and their location. Therefore, we continue with a more detailed description of DC types based on their tissue distribution.

3. Dendritic Cell Types Based on Their Location and Function

Immune cells are not often classified according to their distribution in the body (macrophages being an exception). So, why are DCs different in this regard? DCs are present in all lymphoid and non-lymphoid organs of the body where they have been studied even in the absence of infections. Although in general terms most of them have a similar function, i.e., regulation of innate and adaptive immune responses, they show tissue-specific functions that argue for a study of DC types based on location and function. These tissue-specific functions contribute, among others, to one of the most important basic aspects of the immune system, i.e., the mobility of its cellular constituents. This mobility is required to bring antigen from sites of infection to lymphoid organs by DCs where lymphocytes are first activated. For these effector T cells to protect us against pathogens, they need to mobilize to the sites of infection (e.g., skin, intestinal tract, respiratory tract). Lymphocyte trafficking is highly regulated by an endless repertoire of chemokines and their receptors. Furthermore, DCs contribute to maximizing the homing of effector T cells to relevant

sites because they imprint T cells with organ tropism during the priming process in the lymph nodes. This makes sense because, in general, a pathogen that causes skin infection is usually found in the skin but not in internal organs (except during systemic infections). For example, DCs from the gut-associated lymphoid organs produce retinoic acid, which promotes expression of gut-homing receptors α4β7 and CCR9 by T cells (40–42). Similarly, skin DCs metabolize sun-induced vitamin D_3 resulting in increased expression of the skin-homing receptor CCR10 on T cells and reduced expression of gut-homing α4β7 and CCR9 by T cells (43). Therefore, effector T cells primed against gut or skin antigens will be enriched in the gut or skin areas, respectively.

A summary of the phenotype and location of the main DC types is shown in **Table 2.1**.

3.1. Dendritic Cells in Lymphoid Organs

3.1.1. Bone Marrow

The bone marrow (BM) is a primary lymphoid organ that hosts the hematopoietic stem cells as well as downstream hematopoietic progenitors, resulting in the generation of all cellular lineages composing the immune system. Regarding T- and B-cell primary responses to antigen, the BM has recently been identified as a secondary lymphoid organ (44–47). Although DCs in the BM have been shown to prime T cells against tumor and blood-borne antigens (48), there is very little information regarding their phenotype. DCs in the bone marrow are mostly of the plasmacytoid phenotype ($CD11c^{int}$ MHC-$II^{-/int}$ $B220^{+}$). In comparison to spleen, fewer but more activated $CD11c^{hi}$ MHC-II^{+} $B220^{-}$ cDCs exist in the BM (49), which may explain their ability to prime T-cell responses (48). BM cDCs are organized in perivascular clusters around blood vessels occupying specific niches (49, 50), where they provide survival signals to circulating B cells (49).

3.1.2. Thymus

The thymus is the organ where bone marrow-derived thymocyte precursors finally differentiate into T cells. During this differentiation process the TCR repertoire is shaped by central T-cell tolerance mechanisms where self-reactive thymocytes are deleted by negative selection. In addition, naturally occurring regulatory T (Treg) cells with high affinity for self-antigen presented onto MHC-II are selected and generated in the thymus. A number of reports indicate that DCs in the thymus participate in the negative selection process (31, 51–53), including those DCs bringing antigen from peripheral sites (31, 53). The significance of DCs in thymic Treg selection and negative T-cell selection has been, however, challenged recently by the findings that medullary thymic epithelial cells select the Treg repertoire (54) and that mice with a strong (but not complete) reduction in DC numbers show normal Treg generation and negative selection in the thymus (55).

Table 2.1
Mouse DC subsets and their location

Organs	Phenotype	DC type	References
	Lymphoid organs		
Bone marrow	$CD11c^+MHC\text{-}II^+CD103^+F4/80^+$	Resident DCs	(49)
Thymus	$CD11c^+MHC\text{-}II^+CD8\alpha^+Sirp\text{-}\alpha^-$ $CD11c^+MHC\text{-}II^+CD8\alpha^{-/lo}Sirp\text{-}\alpha^+$	Resident DCs	(53)
Spleen	$CD11c^+MHC\text{-}II^+CD8\alpha^-CD4^-$ $CD11c^+MHC\text{-}II^+CD8\alpha^-CD4^+$ $CD11c^+MHC\text{-}II^+CD8\alpha^+CD4^-$	Resident DCs	(26)
LN	$CD11c^+MHC\text{-}II^+CD8\alpha^-CD4^-$ $CD11c^+MHC\text{-}II^+CD8\alpha^-CD4^+$ $CD11c^+MHC\text{-}II^+CD8\alpha^+CD4^-$ $CD11c^+MHC\text{-}II^+CD8\alpha^{lo}CD205^{int}$ $CD11c^+MHC\text{-}II^+CD8\alpha^{lo}CD205^{hi}$	Resident DCs Migratory DCs	(77)
	Non-lymphoid organs		
Skin	*Langerhans cell:* $CD11c^+MHC\text{-}II^+langerin^+EpCAM^-CD103^-F4/80^+$ *Dermal dendritic cell:* $CD11c^+MHC\text{-}II^+langerin^+CD11b^{hi}EpCAM^-CD103^-F4/80^+$ $CD11c^+MHC\text{-}II^+langerin^+CD11b^{low}EpCAM^-CD103^+F4/80^-$	Migratory DCs	(92)
Intestine	$CD11c^+MHC\text{-}II^+CD8\alpha^-$ $CD11c^+MHC\text{-}II^+CD8\alpha^+$	Migratory DCs	(96, 97)
Kidney	$CD11c^+MHC\text{-}II^+CD8\alpha^-$ $CD11c^+MHC\text{-}II^+CD8\alpha^+$	Migratory DCs	(105)
Liver	$CD11c^+MHC\text{-}II^+CD8\alpha^-$ $CD11c^+MHC\text{-}II^+CD8\alpha^+$	Migratory DCs	(111)

DCs in the thymus can be subdivided into two subsets based on the surface expression of CD8α and Sirp-α (53, 56). The $CD8^{+}$Sirp-α^{-} are generated from intrathymic DC precursors (57, 58) and represent around 70% of total cDCs in the thymus (59), whereas the $CD8^{-/lo}$Sirp-α^{+} are believed to be migratory cDCs that come from the periphery (59). The role for thymic cDCs in negative selection has been demonstrated (31, 51, 60, 61); however, the role of the respective cDC subtypes in T-cell selection has to be determined. Moreover, it has recently been shown that thymic $CD8^{-/lo}$Sirp-α^{+}cDCs are more efficient in generating regulatory T cells in vitro than other DC subtypes (53), suggesting their role in the tolerization of peripheral antigen by generation of regulatory T cells.

3.1.3. Spleen

$CD11c^{hi}$ MHC-II^{+} cDCs in the spleen are the best characterized in terms of function and phenotype. This is because the spleen is the organ where larger numbers of cDCs can be obtained (about 3×10^{6} cells per mouse spleen). Surface expression of CD8α and CD4 allows the identification of three different subsets within cDCs: $CD4^{-}CD8^{+}$, $CD4^{-}CD8^{-}$, and $CD4^{+}CD8^{-}$ (26) (*see* **Table 2.1**). $CD4^{-}CD8^{+}$ cDCs represent 20% of the splenic DCs and are localized in the T-cell area (62). The $CD4^{+}CD8^{-}$ cDCs constitute the majority of splenic cDCs and are mainly localized in the marginal zone together with the $CD4^{-}CD8^{-}$ (62) and migrate to T-cell areas of spleen upon stimulation (63, 64).

$CD8^{+}$ cDCs are particularly good at taking up antigen from cellular debris and they have been shown to be required for CD8 T-cell priming against viruses and tumors (65, 66). The role of $CD8^{-}$ cDCs in spleen is not clear. They have been suggested to direct Th2-type immune responses by stimulating the production of IL-4 (43, 67). Furthermore, a division of labor between $CD8^{+}33D1^{-}CD205^{+}$ DCs and $CD8^{-}33D1^{+}CD205^{-}$ DCs in spleen has been proposed in which the former are specialized in priming CD8 T-cell responses, whereas the latter promote CD4 T-cell responses (68). However, whether this comes as a result of the antibody-based strategy to deliver antigen to either $CD205^{+}$ or $33D1^{+}$ DCs rather than a specific function of these cells remains to be elucidated. A coordinated function of different DC types for immune regulation has been proposed which may allow the DC system to adapt to different infections (17).

After cDCs, pDCs constitute another significant population of DCs in the spleen. Although the role of pDCs in immune responses is more elusive, they are the major producers of IFN-α following viral infection (34, 69), a cytokine that has been shown to increase T- and B-cell responses (70, 71). pDCs show a different strategy of MHC-II antigen presentation than cDCs. The latter reduce de novo antigen presentation following activation by inflammatory stimulus (22) and, by doing so, present an

"antigenic snapshot" of the extracellular material that was taken up at the time of activation. However, pDCs proceed with de novo antigen presentation onto MHC-II after maturation, which reduces their ability to present extracellular material taken up at the time of activation but enhances their ability to present endogenous antigens (22). The role of pDCs in induction of T-cell tolerance vs. immunity appears to be similar to that of cDCs in that resting pDCs have been shown to induce tolerance (72), whereas pDCs activated by inflammatory stimuli are able to prime T-cell responses (73, 74).

3.1.4. Lymph Nodes

The three cDC subsets described in the spleen based on CD8α and CD4 expression are also present in the lymph nodes (LN). The $CD8^+$ cDCs are localized in the paracortical region of the LNs (75), whereas the $CD8^-$ cDCs are in the subcapsular sinus and immediate perifollicular zone of LNs (76). Besides these populations of cDCs, LNs also contain two other DC populations representing the mature form of tissue-derived cDCs which migrate into the draining LNs. These are $CD8^{lo}CD205^{hi}$ and $CD8^{lo}CD205^{int}$ DCs (77). These cells are migratory DCs that may constitute about 50% of the total cDCs in LNs (78). The most characterized migratory DCs are the $CD8^{lo}CD205^{hi}langerin^+$, a marker for Langerhans cells (LC), and dermal dendritic cell (DDC) and are believed to be their mature form migrating from the skin (77). These are mainly present in skin-draining lymph nodes, whereas the $CD8^{lo}CD205^{int}$ are present in most other lymph nodes (79), including lung-draining mediastinal LNs, renal LNs, and hepatic LNs and are thought to migrate from the peripheral tissues (80). Upon activation, tissue-derived cDCs collect antigen at the peripheral sites and migrate to the LNs where they present it to naïve T cells (1). In addition, it has been reported that migration of tissue-derived cDCs occurs also during the steady state even in the absence of microbes (77, 81, 82), suggesting a contribution to peripheral tolerance (83).

3.2. Dendritic Cells in Non-lymphoid Organs

The non-lymphoid organs that are covered in this review are those having barrier function and filter function, because of their prominent role in protection against invading pathogens. DCs in sensory organs such as the eye or in the brain are outside of the scope of this review.

3.2.1. Organs with Barrier Function

Skin and mucosa of the respiratory, reproductory, and gastrointestinal tract constitute the first line of defense against invading pathogens. Due to the exchange function of most mucosa, only a thin epithelial layer of cells separates the body from the *outside*. This is required for the mucosa to perform its basic functions (e.g., gas exchange in the respiratory mucosa, food exchange in the gut mucosa). However, it makes these tissues

particularly vulnerable to invasion by pathogens. Migratory DCs in mucosal tissues take up local antigen and transport it to the draining lymph nodes for the initiation of T-cell responses (e.g., see Ref. (29)).

3.2.1.1. Skin

The skin is the largest organ of the body and one of the first barriers against pathogens. Two main populations of DCs occur in normal skin: epidermal DCs named Langerhans cells (LCs) and dermal DCs (DDCs) (84). LCs represent 3–5% of the total epidermal cells and build a large network to survey the epidermis for foreign antigens. Although langerin was first used to specifically mark LCs, detailed studies have shown that DDCs and certain DCs in several lymphoid and non-lymphoid organs also express langerin (85–90). However, it is not clear whether langerin$^+$ DCs in other organs are derived from skin DCs. Although at first this may seem unlikely, there is evidence that skin DCs enter systemic circulation under experimental conditions. Mutant mice lacking lymph nodes have accumulation of skin-derived DCs in spleen, liver, and lung (91), indicating that skin DCs can seed organs systemically if they are not retained by skin-draining LNs. Furthermore, upon skin painting with FITC, skin DCs have been detected in the thymus. Because langerin is not a suitable marker for differentiating LCs and dermal DCs, a more detailed phenotype is used to differentiate the three main DC subpopulations in the skin (epidermal Langerhans cells, dermal langerin$^+$ or langerin$^-$ DCs) (*see* **Table 2.1**) based on differential expression of EpCAM, CD103, and F4/80 (92). Although much has been learned in the last few years regarding phenotype and migration of skin DCs, their role in immune responses remains unresolved (for reviews on this issue see Refs. (20, 92, 93)). A picture is emerging in which langerin$^+$ skin DCs play an important role in priming T-cell responses in some but not all immunizing regimes (94, 95).

3.2.1.2. Intestine

The gastrointestinal track hosts a large number of commensal bacteria that are beneficial for the body but it is also an entry site for pathogens. Therefore, an important role of the immune system in this organ is to protect against infection along with avoiding the destruction of normal microbiota. The CD8$^-$ cDC constitute the main cDC subsets in the lamina propria of the small intestine, whereas the presence of CD8$^+$ cDC subsets is still controversial (96–98). In the colon, DCs are mainly located in the lymphoid follicles and only few were observed in the lamina propria (99–101). It is now clear that DCs have an important role in choosing the nature of the immune response that takes place in the gut. Some mechanisms have been resolved and retinoic acid seems to be a key factor in their functional regulation (18). In addition, mucosal DCs might also have an important function in

induction, maintenance, or downregulation of abnormal mucosal inflammation, as occurs in allergy.

3.2.2. Organs with Filter Function

Spleen, kidney, and liver are organs that fulfill a filter function for blood. As a result, DCs in these organs are easily exposed to protein antigens (innocuous or harmful). Their capacity to migrate to T-cell areas of lymphoid organs suggests that they may be important in the establishment of peripheral T-cell tolerance to self and to participate in the initiation of immune responses against invading pathogens. DCs present in the spleen have been dealt with in **Section 3.1.3**. Below is a summary of some known aspects of DCs in kidney and liver.

3.2.2.1. Kidney

Proteins under 68 kDa can reach the kidney tubular lumen and be reabsorbed and released back into the circulation (102, 103). During this process, kidney DCs also take up antigens that reach the tubular lumen (104). It has recently been shown that kidney DCs are able to either tolerize or prime T cells based on their activation status (104–106). The kidney contains high numbers of DCs, which are characterized by expression of CD11c, MHC class II, and CD11b (27). Most kidney DCs are $CD8^-$ (~95%), whereas a small number are $CD8^+$ DCs and pDCs (104, 105, 107). Altogether they comprise the renal DCs (rDCs), which constitute a true anatomical surveillance network within the renal parenchyma (107). With an average half-life of 35 days, rDCs have a much longer life-span than DCs in lymphoid organs (104). Besides regulating T-cell responses, mounting evidence is accumulating that kidney DCs maintain the homeostasis of the renal parenchyma (27, 108–110).

3.2.2.2. Liver

In vascular terms, the liver is located downstream the gut and, therefore, it is exposed continuously to gut-derived antigens and endotoxin. The lower TLR-4 expression by liver DCs compared with splenic DCs may explain their relative tolerance to endotoxin stimulation (111). Liver DCs have been suggested to be able to induce both T-cell tolerance and immunity depending on numbers and activation status (112). For instance, liver DCs are poor activators of antigen-specific T-cell immunity in response to endotoxin (111). However, experimental administration of Flt3-L increases the number of DCs in liver correlating with increased transplant rejection (113).

4. Dendritic Cells During Tumor Progression

There is evidence that DCs play a key role in the induction of tumor-specific immune responses, specially via cross-priming

through MHC-I antigen presentation (66). However, despite the expression of immunogenic antigens, tumors often induce a state of immune tolerance that allows for uncontrolled growth. Many mechanisms are recognized to contribute to immune tolerance to tumors, ranging from thymic deletion of T cells bearing high-affinity TCR for tumor antigens to local suppressive mediators (for a review see, e.g., Ref. (114)). Increasing evidence indicates that the tumor microenvironment is an important component leading to DC dysfunction (115). Dendritic cells are believed to be attracted into the tumors by several chemoattractants, including CXCL12, CXCL8, VEGF, and β-defensin which are produced by the tumor microenvironment (116). Tumors often contain low numbers of DCs, with a phenotype that usually reflects a *resting*, non-activated DC based on high CD11c and MHC-II expression but low expression of costimulatory molecules. However, these *resting* DCs display several functions that promote tumor growth, either indirectly by promoting immune T-cell tolerance or directly by supporting tumor growth (for recent reviews see, e.g., Refs. (24, 115, 117)). Below we summarize some features of tumor DCs that contribute to tumor growth.

4.1. DC-Induced T-Cell Tolerance in Cancer

Different tumors secrete TGF-β (118) and IL-10 (119). IL-10 and TGF-β are well known for their suppressive actions on T-cell responses and are suggested to be important players in the induction of T-cell tolerance in the tumor. Modulation of DC function by these cytokines is believed to play an important role in the tolerance process. For instance, DCs of tumor-draining lymph nodes promote local generation/expansion of FoxP3+ regulatory cells (120), which are known to inhibit effector T-cell responses (121, 122). On the other hand, IL-10 has been shown to have a strong inhibitory effect on DCs by directing tumor-specific T cells into anergy (123). Other mediators produced by tumors with inhibitory properties on DC function or development include IL-6, VEGF, prostanoids, and reactive oxygen species (for a recent review see, e.g., Ref. (117)).

4.2. Pro-angiogenic Activity of DCs in Tumors

Besides the immunological role of DCs in tumor immunity, DCs can display pro-angiogenic activity that favors tumor growth. This is not a specific function of DCs, since many myeloid cells can support tumor angiogenesis (116). Thus, although DCs are believed to be important players in tumor angiogenesis, their specific contribution to this process is still unclear.

Tumor DCs have pro-angiogenic function at least at two different levels. First, they secrete a range of factors that induce other cell types to secrete pro-angiogenic mediators. This is the case of TNF-α and CXCL8 production by ovarian cancer DCs that prime endothelial cells to secrete a wide range of pro-angiogenic

factors (124). Second and, perhaps, more interesting is the fact that tumor CD11c^{hi} MHC-II^{+} DCs themselves are a source for cells with endothelial cell (EC)-like phenotype that incorporate into the blood vessels of tumor-bearing mice (125). However, whether these cells represent truly DCs is still a matter of debate because they also express phenotypic markers of EC (125). However, results from in vitro experiments may favor the concept that DCs are indeed a source of EC-like cells. When tumor DCs are incubated with VEGF and other pro-angiogenic factors they trans-differentiate leading to upregulation of EC markers and downregulation of leukocyte markers (126, 127).

5. Dendritic Cell Precursors

5.1. Dendritic Cell Development in Steady State

All leukocyte lineages arise from hematopoietic stem cells (HSCs) in the bone marrow with self-renewal capacity (128). Early commitment steps in hematopoiesis result in the development of common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs), with a strong bias for lymphoid and myeloid lineage differentiation (129, 130). DC generation is the result of a remarkable flexibility in their lineage differentiation. For instance, a single CMP or CLP can differentiate into both cDCs and pDCs (131) indicating that DC commitment occurs downstream of CMPs and CLPs. In addition, recent studies indicate that hematopoietic differentiation is not absolutely linear but there exist graded stages of lineage differentiation (for a recent review see (132)).

In the last years, there has been considerable effort to understand and trace the precursors that lead to DC generation as well as the required growth and transcription factors (see excellent reviews on this issue (132, 133)). A picture has emerged in which DC differentiation is a continuous process involving different locations in the body. During steady state, DC differentiation is initiated in the bone marrow and proceeds in other lymphoid organs such as spleen and lymph nodes, which are seeded by precursors from the circulation (38, 134, 135, 150). An emerging grouping of precursors with *restricted* DC differentiation potential has been proposed comprising early DC precursors, which result in the generation of various DC types, and late DC precursors that differentiate into specific populations of DCs. The lineage relationship between these precursors remains to be elucidated. We will summarize the basic findings regarding DC differentiation. Further insight can be obtained in reviews (132, 136).

5.1.1. Early DC Precursors

They are found in the bone marrow and are $CD11c^{-}Lin^{-}$ with high proliferation capacity (*see* **Table 2.2** for phenotype summary of DC precursors). *MDPs* (macrophage and dendritic cell precursors) are c-Kit^{+} and give rise to macrophages, monocytes, and DCs in vitro and in vivo, showing that they are not restricted to the DC lineage (137, 138). *pro-DCs* (139) and *CDPs* (common DC precursors) (140) express intermediate levels of c-Kit and appear to be restricted to the DC lineage, giving rise to both cDCs and pDCs in vitro and in vivo. Pro-DCs differentiate into downstream pre-DC precursor with the potential to give rise to $CD8^{+}$ and $CD8^{-}$ cDCs, but not pDCs (38). Flt3-L is required to differentiate both early and late DC progenitors into DCs (138, 141). $CD11c^{-}Lin^{-}$c-Kit^{+} DC precursors in the spleen with the potential to differentiate into cDCs under certain conditions have also been identified (K. Hochweller and N. Garbi, unpublished).

5.1.2. Late DC Precursors

These precursors are characterized by being $Lin^{-}CD11c^{+}$MHC-II^{-}. They are present in the bone marrow and secondary lymphoid organs, but have a lower proliferation capacity than early DC precursors. Among them, the splenic *pre-DC* is the most studied. Pre-DCs are a different cell type than monocytes and give rise to cDCs but not pDCs (38), in a process that is Flt3-L-dependent (138). $Lin^{-}CD11c^{+}$MHC-II^{-} late DC precursors have also been found in bone marrow, spleen, lymph nodes, Peyer's patches, and thymus with high potential to differentiate into cDCs (134). These precursors may be similar to pre-DCs, although their relationship remains to be elucidated. In the bone marrow, $CD11c^{+}$MHC-II^{-} DC precursors can be separated into $CD45R^{+}$ precursors (restricted to pDC and cDC differentiation) and $CD45R^{-}$ precursors (which differentiate solely into cDCs) (142). Circulating DC precursors have also been found in circulation in the blood (135, 143), although the precise relationship with pre-DCs is not known. A summary of the phenotype of late DC precursors is shown in **Table 2.2**.

5.1.3. Thymic DC Precursors

The majority of DCs in the murine thymus are the phenotypical equivalent to splenic $CD8^{+}$ DCs (58). Surprisingly, thymic $CD8^{+}$ DCs have been shown to have a *lymphoid past* since they are generated from lymphoid precursors in the thymus (144, 145). Accordingly, about 70% of thymic DCs show IgH D-J DNA rearrangement in contrast to DCs in other lymphoid organs, confirming their *lymphoid past*. The small $CD8^{-}$ DC population of the thymus is not generated from lymphoid precursors but they may differentiate from early/late precursors described earlier.

5.1.4. Langerhans Cells Precursors

It has been suggested that LCs are maintained locally for life under non-inflammatory conditions (146), either by self-renewal

Table 2.2
DC progenitor population (adapted from Ref. (133))

Name	Organs	Phenotype	Differentiation potential in vitro	Differentiation potential in vivo	References
		Early DC progenitors			
MDPs	BM	c-Kit$^+$Lin$^-$CD117$^+$CX$_3$CR1$^+$CD11b$^-$	DCs, macrophages	Monocytes, DC, macrophages	(137)
MDP	BM	c-Kit$^+$Lin$^-$CD117$^{+/-}$CD115$^+$	–	DCs, macrophages	(138)
CDPs	BM	c-Kit$^+$Lin$^-$CD117^{int}CD135$^+$CD115$^+$CD127$^-$	pDCs, cDCs	pDCs, cDCs	(140)
Pro-DCs	BM	c-Kit$^+$Lin$^-$CD117^{int}CD135$^+$CD16/32^{lo}	pDCs, cDCs	pDCs, cDCs	(139)
		Late DC progenitors			
B220-precursors	BM	c-Kit$^-$CD11c$^+$MHC-II$^-$B220$^+$	pDCs, cDCs	pDCs, cDCs	(142)
B220+precursor	BM	c-Kit$^-$CD11c$^+$MHC-II$^-$B220$^-$	DC	DC	(142)
Pre-immunocytes	BM	c-Kit$^-$CD11c$^+$CD31$^+$Ly6C$^+$	pDCs, cDCs, macrophages	–	(149)
Pre-DCs	Spleen	c-Kit$^-$CD11c^{int}CD45R^{lo}CD43^{int}Sirp-α^{int}	cDCs	cDCs	(38)
DC precursors	Blood	c-Kit$^-$CD11c$^+$MHC-II$^-$	–	pDC, DCS	(143)

or by differentiation of in situ precursors. This is exceptional in the DC compartment, since all other DCs are known to have relatively short half-lives and to be renewed from circulating precursors in the steady state. However, it was recognized long ago that circulating hematopoietic cells differentiate into skin LCs following disruption of skin homeostasis (9, 10). A recent study has identified blood monocytes as LC precursors under inflammatory conditions (147).

5.2. Development of Inflammatory Dendritic Cells

Certain DCs are generated specifically under inflammation conditions and they are called inflammatory DCs. The best example are $CD11c^{int}CD11b^{int}MAC\text{-}3^{hi}$ Tip-DCs that are generated upon infection with certain bacteria (36, 37).

Besides LCs (see above), monocytes are able to differentiate into inflammatory DCs following strong inflammatory regimes. $CCR2^{+}$ $Ly6C^{+}$ inflammatory monocytes enter sites of inflammation and differentiate into inflammatory DCs, whereas they do not differentiate into DCs under the steady state (38, 39, 148). Whether the Tip-DCs generated during inflammatory conditions have a monocytic origin is not formally proven; however, the fact that they are absent in $CCR2^{-/-}$ mice strongly suggest so.

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Section II

Human DC Protocols

Chapter 3

Isolation of Human Blood DC Subtypes

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Abstract

Human blood dendritic cells (DCs) are a rare, heterogeneous cell population that comprise approximately 1% of circulating peripheral blood mononuclear cells (PBMCs). Their isolation has been confounded by their scarcity and lack of distinguishing markers and their characterisation perplexed by the recent discovery of phenotypic and functionally distinct subsets. Human blood DCs are broadly defined as leukocytes that are HLA-DR positive and lack expression of markers specific for T cell, B cell, NK cell, monocyte and granulocyte lineages. They can be subdivided into the $CD11c^-$ ($CD123^+CD303^+CD304^+$) plasmacytoid DC and $CD11c^+$ myeloid DC, which can be further subdivided into three subsets based on differential expression of CD1c, CD141 and CD16. DC can be isolated from peripheral blood by using an initial density gradient centrifugation step to enrich for mononuclear cells followed by immunomagnetic depletion of cells expressing markers specific for leukocyte lineages and undesired DC subsets. Subsequent flow cytometry-based cell sorting allows the isolation of highly pure individual DC subsets that can then be used for functional studies.

Key words: Dendritic cells, blood, immunomagnetic selection, flow cytometry.

1. Introduction

Human blood DCs are a heterogeneous cell population that originate from bone marrow precursors and comprise approximately 1% of circulating peripheral blood mononuclear cells (PBMCs). Their isolation in sufficient numbers and purity to perform functional studies is challenged by their scarcity and a lack of distinguishing markers. Hence to date they have been poorly

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characterised. Blood DCs have been classically defined as leukocytes that lack markers of other leukocyte lineages CD3 (T cells), CD14 (monocytes) CD19/20 (B cells), CD56 (NK cells), CD15 (granulocytes) and CD34 (haematopoietic progenitors), and express high levels of major histocompatibility complex (MHC) class II molecules (HLA-DR) (1). Early isolation protocols enriched for a lineage negative (lin^-) DC population by density gradient centrifugation and/or immunoselection using cocktails of lineage antibodies (2), but these preparations are now known to comprise several subpopulations. CD11c broadly divides $lin^-HLA\text{-}DR^+$ blood DC into the $CD11c^-$ plasmacytoid (pDC) and $CD11c^+$ myeloid (mDC) subsets (3–5). pDCs comprise approximately 18% of the $lin^-HLA\text{-}DR^+$ population and can also be distinguished from mDC by their expression of CD123, CD303 (BDCA-2) and CD304 (BDCA-4/neuropilin-1) (6). $CD11c^+$ myeloid DC (mDC) comprises over 70% of $lin^-HLA\text{-}DR^+$ cells and can be further subdivided into three distinct subsets (6–8). The $CD1c^+$ (BDCA-1) subset represents around 19% of $lin^-HLA\text{-}DR^+$ cells and is the most extensively studied mDC subset. The $CD16^+$ subset constitutes approximately 50% of $lin^-HLA\text{-}DR^+$ cells and has been largely overlooked due to the inclusion of CD16 in lineage antibody cocktails and their poor viability in vitro. The $CD141^+$ (BDCA-3) subset is the least abundant, constituting around only 3% of $lin^-HLA\text{-}DR^+$ cells and is hence poorly characterised.

The discovery of these new DC surface markers and more sophisticated immunomagnetic selection, including commercially available kits for some DC subsets and flow cytometric cell sorting technologies, has now allowed the isolation of the distinct DC subpopulations and their functional analysis for the first time. The level of purity required is a crucial factor when choosing an isolation protocol. Even small percentages (<2%) of contaminating lineage cells, particularly NK or T cells, can have profound effects on DC phenotype and function and this may lead to spurious conclusions. Positive immunomagnetic selection of such rare cell populations is often confounded by the non-specific binding of dead cells to magnetic beads. Furthermore, despite the usefulness of some of the markers to distinguish individual subsets, their application for direct positive immunoselection is often precluded by direct functionality of antibody binding (e.g. CD303) or by weak expression on other DC subsets or lineage cells (e.g. CD1c is also expressed on a population of B cells, CD141 is weakly expressed by some $CD1c^+$ mDC and pDC and CD123 is weakly expressed by some mDC subsets). It is also important to note that DC viability, phenotype and function can be modulated to various extents by different isolation protocols. For this reason, when establishing an isolation protocol, DCs should be carefully phenotyped and some basic functional studies

performed (e.g. allogeneic mixed lymphocyte reaction) as described elsewhere (7). The identification of novel DC activation markers, CMRF-44 and CMRF-56, has led to the development of positive immunomagnetic selection protocols that are clinically applicable (9–11). These protocols rely on the partial activation of DCs after overnight culture and predominantly consist of the $CD1c^+$ mDC subset along with some B cells and monocytes. Such preparations are potent antigen-presenting cells for the induction of T-cell responses and are also suitable for vaccination, but their heterogeneous nature and partial activation make them unsuitable for studying DC subset biology.

The following protocol is designed for the isolation of highly pure (>99%) individual human blood DC subsets that is essential for their functional characterisation. Mononuclear cells (MNCs) are first isolated by density gradient centrifugation (**Section 3.1**) followed by an initial enrichment of the desired DC subset by depletion of lin^+ cells, residual red blood cells (using CD235a) and, where applicable, other DC subsets, by immunomagnetic selection (**Section 3.2**). Positive selection by fluorescence-activated cell sorting (FACS, **Section 3.3**) then allows for the complete removal of residual lin^+ cells and enables precision in selecting the desired DC subpopulation based on the intensity of expression of the applicable marker. As expected for such rare cell populations, yields are low but the use of leukapheresis products allows for the isolation of multiple subsets from an individual donor and repeating the isolation procedure results in sufficient yields of even the rarest subsets (e.g. $CD141^+$ DC) for most functional analyses.

2. Materials

1. Fresh whole blood or buffy coats obtained by venipuncture or leukapheresis products (*see* **Note 1**).
2. Ficoll–Hypaque (Pharmacia).
3. 1X Phosphate-buffered saline (PBS) pH 7.2 (Invitrogen).
4. Complete medium: RPMI-1640 (Invitrogen) supplemented with 10% human AB serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (Invitrogen).
5. Running buffer: PBS, supplemented with 0.5% bovine serum albumin (BSA) (Invitrogen) and 2 mM ethylene diamine tetra-acetic acid (EDTA) (Merck). Keep buffer cold (4–8°C).

6. Goat anti-mouse IgG microbeads (Miltenyi Biotec).
7. Purified and fluorescent monoclonal antibodies (mAbs) listed in **Tables 3.1–3.3** (*see* **Notes 2** and **3**).
8. Mouse serum (10% in PBS) (Sigma).
9. 30 μm Pre-separation filter (Miltenyi Biotec).
10. autoMACS™ Separator.
11. FACSAria™ or similar FACS instrument capable of a minimum of four-colour sorting.

Table 3.1
Unconjugated primary mouse IgG antibodies used for cell depletion (Section 3.2)

mAb	Clone, supplier	Per 1×10^8 cells (μg)	CD1c$^+$ mDC	CD141$^+$ mDC	CD16$^+$ mDC	pDC
CD3	OKT3, ATCC	2	Yes	Yes	Yes	Yes
CD14	RMO52, Beckman Coulter	2	Yes	Yes	Yes	Yes
CD19	J3-119, Beckman Coulter	2	Yes	Yes	Yes	Yes
CD20	B9E9, Beckman Coulter	2	Yes	Yes	Yes	Yes
CD34	My10, BD Biosciences	2	Yes	Yes	Yes	Yes
CD56	N901, Beckman Coulter	2	Yes	Yes	Yes	Yes
CD235a	GA-R2, BD Biosciences	2	Yes	Yes	Yes	Yes
CD11c	BU15, Beckman Coulter	2	**No**	**No**	**No**	Yes
CD1c[a]	AD58E7, Miltenyi Biotec	2	**No**	Yes	Yes	Yes
CD141	AD5-14H12, Miltenyi Biotec	2	Yes	**No**	Yes	Yes
CD16	3G8, Beckman Coulter	2	Yes	Yes	**No**	Yes
CD304	AD5-17F6, Miltenyi Biotec	2	Yes	Yes	Yes	**No**

[a] *See* **Note 4**

Table 3.2
'Exclusion' fluorescent-conjugated antibodies for FACS sorting (Section 3.3)

mAb	Clone, supplier	Volume per test (μl)	CD1c$^+$ mDC	CD141$^+$ mDC	CD16$^+$ mDC	pDC
Sheep anti-mouse-PE or FITC	Chemicon	2	Yes – –PE	Yes – FITC	Yes – FITC	Yes – FITC
CD15-PE or FITC[a]	MMA, BD Biosciences	5	Yes – PE	Yes – FITC	Yes – FITC	Yes – FITC

[a] *See* **Note 5**

Table 3.3
'Selection' fluorescent-conjugated antibodies for FACS sorting (Section 3.3)

mAb	Clone, supplier	Volume per test (μl)	CD1c$^+$ mDC	CD141$^+$ mDC	CD16$^+$ mDC	pDC
CD1b/c-FITC	B-B5, Diaclone	5	Yes	Yes[a]	**No**	**No**
CD11c-APC	S-HCL-3, BD Biosciences	5	**No**	**No**	Yes	**No**
CD16-PE	B73.1, BD Biosciences	5	**No**	**No**	Yes	**No**
CD141-APC	AD5-14H12, Miltenyi Biotec	10	Yes[b]	Yes	**No**	**No**
CD123-PE	9F5, BD Biosciences	5	**No**	**No**	**No**	Yes
CD304-APC[c]	AD5-17F6, Miltenyi Biotec	10	**No**	**No**	**No**	Yes

[a] *See* **Note 6**
[b] *See* **Note 7**
[c] *See* **Note 8**

3. Methods

3.1. Isolation of MNCs by Density Gradient Centrifugation

1. Aliquot 20 ml whole blood into 50-ml conical tubes and add an equal volume of room temperature (RT) PBS. For leukapheresis products aliquot 10 ml and add 30 ml RT PBS (*see* **Note 9**). Mix well.
2. Using a 10-ml pipette or syringe and cannula slowly layer 10 ml Ficoll–Hypaque underneath the blood/PBS mixture.
3. Centrifuge at 500 × *g* for 20 min at RT without braking.
4. Collect the mononuclear cell layer from the interphase and carefully transfer into fresh 50-ml conical tubes up to a volume of 20 ml.
5. Fill the 50-ml tubes with cold running buffer up to a 50-ml volume.
6. Centrifuge at 300 × *g* for 10 min at 4°C to remove platelets.
7. Pipette off the supernatant and gently resuspend the cell pellets in a small volume of running buffer.
8. Pool cells into a single 50-ml tube and wash with cold running buffer.
9. Centrifuge at 500 × *g* for 5 min at 4°C.
10. Resuspend the cell pellet in 50 ml running buffer and proceed with experiment.
11. Determine cell yield and viability using a haemocytometer and Trypan Blue exclusion.

3.2. Enrichment of lin$^-$ Cells by Immunomagnetic Depletion

1. Resuspend MNCs in a 50-ml tube at 10^8 cells per ml in cold running buffer with a maximum of 5×10^8 MNCs per separation run (*see* **Notes 3** and **10**).
2. Add 2 μg of each primary mouse IgG antibody per 10^8 total cells as indicated in **Table 3.1** for the required DC subpopulation. Mix well and incubate for 20 min at 4–8°C with occasional mixing (*see* **Notes 10** and **11**).
3. Wash the cells once with 10–20X labelling volume of running buffer at 500 × *g* for 5 min at 4°C to remove unbound primary antibody.
4. Pipette off supernatant completely and resuspend the cell pellet in 900 μl running buffer per 10^8 total cells.
5. Add 100 μl goat anti-mouse IgG microbeads per 10^8 total cells. Mix well and incubate for 15 min at 4–8°C, with occasional mixing.
6. Wash cells with 10–20X labelling volume of running buffer and centrifuge at 500 × *g* for 5 min at 4°C. Pipette off supernatant completely.
7. Resuspend the cell pellet in 0.5 ml cold running buffer per 10^8 total cells.
8. Prepare a pre-separation filter by applying 1 ml running buffer and discarding the flow-through. Pass cells through filter to remove cell aggregates.
9. Prepare and prime the autoMACS^{TM} separation device according to the manufacturer's instructions (*see* **Note 12**).
10. Place tube containing the magnetically labelled cells in the autoMACS^{TM} separator. Choose 'Depl025' program.
11. Collect negative fraction (outlet port 'neg1'). This fraction contains the enriched lin$^-$ DC population. (If using an LD column collect flow-through.)

3.3. Positive Selection of DC Subpopulations by FACS Sorting

1. Centrifuge negative fraction at 500 × *g* for 5 min at 4°C. Pipette off supernatant completely.
2. Label cell pellet with appropriate fluorescent sheep anti-mouse Ig and CD15 antibody for desired DC population (**Table 3.2**).
3. Mix well and incubate for 15 min at 4–8°C, with occasional mixing.
4. Wash the cells once with running buffer and centrifuge at 500 × *g* for 5 min at 4°C. Pipette off supernatant completely.
5. Resuspend the cell pellet in 50 μl 10% mouse serum.
6. Repeat Steps 3 and 4.

7. Label the cell pellet with appropriate 'selection' antibody mixture for identification of desired DC population (**Table 3.3**).
8. Repeat Steps 3 and 4.
9. Resuspend the cell pellet in running buffer (10^7–10^8 cells/ml).
10. Sort desired DC population by flow cytometry FACSAria^{TM} or similar device according to the criteria in **Fig. 3.1** (*see* **Note 13**).

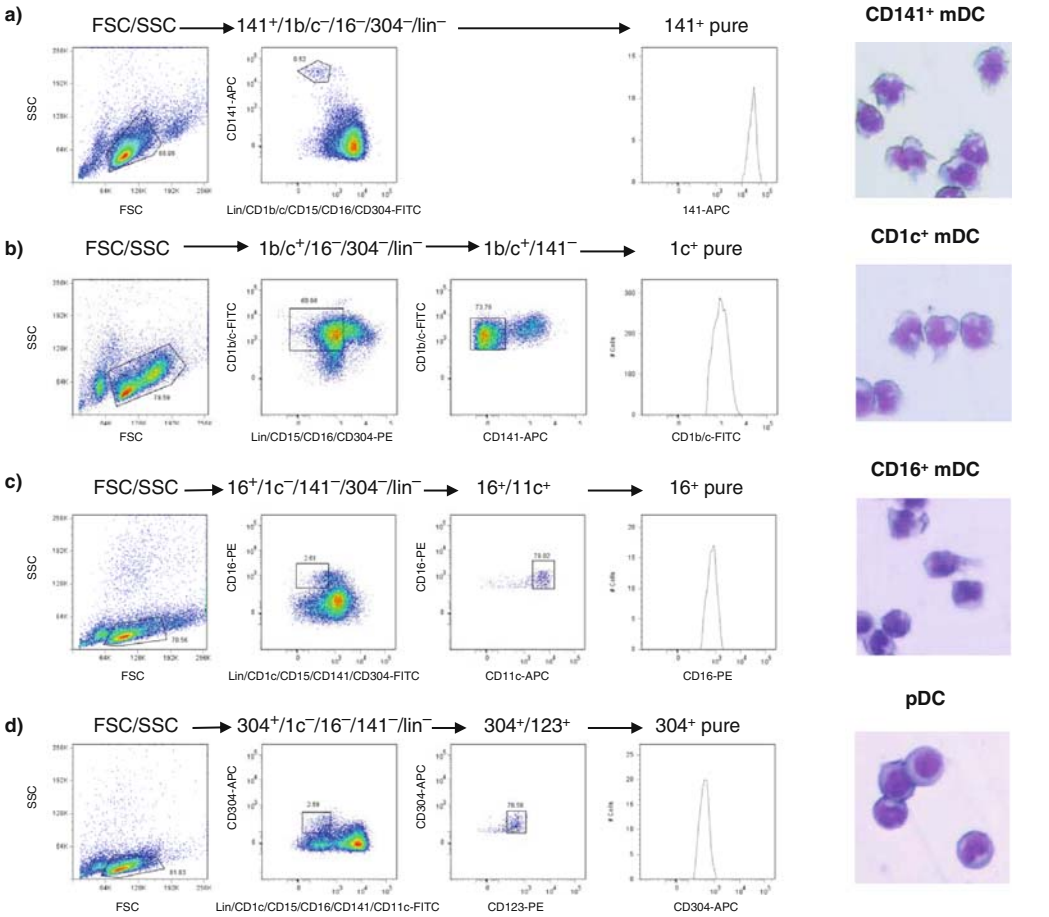

Fig. 3.1. Gating criteria for FACS sorting and morphology of human lin$^-$ DC subpopulations. For all populations cells are first gated by FSC and SSC to remove dead cells and debris (left of marked region) and cell aggregates (right and top of marked region). (**a**) Isolation of the CD141$^+$ DC population. As CD141 is weakly expressed on some CD1c and PDC, this population is sorted as lin-, CD1c- and CD304-FITC negative, and CD141-APC bright. (**b**) The CD1c$^+$ DC population is sorted as lin-, CD16- and CD304-PE negative, CD141-APC negative and CD1b/c-FITC positive. (**c**) The CD16$^+$ DC population is sorted as lin-, CD1c-, CD141- and CD304-FITC negative, CD11c-APC bright and CD16-PE positive. (**d**) PDCs are sorted as lin-, CD1c-, CD141- and CD11c-FITC negative, CD304-APC positive and CD123-PE positive.

11. Collect cells into complete medium for phenotypic or functional analysis. Expected yields per 5×10^8 starting MNCs are 5–8 $\times$ 10^5 CD1c$^+$ mDC, 0.5–1.0 $\times$ 10^5 CD141$^+$ mDC, 0.5–3.5 $\times$ 10^5 CD16$^+$ mDC and 3–5 $\times$ 10^5 pDC (*see* **Note 14**).

4. Notes

1. Acquisition of blood products requires approval from an appropriate ethics committee and written informed consent from the donor. When working with human blood appropriate biosafety practices (PC2 facilities) must be followed. Ideally DCs should be freshly isolated, however, good cell yields and viabilities can be obtained from whole anti-coagulated blood or leukapheresis products left sealed in their collection bag at RT for up to 20 h.
2. These antibody combinations are based on the isolation of a single DC subset per experiment and are designed for maximum purity of individual subsets. By using only CD3, CD14, CD19, CD20, CD34, CD56 and CD235a in the initial depletion step (**Section 3.2**) it is possible to then sort multiple DC subsets simultaneously by flow cytometry (**Section 3.3**) by excluding residual lineage cells (using PE-SAM and CD15-PE)) and staining with CD11c in combination with CD1c, CD141, CD16, CD123 or CD304, depending on the instrument's sorting capacity and fluorescent antibody combinations available. This practice is not recommended where high purities and maximum yields of individual subsets are required. Cell depletion kits and Ab-conjugated magnetic beads that use similar immunomagnetic separation technologies are also available (e.g. StemCell Technologies, Dynal®) and can alternatively be used for **Section 3.2**.
3. Antibody concentrations are optimal for starting populations of $\leq 5 \times 10^8$ MNCs. Do not exceed this cell number as it reduces binding efficiencies and increases sorting time, resulting in a reduction in the final yield, purity and cellular viability. When isolations from larger starting populations are required repeat the procedure using a maximum of 5×10^8 MNCs in each run.
4. Do not use CD1b/c antibody in this step.
5. The CD15 antibody (clone MMA) is mouse IgM and cannot be used in the initial depletion step (**Section 3.2**) as it will not bind goat anti-mouse IgG microbeads.

6. CD1c is not highly expressed on the cell surface. The addition of CD1b/c-FITC is recommended for the CD141^{+} mDC isolation procedure to absolutely exclude the CD141^{dim} CD1c^{+} cells.
7. Include CD141-APC into the CD1c^{+} mDC isolation procedure to ensure exclusion of CD1c/CD141 double positive cells.
8. CD303 (BDCA-2) also defines the pDC population but should not be used to isolate them as it is internalised upon receptor engagement.
9. Ficoll–Hypaque and PBS must be at RT before proceeding. For smaller blood or leukapheresis products density gradient centrifugation can be performed in 15-ml tubes provided the volumes are scaled down accordingly to maintain ratios of 1:2 whole blood:PBS, 1:4 leukapheresis:PBS and 1:5 Ficoll–Hypaque: blood product/PBS mixture.
10. Work fast, keep cells cold and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labelling.
11. Use a standard refrigerator rather than ice for 4–8°C incubations.
12. Separations (Steps 9–11) can alternatively be performed manually using a VarioMACS^{TM} Separator with LD or D columns (Miltenyi Biotec) or similar manual magnetic bead separation device according to the manufacturer's instructions.
13. Keep sorting times to less than 50 min to maintain cell viability.
14. These figures are donor dependent and are highly variable.

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Chapter 4

Isolation of Immature Primary Langerhans Cells from Human Epidermal Skin

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Abstract

Langerhans cells (LCs) are the main population of antigen-presenting cells lining the epidermis and stratified mucosal epithelia (1). Therefore, they play an important role in the first line of defense against invading pathogens. Upon capture of these pathogens, LCs subsequently migrate to the lymph nodes where they present pathogen-derived antigens to T cells to initiate an adaptive immune response. During this migration, LCs up-regulate cell surface marker HLA-DR and co-stimulatory molecules, while the LC-specific C-type lectin Langerin is down-regulated (reviewed in Refs. (2,3)). In the epidermis, LCs are the only cell population expressing CD1a and this marker is therefore extremely useful to isolate LCs from epidermis (4). Here we discuss a method to isolate human primary LCs from the epidermis in an as immature state as possible. The use of immature LCs is especially important in the investigation of the function of these cells, since few acceptable LC models are available. Immature LCs can be used to further elucidate the function of LCs in pathogen interactions and adaptive immunity.

Key words: Langerhans cells, CD1a, langerin, MACS isolation, primary cells, epidermis.

1. Introduction

Langerhans cells (LCs) are the main antigen-presenting cells lining the epidermis and stratified mucosal epithelia (1, 5). They play an important role in the protection against invading pathogens and are characterized by their expression of CD1a, Langerin, and Birbeck granules (4, 6, 7). The function of these cells in adaptive immune responses has not been completely elucidated. They might have an anti-viral function since recently it has been shown that LCs capture and degrade HIV-1 by C-type lectin Langerin, thereby preventing further infection and dissemination of

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HIV-1 (6). Upon activation in the epidermis, LCs migrate to the lymph nodes. There they present antigens to induce a proper T-cell response against the invading pathogens. During this maturation process, Langerin expression is down-regulated, while co-stimulatory molecules CD80, CD83, and CD86 are up-regulated (5, 7, 8).

In order to investigate the function of receptors such as Langerin and more in general the function of primary LCs it is important to use immature LCs. In previous editions of this book, a model to isolate LCs using their migratory capacity has been described. LCs obtained using this method have a more mature phenotype with high expression of CD80, CD83, and CD86 and an intermediate expression of Langerin (6, 8). The use of immature LCs in experimental research is especially important when investigating the pathogen uptake capacity of LCs, the function of LCs in the periphery, and the maturation of LCs. Currently, few appropriate model systems are available to study LC function in vitro. Therefore, the use of human primary material is still the most appropriate source of LCs for research purpose. In this chapter, we describe a method to isolate immature primary LCs from the epidermis based on CD1a isolation.

2. Materials

For the isolation of LCs it is important to use sterile and endotoxin-free reagents and material to prevent activation via Toll-like receptors. Soluble reagents can be sterilized by passing them over a 0.22-μm filter. The use of autoclaved reagents is not recommended, since they are often contaminated with endotoxins. Company-bought culture grade reagents are most often endotoxin free. Skin itself is per definition colonized with microorganisms. However, to prevent outgrowth of unwanted microorganisms, broad-spectrum antibiotics can be used, which can also be supplemented with a fungizone.

2.1. Epidermis Isolation

1. Dermatome, sterile blades (*see* **Note 1**).
2. Forceps. Sterilized by draining in 96% ethanol. Air-dry before use.
3. Self-made board to fix skin. Sterilized by draining in 96% ethanol. Air-dry before use. *See* **Fig. 4.1**.
4. Surgical sheets.
5. Petri dishes 145 mm/20 mm.
6. Gentamicin.

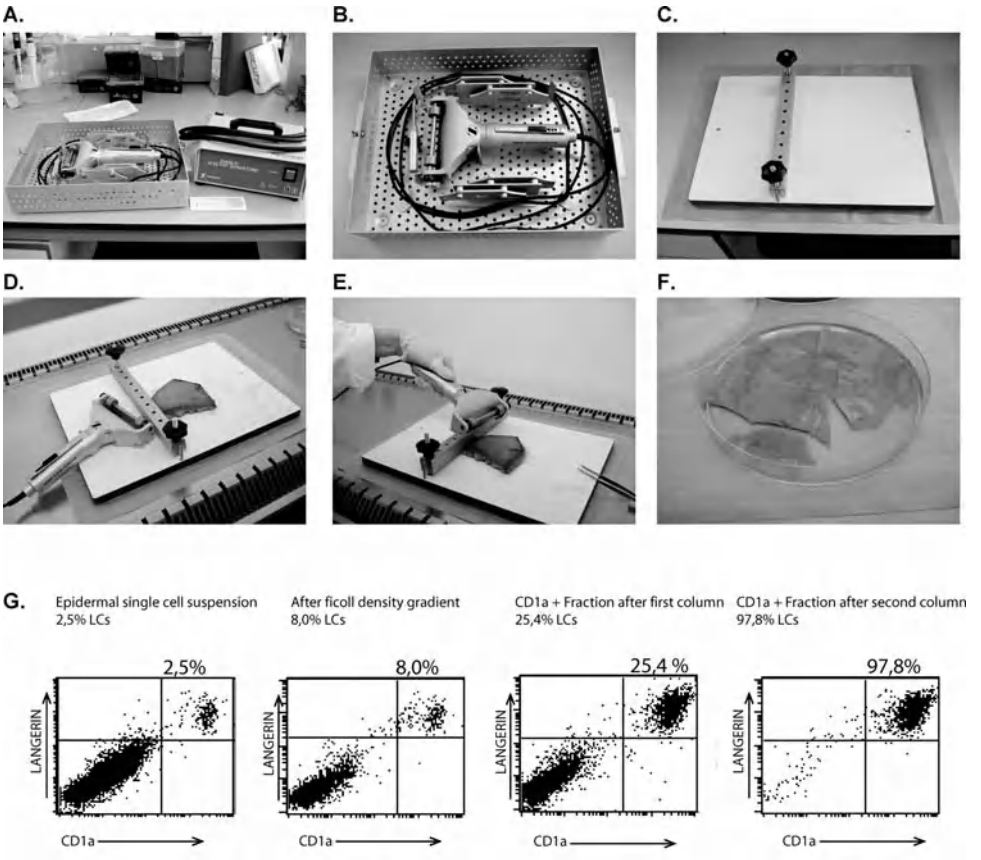

Fig. 4.1. Purification of Langerhans cells from epidermal cell suspension. (**A–F**) Materials needed for skin isolation. (**A**, **B**) Electric dermatome. (**C**) Board to fix skin. (**D**, **E**) Set-up for isolation of skin. (**F**) Skin floating in dispase/medium mixture. (**G**) Sequential enrichment steps lead to >90% pure LC population as determined by CD1a/Langerin expression.

7. PBS/gentamicin: Phosphate-buffered saline (PBS), sterile and endotoxin-free, is supplemented with 10 μg/ml gentamicin.
8. Iscove's Modified Dulbecco's Medium (IMDM).
9. Penicillin, streptomycin, and L-glutamine.
10. Heat-inactivated fetal calf serum (FCS).
11. Complete IMDM: IMDM supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM L-glutamine, and 20 μg/ml gentamicin, store at 4°C.
12. Dispase is dissolved at 2.5 mg/ml stock solution in PBS and stored at −20°C. Dispase should first be completely dissolved in PBS to a concentration of 10 mg/ml. Slowly add PBS to obtain a homogenous solution at a concentration of 2.5 mg/ml. Let the solution rotate for 4 h. Sterilize

dispase over a 0.22-μm filter membrane using a vacuum pump.

2.2. Single-Cell Suspension Preparation

1. Trypsin, 0.5% in PBS. Sterilize over a 0.22-μm filter membrane. Store at −20°C (10X stock).
2. DNase I: 10 mg/ml stock in sterile H_2O (100 mg in 10 ml Milli Q H_2O). Dilute further 1 to 1 in PBS to reach a 10,000 units/ml stock solution in PBS. Store reconstituted solution at −20°C.
3. Heat-inactivated FCS.
4. Complete IMDM medium.
5. 100 μm Cell strainer.
6. Lymphoprep Ficoll (Nycomed, Pharma, Oslo, Norway).

2.3. CD1a Isolation Using Magnetic Cell Sorting

1. CD1a microbeads (human) (Miltenyi Biotec, Bergisch Gladbach, Germany). Store at 2–8°C.
2. MACS separator: metal board and magnet (Miltenyi Biotec).
3. Large cell separation columns including needles (23 G 0.6 × 30 mm) (Miltenyi Biotec).
4. MACS buffer: 10 mM EDTA, 1% heat-inactivated FCS in PBS. Sterilize over a 0.22-μm filter membrane.
5. Complete IMDM medium.

2.4. Flow Cytometry Analysis

1. 96-Well V-bottom plate.
2. Antibodies against CD1a (CD1a-FITC, Pharmingen, San Diego, CA, USA), Langerin (DCGM4-PE, Beckman Coulter Inc., Miami, FL, USA) (calcium dependent), and other markers of choice (e.g., HA5.2B7-PE (CD86), Immunotech, Marseille, France). Store at 4°C and protect from light (*see* **Note 2**).
3. PBS/BSA: PBS, 0.5% BSA (bovine serum albumin fraction V, Roche Applied Science, Mannheim, Germany) and 0.02% sodium azide (very toxic, use gloves). Store at 4°C.

3. Methods

In the epidermis Langerhans cells constitute 1–3 % of the epidermal cells and approximately 460 LCs per mm^2 can be found (6, 9). Therefore, sequential isolation procedures are necessary to obtain a pure population. The procedure to isolate LCs is time-consuming. However, it is recommended to complete the isolation procedure within 24 h after surgery to ensure that the

LCs remain as immature and as little activated as possible. Where applicable, acceptable shortcuts in the procedure are noted. Since human material is used, it is important to follow your own institutional ethical guidelines for working with human materials (*see* **Note 3**).

In the first part of the procedure, the epidermis is isolated from patient material obtained from plastic surgery such as abdominal reconstructive surgery and abdominoplasty. Next, the epidermis is digested and a single-cell suspension is obtained. From this suspension, LCs can be selected using MACS isolation with magnetic anti-CD1a beads. If available at the institute, flow cytometry sorting using CD1a (or Langerin) antibodies can also be used. However, this method is not discussed here. Finally, the isolation procedure is evaluated using flow cytometry staining and the LCs can be used for cellular or other assays. It is recommended to collect a sample (100,000 cells) from each isolation step for analysis.

3.1. Epidermis Isolation

1. Skin from plastic surgery department is obtained within 3 h after surgery (*see* **Note 4**).
2. Place surgical sheets in a sterile cabinet and disinfect all devices with 96% ethanol before use and let them air-dry.
3. Fix the human material onto a board and use an electric dermatome to shave off 0.3-mm slices of the skin. This layer contains the epidermis and a small part of the dermis (*see* **Fig. 4.1A–F**).
4. Place the skin in 145-mm Petri dish with 50 ml PBS/gentamicin.
5. Rinse the skin twice in 100 ml PBS/gentamicin.
6. Dilute dispase 1 to 2 in IMDM medium.
7. Pipet 50 ml of dipase/medium mixture in a Petri dish and add the skin with the epidermis facing up. Dispase will cleave the basement membrane between the epidermis and dermis, which allows separation (*see* **Note 5**).
8. Incubate overnight at 4°C (*see* **Note 6**).
9. Using fine forceps detach the epidermis from the dermis (*see* **Note 7**).
10. Rinse the epidermis twice in complete IMDM to remove the dispase.

3.2. Single-Cell Suspension Preparation

1. Place the epidermis in two 50-ml tubes and fill the tubes with PBS.
2. Centrifuge at 700 × *g* for 10 min.
3. Discard the PBS and use sterile scissors to cut the epidermis into small pieces (10–50 mm^2).

4. Fill the tubes with 45 ml PBS, 5 ml trypsin (final concentration 0.05%), and 500 μl DNAse I (final concentration 100 U/ml). Trypsin acts as a proteolytic enzyme while DNAse I will prevent unwanted cell clumping as a result of DNA release during disaggregation procedures (*see* **Note 8**).
5. Incubate in a 37°C water bath for 30 min.
6. Prepare four 50-ml tubes with 25 ml FCS.
7. Add the epidermis suspension to the FCS tubes to inactivate trypsin.
8. Pipette up and down for at least 15 min using first a 25-ml pipet, followed by a 10-ml pipet (*see* **Note 9**).
9. Filter the cell suspension over a 100-μm cell strainer to obtain a single-cell suspension.
10. Centrifuge at 600 × *g* for 10 min and resuspend the pellet in 70 ml complete IMDM (room temperature). Filter the cells again over a 100-μm cell strainer. Count the cells and keep a sample for flow cytometry analysis. At this time, the population will consist of approximately 1–3% LCs (*see* **Note 10**).
11. Fill two tubes with 15-ml Lymphoprep and layer 35 ml of cell suspension carefully onto the Lymphoprep (*see* **Note 11**).
12. Centrifuge at 1,000 × *g* for 25 min at room temperature with low acceleration and no brakes, otherwise the separation gradient will be distorted. During this centrifugation based on density separation, LCs will remain on top of the Lymphoprep layer.
13. Carefully collect the cells from the interphase of the Lymphoprep layer.
14. Count the cells and keep a sample for flow cytometry analysis
15. Wash the cells in medium by centrifuging for 10 min at 600 × *g*.

At this point, the cell suspension will contain 5–10% LCs. Further isolation can be achieved by using (magnetic) cell sorting. However, for some experiment a 5–10% pure LC population may be sufficient to perform experiments, since LCs can easily be identified using staining for CD1a by flow cytometry. In addition, others have previously shown that consecutive density gradient centrifugation can be used to obtain a 75% pure LC population (10). Other separation techniques such as flow cytometry sorting or auto-MACS systems can also be used at this point. However, here we discuss CD1a isolation based on magnetic cell labeling, since no additional (expensive) equipment is needed.

3.3. CD1a Isolation Using Magnetic Cell Sorting

This protocol is adopted from the Miltenyi Biotec manual. First the CD1a-positive cells in the cell suspension are magnetically labeled with CD1a microbeads. Next, the cells are loaded onto a MACS column, which is placed in the magnetic field of a MACS separator. Unlabeled cells will pass through the column, while CD1a-labeled cells are retained in the column. After several washes, the column is removed from the magnetic field and the CD1a-positive cells are eluted from the column.

1. Resuspend the cells in cold MACS buffer and transfer them to a 15-ml tube (*see* **Note 12**).
2. Wash cells by centrifuging for 10 min at 250 × *g*.
3. Count cells and resuspend the cells in the appropriate amount of MACS buffer and CD1a beads (80 μl buffer and 20 μl beads per 10^7 total cells; scale-up accordingly) (*see* **Note 13**).
4. Mix well but gently and incubate in the refrigerator for 15–30 min, while gently shaking them every 10 min.
5. Add 2 ml of buffer per 10^7 cells, run cells over a 100-μm cell strainer, and centrifuge at *250* × *g* for 10 min (*see* **Note 14**).
6. Resuspend the cells in 500 μl buffer (for maximal 10^8 cells). Scale-up accordingly for higher cell numbers.
7. Place a large cell separation column in the magnetic field of a MACS separator in a sterile flow cabinet. Attach a needle to the tip of the column to increase separation efficiency by decreasing flow rate. Place a 15-ml tube under the column.
8. Pre-wet the column with 500 μl buffer. Discard the flow-through.
9. Apply the cell suspension onto the column. At this time the unlabeled cells will pass through the column into the 15 ml-tube (*see* **Note 15**).
10. Wash the column three times with 500 μl buffer. Now you have collected the CD1a-negative fraction (*see* **Note 16**).
11. Remove the column from the magnetic field into a new 15-ml tube and add 1 ml buffer. Use the plunger to flush out the magnetically labeled cells. This fraction will contain 15–40% CD1a-positive cells.
12. Repeat Steps 7–11 over a new column: Pre-wet the column, add the CD1a-positive fraction from column 1. Rinse the column three times and remove the column from the magnetic field. Add 1 ml buffer and flush out magnetically labeled cells. This fraction should contain more than 90% pure CD1a-positive cells.

13. Keep a sample from the flow-through and the CD1a-positive fraction for flow cytometry analysis and count the cells.
14. Wash the cells in complete IMDM by centrifuging for 5 min at 450 × *g* before using them in cellular assays (*see* **Note 17**).

3.4. Flow Cytometry Analysis

See **Fig. 4.1G** and **Table 4.1** for a representative CD1a isolation.

Table 4.1
Purification efficiency

	Total number of cells ($\times 10^6$)	Purity (% CD1a positive)	Total nr LCs present ($\times 10^6$)	Yield (%) LCs from single-cell suspension
Single-cell suspension	80	2.5	2.0	100
After Ficoll density gradient	20	8.0	1.6	80
After first column CD1a MACS isolation	6	25.4	1.5	75
After second column CD1a MACS isolation	1.4	97.8	1.4	70

1. Add 50,000 cells of each enrichment step in a V-bottom 96-well plate.
2. Wash cells in PBS/BSA by centrifuging for 2 min at 400 × *g*. Vortex briefly afterward.
3. Add 2 μl anti-CD1a-FITC, anti-Langerin-PE, or other markers of choice in a total volume of 25 μl per well. Incubate at 4°C for 30 min (*see* **Notes 18** and **19**).
4. Wash cells once with 100 μl PBS/BSA by centrifuging for 2 min at 400 × *g* and resuspend in 100 μl PBS/BSA.
5. Determine expression levels by flow cytometry.

4. Notes

1. Other companies also have dermatomes available.
2. Some clones of Langerin antibodies (e.g., DCGM4) are calcium dependent. When utilizing these antibodies for

flow cytometry analysis prepare PBS/BSA by using PBS supplemented with calcium and magnesium).

3. Take proper care to protect yourself against contamination with human material; wear protective gloves, lab coat, and eye goggles.
4. If necessary, material can be stored overnight at 4°C before isolation.
5. The epidermis has the tendency to float, since the top layer is water repellent while the white-colored dermis is not water repellent.
6. Incubation can also be performed in pure dispase and at 37°C. The epidermis will detach within 1 h. Cells will die if left longer at 37°C. To limit activation and due to logistics (most often material is obtained in the late afternoon), overnight incubation at 4°C is more often performed.
7. Gentle pulling should be sufficient to separate the epidermis from the dermis. If this is not the case, increasing the dispase concentration or incubation at 37°C could help.
8. Trypsin treatment should be optimized to find the balance between highest cell yield and cell death. Care should be taken when using trypsin, since it may cleave off cell surface proteins, which may be important in cellular assays (11).
9. This is an essential step since the longer you pipet up and down, the higher the yield will be.
10. To shorten the time, the density gradient centrifugation could be bypassed and CD1a isolation can be performed using the whole single-cell suspension. However, this is cost inefficient since a large amount of CD1a beads will be needed.
11. This should be done carefully without disturbing the Lymphoprep layer to ensure proper separation.
12. Keep the cells and reagents at 4°C at all times to prevent capping of antibodies on the cell surface and non-specific cell labeling.
13. It is important to use the large cell separation column. The maximal number of cells that can be loaded onto a large cell separation column is 2×10^8, while maximally 10^7 labeled cells can be selected.
14. Cell clumps will clog the column.
15. To optimize the yield, the flow-through can be loaded for the second time over the column before washing.
16. Only add new buffer when the reservoir is empty to ensure efficient washing.

17. Cells can also be frozen by resuspending cells in 500 µl ice-cold complete IMDM. Slowly add freezing medium containing 80% FCS and 20% DMSO (sterilized over a 0.22-µm filter membrane). Add 1 ml of cell suspension to freezing vials and store them in a freezing box containing isopropanol at −80°C for 24 h before transferring them to liquid nitrogen storage.
18. Cells and reagents should be protected from light throughout staining and storage.
19. Double staining with anti-CD1a-FITC and anti-Langerin-PE can also be performed by adding the antibodies to the same sample. When measuring the samples using flow cytometry, make sure to compensate for spectral overlap of fluorescent emissions.

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Chapter 5

Isolation and In Vitro Generation of Gene-Manipulated Human Plasmacytoid and Conventional Dendritic Cells

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Abstract

Our understanding of human lymphocyte development has increased significantly over the past 20 years. In particular, our insight into human T- and B-cell development has improved (1, 2). Nonetheless, there are many gaps in our understanding, particularly regarding the early stages of development of hematopoietic progenitor cells (HPCs) into downstream lineage-biased and lineage-restricted precursors and the molecular mechanisms underlying these activities. The same holds true for our knowledge of human dendritic cell (DC) development. While the amount of data on the different subsets of conventional DCs (cDCs) and plasmacytoid DCs (pDCs) rapidly increases in mice (3, 4), the developmental stages of different DC subsets in humans remain poorly defined (2). The relatively easy access to patient material and therefore human precursor cells that can be isolated from these tissues combined with the availability of in vitro and in vivo differentiation assays allows studies in the field of human hematopoietic development, including that of DCs. In addition, the opportunities to manipulate gene expression, by stable overexpression of a gene of interest or RNA interference-mediated knockdown, generate valuable information about the mechanisms underlying lineage commitment and differentiation.

Key words: Human immune system, $CD34^+$ hematopoietic stem cells, OP9, differentiation, plasmacytoid dendritic cells, conventional dendritic cells.

1. Introduction

To date our knowledge of the mechanisms that regulate development and function of the hematopoietic system is largely derived from experiments performed in mouse models. While these studies are of the utmost importance, one has to take into account that considerable differences between mice and humans exist. This

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discrepancy invites for more extensive research on fundamental processes in human biology. Access to human tissues such as umbilical cord blood, buffy coats extracted from blood donations, and left-over samples from surgical procedures including postnatal thymus and fetal tissues has greatly facilitated research on development of the human hematopoietic system. These biological materials allow isolation of (subsets of) cell populations of the immune system and moreover they provide a source of human HPCs. Using in vitro assays we are able to stimulate the differentiation of isolated human progenitor cells into most hematopoietic lineages (5–7). In addition we are able to modify the genetic profile of progenitor cells by means of retro- (8, 9) and lentiviral transduction (see chapter by van Lent et al. in this issue). Combined these techniques allowed the identification of factors that control the development and function of the cells that make up the human hematopoietic system.

The cytokine Flt3L (10) is vital for the development of human pDCs in vitro from $CD34^+$ progenitor cells present in fetal liver, cord blood, and bone marrow (11). The subsequent stages of development of the type-I interferon (IFN)-producing pDCs in these compartments have been well characterized (11). The human postnatal thymus also contains $CD34^+$ progenitor cells with pDC potential, however, the stages of development appear to be slightly different (11, 12). In recent years our lab has concentrated on gaining insight into the complex network of transcription factors that controls progenitor cell differentiation and the regulation of human pDC development. Using the methods described in this chapter we have identified the ETS transcription factor Spi-B as a key regulator in human pDC development (6, 13). Furthermore, we showed that the DeltaLike1/Notch1 signaling pathway controls the T/pDC lineage decision by controlling the expression of downstream lineage-specific transcription factors. Activation of the T-cell lineage-specific receptor Notch1 by its ligand DeltaLike1 negatively regulates Spi-B expression and thereby inhibits pDC development (7). In addition, we recently found that the E-protein TCF4/E2-2 is highly expressed in pDCs as compared to the $CD34^+$ progenitor cells and that development of pDCs depends on E2-2 expression (14). This is in line with our previous observation that forced expression of Id2 and Id3, which are negative regulators of E-proteins, in $CD34^+$ progenitor cells induces a profound block in pDC differentiation (15). This, however, left the development of conventional DCs unaffected (15). The Id2-induced block in pDC development can be released by co-transduction of E2-2 (14). Notably, Spi-B was unable to overcome this developmental block. Also, we observed that Spi-B downregulates Id2 expression likely allowing E2-2 to stimulate pDC development. Together a model is emerging in which Spi-B and E2-2 co-operate in the development of human pDCs.

The in vitro generated pDCs are fully functional as addition of the Toll-like receptor 9 (TLR-9)-specific ligand CpG or virus, such as Herpes simplex virus, Influenza virus, and HIV, induces production and secretion of type-I IFN (7, 16). In vitro generated pDCs stimulated with CpG or virus inhibit thymic T-cell development at an early stage (16). This suggests that the anatomical localization of T-cell progenitor cells in the cortex and pDCs in the medulla of the thymus (17, 18) is an important requirement to allow proper development of T cells.

Although in vitro systems have merit for investigations on development of particular lineage branches they never allow for studying the complexity of the entire hematopoietic system in one assay. Tremendous progress has been made in the generation of humanized mouse models allowing the simultaneous development of the different hematopoietic lineages and their function in an in vivo setting (6, 19, 20). These issues, however, are addressed in this issue by van Lent et al. Here we will focus on the techniques currently employed in our lab to isolate pDCs and cDCs from primary human material and to differentiate human HPCs into pDCs in vitro.

2. Materials

2.1. Isolation of Mononuclear Cells

1. A source of human hematopoietic cells (*see* **Note 1**).
   (a) Buffy coats isolated from human donor whole blood are obtained from the blood bank.
   (b) Umbilical cord blood is obtained from pregnant patients at delivery.
   (c) Postnatal thymus is obtained from surgical specimens removed from children up to 3 years of age undergoing open heart surgery. Tissue is processed the same day (*see* **Note 2**).
   (d) Fetal liver and fetal thymus are obtained from elective abortions with gestational age ranging from 13 to 17 weeks. The raw material can be maintained overnight in a cold room or fridge. Phosphate-buffered saline (PBS) buffer (Invitrogen, Grand Island, NY, USA).
2. RPMI-1640 (Invitrogen) supplemented with 2% fetal calf serum (FCS). Store at 4–8°C.
3. Penicillin and streptomycin (Invitrogen).
4. Stomacher-80 Biomaster lab system (Seward Ltd., Worthing, UK), Stomacher bags, and a bag-sealing device (e.g., Sealboy 236 Audion Elektro).

5. Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Store away from light at 4–8°C.
6. Fine-toothed tweezer (VWR, West Chester, PA, USA).
7. Regular tweezer (VWR).
8. Small spoon (optional) (VWR).
9. Scalpel (VWR).
10. Stainless steel mesh (VWR).

2.2. Enrichment and Sort of Hematopoietic Populations

1. MACS isolation kit depending on desired population (*see* **Note 3**).
   (a) Direct CD34 human MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).
   (b) Indirect CD34 human MicroBead Kit (Miltenyi Biotec).
   (c) CD304 (BDCA4/neuropilin-1) MicroBead Kit (Miltenyi Biotec).
2. MACS pre-separation filters and recovery tubes (Miltenyi Biotec).
3. MACS buffer is prepared as follows: PBS pH 7.2, 0.5% bovine serum albumin, 2 mM ethylenediaminetetraacetic acid (EDTA). Store at 4–8°C.
4. MACS separator, Large Scale (LS) MACS separation columns (Miltenyi Biotec).
5. Anti-PE microbeads (Miltenyi Biotec).
6. 5-ml Polystyrene round-bottomed 12 mm × 75-mm tubes (Becton Dickinson (BD), Franklin Lakes, NJ, USA).
7. Fluorochrome-coupled monoclonal antibodies depending on intended population:
   anti-human CD1a (BD), anti-human CD11c-PE (BD), anti-human CD3 (BD), anti-human CD34 (BD), anti-human CD38 (BD), anti-human CD45RA (BD), anti-human CD56 (BD), anti-human CD123 (BD), anti-human BDCA2 (Miltenyi Biotec), anti-human HLA-DR (BD).
8. Fluorescence-activated cell sorter for isolation of pure cell populations, e.g., FACS Aria (BD).

2.3. Virus Production

1. Retroviral construct, e.g., pMX, LZRS expressing the gene of interest.
2. Retroviral producer cells, e.g., Phoenix-ampho or Phoenix-GALV (*see* **Note 9**).
3. 70 μm Cell strainer (BD).
4. Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen) supplemented with 8% FCS.
5. Penicillin and streptomycin (Invitrogen).

6. Puromycin (Sigma–Aldrich, St. Louis, MO, USA) (when LZRS is used).

2.4. Progenitor Transduction

1. Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen).
2. Yssels supplement (21).
3. Pooled human serum (Invitrogen).
4. SCF, IL-7, and TPO (PeproTech EC, London, UK).
5. Retronectin (30 μg/ml; Takara Biomedicals, Otsu, Shiga, Japan).
6. Multiwell 24-well plate non-tissue culture treated (BD).

2.5. In Vitro DC-Differentiation Assay

1. Complete MEMα: Minimum Essential Medium (MEM) α is prepared from MEMα medium powder without $NaHCO_3$ (Invitrogen). One vial powder medium is reconstituted in 1 l MilliQ water together with 2.2 g $NaHCO_3$ (Merck, Darmstadt, Germany) and filtered over a 500-ml 0.22-μm Bottletop filter (Corning, Corning, NY, USA). Medium is supplemented with 20% Hyclone (Logan, UT, USA) serum and penicillin/streptomycin (Roche).
2. OP9 cell line (*see* **Note 21**).
3. Phosphate-buffered saline (PBS) (Invitrogen).
4. Trypsin (Invitrogen).
5. 6-Well tissue culture plates (Corning).
6. IL-7 and Ftl3L (PeproTech EC, London, UK).
7. 70-μm Nylon mesh (Spectrum Laboratories, Inc., CA, USA). Cut into 1 × 1 cm square pieces and autoclave before use.
8. Fluorescence-activated cell analyzer, e.g., FACS LSRII (BD).

2.6. Stimulation of In Vitro Generated pDCs

1. pDCs generated in vitro from $CD34^+$ progenitors in OP9 cultures with IL-7 and Flt3L.
2. CpG oligodeoxynucleotide (CpG-ODN) 2216 ggGGGACGATCGTCgggggG and CpG oligodeoxynucleotide (CpG-ODN) 2243 ggGGGAGCATGCTCgggggG (Sigma–Aldrich, St. Louis, MO, USA).
3. Irradiated Herpes simplex virus, Influenza virus, or HIV.
4. Human IFN-α enzyme-linked immunosorbent assay (ELISA; Biosource International, Camarillo, CA, USA).
5. Brefeldin A (Sigma).

6. Fix and Perm Kit (Caltag, Burlingame, CA, USA).
7. FITC-labeled anti-IFN-α2 mAb (Chromaprobe, Maryland Heights, MO, USA).

3. Methods

3.1. Isolation of Nucleated Cells from Human Peripheral (or Umbilical Cord Blood)

1. Open the collection bag with sterile scissors.
2. Empty the bag in a T75-ml flask, the collected volume is around 25–50 ml.
3. The amount of collected umbilical cord blood is usually smaller, adjust all volumes accordingly.
4. Add PBS to a total of 200 ml PBS.
5. Divide the suspension over 50-ml Falcon tubes at 25 ml/tube. Four tubes in general generate enough cells for downstream applications.
6. Add 12 ml Ficoll-Paque and isolate nucleated cells from a regular density gradient by standard procedures.
7. Wash cells in RPMI-1640.

3.2. Isolation of Nucleated Cells from Human Postnatal Thymus

1. The collected tissue is processed the same day.
2. Put the thymus tissue in a sterile Petri dish and pull off the capsule layer with a set of fine-toothed tweezers. Subsequently, cut the tissue into small pieces using a sterile disposable scalpel.
3. Transfer the pieces into a sterile Stomacher bag with either tweezers or a small spoon, wash the Petri dish with a small amount of RPMI medium (with 8% FCS, penicillin, and streptomycin) and add the rinsing to the bag. The bag is sealed twice to avoid leakage and put into a Stomacher device for further disruption of the tissue.
4. Clean the bag with 70% ethanol and open the bag with sterile scissors. Empty the contents on an autoclaved stainless steel mesh and press the cells through the filter to obtain a single-cell suspension. Wash the filter and plate with RPMI 8% FCS, suspension is left overnight in a 50-ml tube at 4°C.
5. The following day thymocytes are isolated from a Ficoll-paque density gradient at a maximum of 600×10^6 cells/50-ml Falcon tube.
6. Wash the cell suspensions by centrifugation in RPMI-1640 2% FCS medium.

3.3. Isolation of Nucleated Cells from Human Fetal Liver

1. Transfer one fetal liver into one Stomacher bag, add 10–15 ml RPMI-1640 medium, and seal the bag twice to avoid leakage.
2. Disrupt the liver pieces mechanically in a Stomacher device for 1 min at high speed. The bag now contains a cell suspension mixed with non-dissociated liver stroma.
3. Wipe the bag with ethanol 70% and open it with sterile scissors. Transfer the contents of the bag into a 10-cm Petri dish.
4. Dilute the cell suspension with RPMI-1640 and recover the supernatant from the dish avoiding the pieces of stroma at the bottom of the dish. Pour new RPMI-1640 medium into the plate and repeat this procedure until the recovered medium is clear. Usually, pouring fresh medium three times is enough.
5. Add RPMI-1640 medium to the tubes until you reach approximately 20 ml. Wash the cell suspensions by centrifugation. Aspirate the fat-containing supernatant.
6. Isolate the cells from a Ficoll-paque density gradient.
7. Wash the cell suspensions by centrifugation and pool the cell pellets in RPMI-1640 2% FCS medium.

3.4. Isolation of $CD123^+CD45RA^+$ Plasmacytoid Dendritic Cells

1. Cells recovered from Ficoll-paque density gradient are enriched for $BDCA4^+$ cells by immunomagnetic cell sorting, using a $BDCA4^+$ cell separation kit (*see* **Note 5**).
2. Label the cells according to manufacturer's protocol (*see* **Note 6**).
3. Install the magnetic separation column in the MACS magnet and put a pre-separation filter on top of it. Prepare the collection tube for the column flow-through. Proceed to filter/column washing with MACS buffer, following the manufacturer's instructions.
4. After extensive washing of the column take it from the magnet. Add 5 ml MACS buffer to the column and flush out the $BDCA4^+$ cells into a 15-ml tube using the plunger.
5. Add 5 ml of RPMI-1640 2% FCS medium to the tube and wash the cell suspension by centrifugation.
6. Resuspend the pellet in a volume of 1 ml of RPMI-1640 2% FCS medium and count the amount of nucleated cells in the suspension.
7. The $BDCA4^+$ cell fraction is labeled with anti-CD123, anti-CD45RA, and lineage markers (*see* **Note 7**).

8. After washing-off excess antibodies in RPMI-1640 2% FCS medium cells are filtered over 70-μm autoclave mesh to remove potential clumps.
9. On a flow cytometry sorter $CD123^{hi}CD45RA^{+}$cells are sorted to purity (**Fig. 5.1**, *see* **Note 8**).

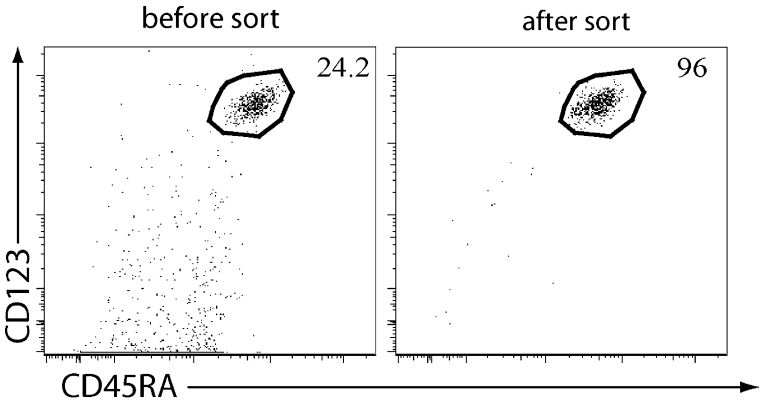

Fig. 5.1. Sorting of $CD123^{+}CD45RA^{+}$ pDCs from $BDCA4^{+}$ MACS-enriched cells. Dotplot "Before sorting" is gated lineage negative and doublets are excluded.

3.5. Isolation of $CD11c^{+}HLA\text{-}DR^{+}$ Conventional DCs

1. Cells recovered from Ficoll-paque density gradient are enriched for $CD11c^{+}$ cells by a two-step immunomagnetic cell protocol (*see* **Note 5**).
2. Cells are stained with anti-human CD11c-PE antibodies.
3. Incubate at 4°C for 15 min and wash.
4. Add anti-PE microbeads.
5. Incubate at 4°C for 15 min and wash.
6. Install the magnetic separation column in the MACS magnet and put a pre-separation filter on top of it. Prepare the collection tube for the column flow-through. Proceed to filter/column washing with MACS buffer, following the manufacturer's instructions.
7. After extensive washing of the column take it from the magnet. Add 5 ml MACS buffer to the column and flush out the $CD11c^{+}$-enriched cells into a 15-ml tube using the plunger.
8. Resuspend the pellet in a volume of 1 ml of RPMI-1640 2% FCS medium and count the amount of nucleated cells in the suspension.
9. The $CD11c^{+}$ cell fraction is labeled with CD3, CD19, CD56, CD11, and HLA-DR antibodies for 30 min at 4°C.

10. After washing-off excess antibodies in RPMI-1640 2% FCS medium cells are filtered over 70-μm autoclave mesh to remove potential clumps.
11. By flow cytometry $Lin^{-}CD11c^{+}HLA\text{-}DR^{+}$ cDCs are sorted to purity (*see* **Fig. 5.2**, *see* **Note 8**).

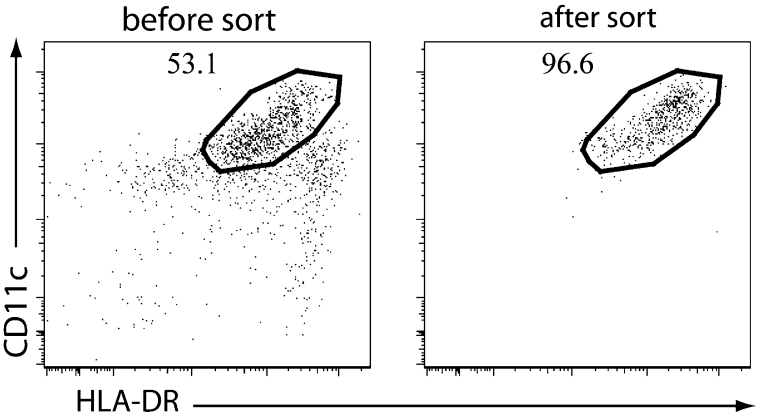

Fig. 5.2. Sorting of $CD11c^{+}HLA\text{-}DR^{+}$ cDCs from $CD11c^{+}$ MACS-enriched cells. Dotplot "Before sorting" is gated lineage negative and doublets are excluded.

3.6. Isolation of $CD34^{+}$ Cells from Postnatal Thymus

1. Cells recovered from Ficoll-paque density gradient are enriched for $CD34^{+}$ cells by immunomagnetic cell sorting, using a direct $CD34^{+}$ cell separation kit (*see* **Note 3**).
2. Label the cells according to manufacturer's protocol (*see* **Note 3**).
3. Install the magnetic separation column in the MACS magnet and put a pre-separation filter on top of it. Prepare the collection tube for the column flow-through. Proceed to filter/column washing with MACS buffer, following the manufacturer's instructions.
4. After extensive washing of the column take it from the magnet. Add 5 ml MACS buffer to the column and flush out the $CD34^{+}$ into a 15-ml tube using the plunger.
5. Add 5 ml of RPMI-1640 2% FCS medium to the tube and wash the cell suspension by centrifugation.
6. Resuspend the pellet in a volume of 1 ml of RPMI-1640 2% FCS medium and count the amount of nucleated cells in the suspension.
7. Stain the $CD34^{+}$-enriched thymocytes with antibodies against CD34, CD1a, CD56, and BDCA2.
8. After washing-off excess antibodies in RPMI-1640 2% FCS medium cells are filtered over 70-μm autoclave mesh to remove potential clumps.

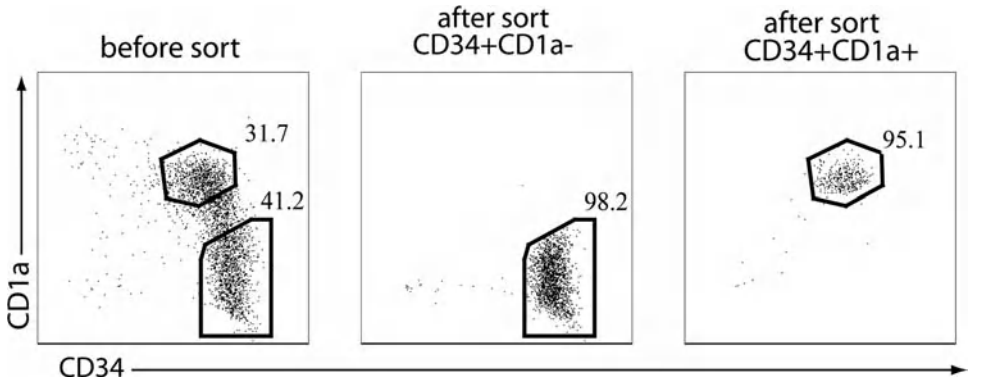

Fig. 5.3. Sorting of $CD34^+CD1a^-$ and $CD34^+CD1a^+$ subsets from $CD34^+$ MACS-enriched cells. Dotplot "Before sorting" is gated lineage negative and doublets are excluded.

9. $Lin^-CD34^+CD1a^-$ cells with T/NK/pDC potential are sorted to purity by flow cytometry (**Fig. 5.3**) (*see* **Notes 4** and **8**).

3.7. Enrichment for $CD34^+$ Hematopoietic Stem Cells (HSCs)

1. Cells recovered from Ficoll-paque density gradient are enriched for $CD34^+$ cells by immunomagnetic cell sorting, using an indirect $CD34^+$ cell separation kit (*see* **Note 3**) consisting of (1) anti-CD34 hapten-coupled antibody; (2) anti-hapten antibody conjugated with colloidal paramagnetic beads.
2. Label the cells according to manufacturer's protocol (*see* **Note 3**).
3. Install the magnetic separation column in the MACS magnet and put a pre-separation filter on top of it. Prepare the collection tube for the column flow-through. Proceed to filter/column washing with MACS buffer, following the manufacturer's instructions.
4. After extensive washing of the column take it from the magnet. Add 5 ml MACS buffer to the column and flush out the $CD34^+$ into a 15-ml tube using the plunger.
5. Add 5 ml of RPMI-1640 2% FCS medium to the tube and wash the cell suspension by centrifugation.
6. Resuspend the pellet in a volume of 1 ml of RPMI-1640 2% FCS medium and count the amount of nucleated cells in the suspension.
7. Stain the $CD34^+$-enriched fetal liver cells with antibodies against CD34, CD38, CD56, CD3, and BDCA2.
8. After washing-off excess antibodies in RPMI-1640 2% FCS medium cells are filtered over 70-μm autoclave mesh to remove potential clumps.

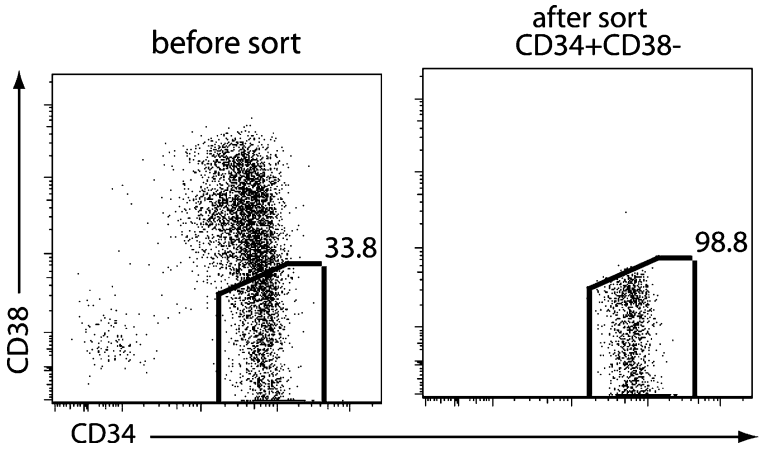

Fig. 5.4. Sorting of $CD34^+CD38^-$ from $CD34^+$ MACS-enriched fetal liver cells. Dotplot "Before sorting" is gated lineage negative and doublets are excluded.

9. Multipotent $CD34^+CD38^-$ HSCs are sorted to purity by flow cytometry (**Fig. 5.4**, *see* **Note 8**).

3.8. Production of High-Titer Amphotropic Retroviral Stocks (see Note 9)

1. Day 1: Near confluent Phoenix-ampho or Phoenix-GALV cells are split 1:5 to have the cells growing in log phase (*see* **Note 10**).
2. Day 2: Phoenix cells are plated at 1.5×10^6 cells/10-cm Petri dish in 8 ml IMDM 8% FCS.
3. Day 3: 25 μl FuGene is added to 400 μl RPMI without serum (the absence of serum is important). Under shaking or vortexing the RPMI/FuGene mixture is added dropwise to 10 μg retroviral plasmid DNA in a 2-ml eppendorf tube (*see* **Note 11**).
4. Incubate the DNA/FuGene mixture at RT for 20 min and divide it dropwise over the Phoenix cells.
5. If LZRS constructs were transfected continue from Step 9, otherwise continue from Step 6.

3.8.1. Retroviral Production from Non-LZRS Constructs

6. Day 5: For non-LZRS constructs the medium is overnight replaced with 4 ml IMDM 8% FCS medium.
7. Day 6: If desired repeat the same procedure as described on day 4 (*see* **Note 12**).
8. Continue from Step 14.

*3.8.2. Retroviral Production from LZRS Constructs (see **Note 13**)*

9. Day 4: Transfer the Phoenix cells from the 10-cm dish into a large T175 flask containing 20 ml IMDM 8% FCS.
10. Day 5: Add 40 μl puromycin 1 mg/ml to select for transfected-Phoenix cells (*see* **Note 14**).

11. Day 8: If the T175 flask is nearly confluent, remove half of the Phoenix cells by trypsinzation from the flask and add fresh medium and puromycin.
12. Days 10–12: Around this time the puromycin selection is completed and over 90% of the Phoenix cells contain the LZRS plasmid. When the flask is around 70% confluent, the medium is replaced in the morning to wash away the puromycin. Handle the flask carefully, the Phoenix cells easily detach when grown at high density.
13. Put 16 ml IMDM 8% FCS overnight on the Phoenix cells.
14. Next day harvest the retroviral supernatant from the Phoenix cells. Spin the supernatant for 5 min at 1,250 rpm without brake to pellet detached cells and cell debris.
15. A typical transduction of $CD34^+$ progenitor cells requires 300 µl virus, therefore virus stocks are frozen in 400-µl aliquots. Snap-freeze the virus on dry ice and store at −80°C. The virus titer remains stable for years.

3.9. Retroviral Transduction of Human $CD34^+$ Progenitor Cells (see Note 15)

1. Sorted $CD34^+CD38^-$ fetal liver or $CD34^+CD1a^-$ postnatal thymocytes are cultured overnight at a density of 250,000–800,000 cells/1 ml in a standard 24-well tissue culture plate in IMDM with Yssels supplement, 5% human serum, and cytokines. Cytokines are 20 ng/ml SCF + 10 ng/ml IL-7 + 20 ng/ml TPO for fetal liver or 20 ng/ml SCF + 10 ng/ml IL-7 for postnatal thymocytes, respectively.
2. Cells are cultured overnight at 37°C to induce cell cycle progression.
3. Simultaneously, a non-tissue culture treated 24-well plate is coated with 0.5 ml/well retronectin overnight a 4°C (*see* **Note 16**).
4. The next morning the retronectin is collected from the plate (*see* **Note 17**). Retronectin-coated wells are incubated with 0.5 ml/well PBS 2% HSA for 20 min at room temperature to block non-specific sites. Just before use the plates are washed with PBS.
5. In the meantime the progenitor cells are collected from the overnight culture by resuspending the cells in the well and subsequent washing of the wells with IMDM with Yssels supplement. Progenitor cells are spun and taken up in the desired volume of IMDM with Yssels supplement and cytokines (no serum, *see* **Note 18**).
6. Virus is taken from the −80°C storage and quickly thawed in hot tap water.

7. Cells and virus are added onto the retronectin plate in a 1:1 ratio, 300 μl each with a final concentration of cytokines of 20 ng/ml SCF + 10 ng/ml IL-7 + 20 ng/ml TPO for fetal liver or 20 ng/ml SCF + 10 ng/ml IL-7 for postnatal thymocytes, respectively (similar to the concentrations used in Step 1) (*see* **Note 19**).
8. The plates are spun in a plate centrifuge for 90 min at 800 rpm and room temperature without brake.
9. After centrifugation the cells are incubated at 37°C for an additional 5–6 h.
10. Cells are collected from the wells, counted, and resuspended in the appropriate medium for subsequent differentiation assay.
11. A small amount of transduced cells can be cultured in a 96-well round-bottomed plate in IMDM Yssels 5% human serum with cytokines and analyzed subsequently at day 3 after transduction for transduction efficiency. Transduction efficiencies up to 20% are anticipated (**Fig. 5.5**, *see* **Note 20**).

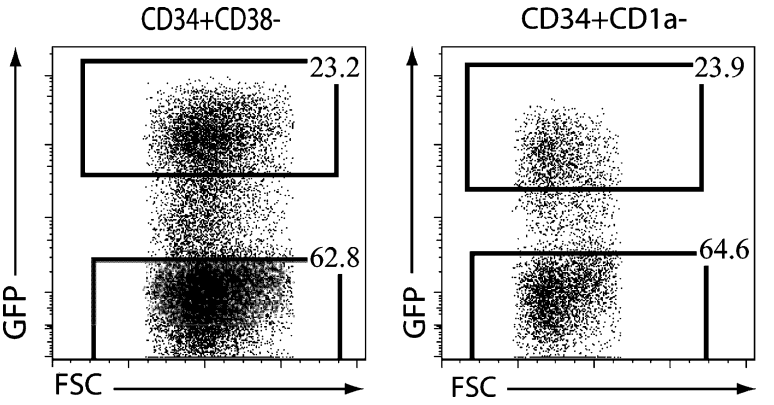

Fig. 5.5. Transduction efficiency measured 3 days after transduction.

3.10. In Vitro pDC and cDC Differentiations

1. OP9 cells (or MS-5 or S17) are plated in a 6-well at 50,000 cells/well in 1 ml complete MEMα, preferably up to 24 h before starting co-cultures (*see* **Note 21**).
2. Sorted progenitor cells either $CD34^+CD38^-$ or $CD34^+CD1a^-$ (transduced or not-transduced) are counted, resuspended in 1 ml MEMα, and carefully added to the OP9 cells to a total culture volume of 2 ml (*see* **Note 22**).
3. The medium is supplemented with 5 ng/ml IL-7 and 5 ng/ml Flt3L (*see* **Notes 23 and 24**).

4. Fresh medium and cytokines (1 ml complete MEMα with 10 ng/ml IL-7 and 10 ng/ml Flt3L) are added every 2–3 days.
5. At the day of analysis the cultures are resuspended and filtered over a piece of autoclaved 70-μm nylon mesh into a 15-ml tube. The peak in percentage and number of pDCs from $CD34^+CD38^-$ and $CD34^+CD1a^-$ is reached between day 5 and day 7, respectively (16).
6. Spin the cells, resuspend in 1 ml medium, and count the number of cells.
7. The cultures are analyzed by flow cytometry. pDCs stain double positive for CD123 (IL3Rα) and BDCA2 antigens (**Fig. 5.6**). CD4, CD45RA, and HLA-DR staining may also be included. Although the percentage of OP9 falling into the lymphocyte gate of an FSC–SSC dotplot is usually neglectable, an antibody against human CD45 can be used to exclude contaminating OP9 cells.
8. Cells recovered from OP9 cultures started from $CD34^+CD38^-$ progenitor cells may be replated on fresh OP9 cells with cytokines as described above. Around day 11 $CD10^+CD19^+$ pro-B cells and $CD11c^+CD14^+$ cells of myeloid origin can be detected (6) (*see* **Note 25**).

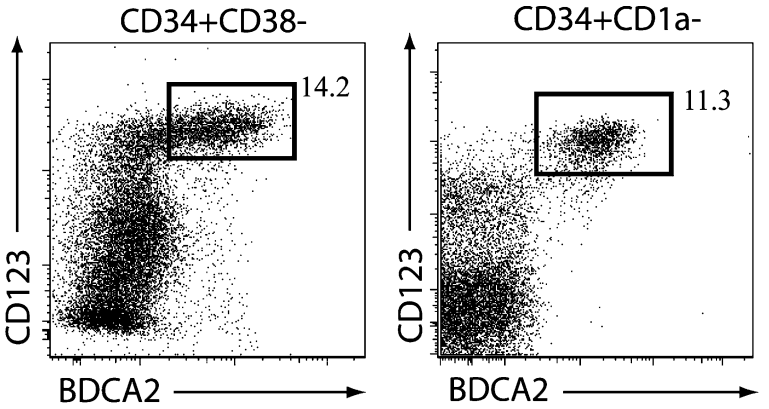

Fig. 5.6. $BDCA2^+CD123^+$ pDCs derived from $CD34^+CD38^-$ fetal liver cells or $CD34^+CD1a^-$ postnatal thymocytes on OP9 in the presence of Flt3L and IL-7.

3.11. Stimulation of In Vitro Generated pDCs

1. After 5–7 days of culturing $CD34^+$ progenitors on OP9 cells with IL-7 and Flt3L the pDCs that have developed in the culture are stimulated by adding 10 μg/ml (CpG-ODN) 2216 or the negative control (CpG-ODN) 2243 or virus at 10 PFU/cell to bulk cultures. Alternatively, $CD123^+BDCA2^+$ pDCs are sorted from the bulk cultures before stimulation.

2. After 24–48 h of stimulation IFN-α production is measured by analyzing the supernatant by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instruction.
3. Intracellular IFN-α can be detected by FACS after 8–12 h of stimulation.
   To optimize the detection of intracellular cytokines Brefeldin A (10 μg/ml) may be added during the last 2 h of stimulation. After stimulation, pDCs are, if required, labeled with CD123 and BDCA2 and subsequently fixed and permeabilized using the Fix and Perm Kit according to the manufacturer's instructions and are labeled with anti-IFN-α2 monoclonal antibody.

4. Notes

1. Human fetal tissues are obtained from elective abortions. The use of fetal tissue was approved by the Medical Ethical Committee of the Academic Medical Center and was contingent on obtaining informed consent, in accordance with the Declaration of Helsinki. Gestational age was determined by ultrasonic measurement of the diameter of the skull and ranged from 13 to 17 weeks.
   The use of postnatal thymus tissue, donor blood, and cord blood was approved by the Medical Ethical Committee of the Academic Medical Center and used with informed consent in accordance with the Declaration of Helsinki.
2. Postnatal thymus samples are taken from children undergoing open-heart surgery ranging from 0 to 3 years of age. Although samples from older children can be obtained they typically contain severely reduced numbers of $CD34^+$ progenitor cells as compared to children below the age of 3 years.
3. According to the manufacturer direct and indirect $CD34^+$ progenitor cell isolations have identical efficiencies. However, in our hands the direct isolation kit works significantly better for enrichment of $CD34^+$ from postnatal thymus as compared to cells from fetal liver. It may be considered to use the indirect isolation kit for fetal liver cells due to the lower pricing compared to the direct kit. Of note, we use only about 25–50% of the amount of antibodies and beads that are recommended by the manufacturer.
4. If required $Lin^-CD34^+CD1a^+$ cells can simultaneously be sorted from the $CD34^+$ MACS-enriched cells (**Fig. 5.3**). These cells are strongly biased to the T-cell lineage.

Although they still have little NK-cell potential, they have completely lost their capacity to develop into pDCs (15).

5. Human pDCs, as opposed to murine pDCs, do not express CD11c. Therefore, the described CD11c MACS protocol does not enrich for human pDCs.
6. When using the BDCA4 cell separation kit it is advisable to pre-incubate the cells for several minutes with the FcR-blocker before adding the BDCA4-beads. This will increase the enrichment rate compared to simultaneous addition of FcR-blocker and BDCA4-beads to the cells.
7. Although BDCA2 is the specific marker for human pDCs it is best not be used for cell sorting as it activates the pDCs (22).
8. Typically, the tubes used for FACS sorting are polystyrene as cells are less likely to stick to the tube as compared to regular tubes.
9. More detailed information on the Phoenix retroviral system can be obtained on http://www.stanford.edu/group/nolan/.
10. In case of low transfection efficiency it may be worthwhile to try using more or less plasmid DNA, as less DNA may also give higher transfection efficiency. If transfection efficiency stays low it is advised to redo the plasmid DNA isolation to obtain plasmid DNA of better quality.
11. Amphotropic- and gibbon-ape-leukemia-virus (GALV)-pseudotyped retrovirus are much more effective in transducing human cells than the commonly used ecotropic-pseudotype virus to transduce murine cells. We never observed a clear advantage of either amphotropic- or GALV-typed virus for transduction of $CD34^+$ progenitor cells, also not in double transduction experiments.
12. The virus titers of the second harvest are generally somewhat increased from the first harvest. The second harvest typically gives increased transduction efficiencies.
13. The LZRS retroviral plasmid contains a puromycin resistance and EBNA (for replication of the plasmid in human cells) sequences in the backbone. This combination allows positive selection and expansion of transfected and thus puromycin resistant Phoenix cells resulting in the easy generation of large amounts of high titer virus. The plasmid is thought to be present in the Phoenix cells up to 3 months.
14. It takes about 2 days before the non-transfected Phoenix cells start to die due to the puromycin treatment.
15. Although $CD34^+CD38^-$ isolated from fetal liver can be efficiently transduced with lenti-viral constructs (see van Lent et al., this issue) this has been problematical for

$CD34^+CD1a^-$ postnatal thymocytes. The reason for this is still unclear and may depend on both the lenti-viral construct (e.g., promoters used) or the virus titer.

16. Instead of coating a 24-well plate with retronectin overnight at 4°C, 2 h at room temperature or in the incubator is also sufficient.
17. Retronectin can be re-used up to three times without loss of transduction efficiency.
18. The presence of high serum dose in the transduction cultures inhibits transduction efficiency (23).
19. For transductions virus and cells are mixed at a 1:1 volume ratio. In case of double transduction with two different viral constructs 300 μl cells is mixed with 150 μl of each of the respective virus.
20. Typically, we observe lower transduction efficiencies when $CD34^+CD1a^-$ thymocyte precursors are used as compared to $CD34^+CD38^-$ fetal liver cells. In addition constructs with RNA interference or microRNA-encoding sequences negatively affect transduction of both cell types.
21. OP9 is a bone marrow stromal cell line derived from M-CSF deficient mice (24). OP9 needs to be cultured at relatively high/semi-confluent density and preferably passaged 1:2−1:3 three times a week. When OP9 cells are cultured at low density they tend to change the morphology by stretching out (although we have no indication that this affects the functionality of these cells). For splitting, OP9 cells can be treated similar to other common adherent cell lines. However, during trypsinizing the OP9 cells tend to come off the plate in large sheets. Vigorous pipetting will break up the sheets after which the cells are passed through a 70-μm cell strainer to remove the clumps.
22. At the start of the differentiation assay the genes encoded by the viral constructs are not expressed at detectable levels yet. Therefore, both transduced and non-transduced progenitor cells are cultured concomitantly, which requires the use of a fluorescent marker, such as GFP, expressed from the retroviral construct in order to be able to discriminate transduced and non-transduced cells by flow cytometric analysis.
23. Although it is clear that Flt3L is the cytokine driving DC development we typically use the combination of IL-7 and Flt3L in the differentiation assays to allow a better comparison between OP9 DC and OP9-DeltaLike1 T-cell differentiation cultures (7). Also increased expansion and cell survival of progenitor cells is observed in the presence of IL-7.

24. In addition to Flt3L and IL-7 thrombopoietin (TPO) can be added to the cultures to increase the number of pDCs derived from $CD34^+$ fetal liver or cord blood progenitor cells (25).
25. Under the conditions described here $CD34^+CD38^-$ HSC will give rise to a small percentage of pro-B cells. Lowering or completely leaving out cytokines will increase the percentage (but decrease the absolute cell number) of pro-B cells in the culture. Sorted $CD19^+$ from the OP9 co-cultures can be further differentiated into BCR^+ B-cells with CD40L + IL-2/IL-4 (26).

Acknowledgments

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Chapter 6

In Vivo Modulation of Gene Expression by Lentiviral Transduction in "Human Immune System" Rag2$^{-/-}\gamma_c^{-/-}$ Mice

Anja U. van Lent, Mireille Centlivre, Maho Nagasawa, Julien J. Karrich, Stephan M. Pouw, Kees Weijer, Hergen Spits, Bianca Blom, and Nicolas Legrand

Abstract

Over the last two decades, several humanized mouse models have been used to experimentally analyze the function and development of the human immune system. Recent advances have lead to the establishment of new murine–human chimeric models with improved characteristics, both in terms of human engraftment efficiency and in situ multilineage human hematopoietic development. We describe here the use of newborn BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ mice as recipients of human hematopoietic progenitor cells to produce "human immune system" (HIS) (BALB-Rag/γ) mice, using human fetal liver progenitors. The two major subsets of the human dendritic cell lineage, namely, BDCA2$^+$CD11c$^-$ plasmacytoid dendritic cells and BDCA2$^-$CD11c$^+$ conventional dendritic cells, can be found in HIS (BALB-Rag/γ) mice. In order to manipulate the expression of genes of interest, the human hematopoietic progenitor cells can be genetically engineered ex vivo by lentiviral transduction before performing xenograft transplantation. Using this mouse model, the human immune system can be assessed for both fundamental and pre-clinical purposes.

Key words: Human immune system, BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ mouse, HIS (Rag/γ), CD34$^+$ hematopoietic stem cell, stem cell transplantation, SCID mouse model, fetal liver, lentivirus, transduction, human dendritic cells.

1. Introduction

It is particularly challenging to get experimental access to the human immune system (HIS) in vivo, for practical and ethical reasons. To meet these concerns, "humanized" animal models

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have been established over the last two decades, to specifically address questions regarding human immunology. In particular, mouse models have been privileged, because of easy manipulation, easy breeding, and relatively low cost, as compared for instance to non-human primates. Confronted with xenograft transplantation barriers, several pioneering groups have screened various immunodeficient mouse strains, and multiple human xenograft transplantation models have been developed, depending on the desired outcome and features (1–6). Among these, the SCID-hu (Thy/Liv) system, which combines both human fetal liver and thymus engraftment into severe combined immunodeficiency (SCID) mice, has been shown to be valuable for the study of HIV pathogenesis in vivo (7), but this model is intrinsically limited by poor accumulation of human cells – mainly T cells – in peripheral lymphoid organs (8, 9). As an alternative, immunodeficient mice using the NOD genetic background (e.g., NOD/SCID, NOD/SCID/$\beta 2m^{-/-}$, and NOD/SCID/$\gamma_c^{-/-}$ mice) have been efficiently reconstituted with hematopoietic stem cells (HSC) isolated from human umbilical cord blood (UCB). Despite improvements, human T-cell development and accumulation was described as being limited in such humanized animals, especially in the peripheral lymphoid organs of the recipient mice (10–14). More recently, "BLT" (bone marrow, liver, and thymus) mice have been produced by co-engrafting bone marrow HSC and Thy/Liv organoid into NOD/SCID mice and were proven efficiently repopulated by human cells, although technically more challenging (15).

By reasoning that the extent of engraftment by human progenitor cells could be limited by the use of adult mice, we and others have developed a new experimental strategy, namely, the inoculation of human HSC into newborn immunodeficient mice (16–19). In particular, the use of newborn BALB/c $Rag2^{-/-}\gamma_c^{-/-}$ immunodeficient mice for injection of human HSC-enriched ($CD34^+$) cell populations gives rise to robust human reconstitution, with de novo multilineage hematopoietic reconstitution and marked human thymopoiesis (17, 18). The resulting animals are referred to as "HIS (BALB-Rag/γ) mice" (3) or "human adaptive immune system $Rag2^{-/-}\gamma_c^{-/-}$ mice" (huAIS-RG) (20). Efficient engraftment of BALB/c $Rag2^{-/-}\gamma_c^{-/-}$ mice is age-dependent (18) and necessitates sublethal total body irradiation prior to intra-hepatic inoculation of $CD34^+$ HSC-enriched cell suspensions. HSC suspensions can be prepared from various origins such as fetal liver, umbilical cord blood, fetal bone marrow, adult bone marrow, and mobilized peripheral blood, starting from the richest source of HSC. Before xenograft transplantation, HSC can be manipulated ex vivo by lentiviral vector-mediated transduction to enforce increased or reduced expression of genes of interest in the HSC and their progeny in vivo (18, 21–23).

Besides classical overexpression and functional knock-down systems (e.g., RNA interference-based), HIS (BALB-Rag/γ) mice can be constructed using HSC transduced with "Tet-on" lentiviral vectors, which render gene expression or down-regulation inducible upon in vivo doxycycline treatment (M. Centlivre et al., manuscript submitted). Once HIS (BALB-Rag/γ) mice are produced, their effective engraftment level is monitored by flow cytometry on blood samples, not before 6 weeks post-inoculation, in order to identify the animals with satisfying reconstitution level for subsequent experimental use. Such animals can be constructed to dissect mechanisms of human hematopoiesis. For instance, HIS (BALB-Rag/γ) mice were used in our laboratory as a model to study human plasmacytoid dendritic cell (pDC) development, by demonstrating that the SpiB transcription factor is required for proper pDC ontogeny (21). We showed that HIS (BALB-Rag/γ) mice contain a population of $IgM^+IgD^+CD27^+$ marginal zone-like B cells, which develop in a NOTCH2-dependent fashion (22). We also observed that human hematopoiesis in HIS (BALB-Rag/γ) mice is sensitive to the addition of human cytokines, such as IL-7 (A.U. van Lent et al., manuscript submitted) and IL-15 (50). Furthermore, we described that in vivo treatment of HIS (BALB-Rag/γ) mice with a superagonist anti-human CD28 antibody leads to accumulation of human thymocytes and $CD4^+FoxP3^+$ regulatory T cells (24). These results indicate that HIS (BALB-Rag/γ) mice are particularly adapted to investigate the mechanisms underlying the development of specific human hematopoiesis-derived cell subsets, as well as a pre-clinical model for therapeutic approaches.

The study of the interaction between a human immune system and human cell tropic pathogens is of major interest to better understand the roadblocks during the immune response. Similar to other humanized mouse models, the HIS (BALB-Rag/γ) mice are able to mount cellular and humoral adaptative immune responses, although in a limited fashion, and they contain functional human antigen-presenting cells (17). As expected from the seminal studies with SCID-hu (Thy/Liv) mice, several reports clearly indicate that HIS (BALB-Rag/γ) mice can support productive HIV infection, mimicking some aspects of HIV pathogenesis (25–29). This includes $CD4^+$ T-cell depletion, blood viremia, and spreading of the virus into the lymphoid organs. Still, despite productive and sustained HIV infection, no T cell and only rare B-cell anti-HIV immune responses were reported so far. Of note, similar results have been described in humanized NOD/SCID/$\gamma_c^{-/-}$ mice (30, 31) and BLT mice (32). Interestingly, several studies have focused on the mucosal transmission of HIV in humanized mouse models. Using the BLT mice, it was shown that HIV can be efficiently transmitted to humanized

mice via the rectal or vaginal routes, resulting in a systemic infection (32, 33). Despite significantly less human cells at the mucosal level, similar results have also been obtained in HIS (BALB-Rag/γ) mice (34). These results are very promising, since they give access to in vivo testing of new prophylactic treatments preventing mucosal transmission of HIV infection (33). In this context, it is of interest to note that the proof-of-concept of a gene therapy against HIV using a short-hairpin RNA-based approach was obtained in several humanized mouse models, including HIS (BALB-Rag/γ) mice (23, 35, 36). In the future, it is likely that several other human-specific pathogens will be assessed in humanized mice, as illustrated by a recent study reporting that HIS (BALB-Rag/γ) mice support dengue virus infection (37). A special interest should be given to pathogens responsible for devastating infectious diseases in developing countries, and humanized mice are particularly appropriate as a bridging tool between fundamental work and clinical testing of potential therapies.

In this protocol, we give a detailed description of the construction of HIS (BALB-Rag/γ) mice, including the methods used to genetically engineer the human HSC ex vivo by lentiviral transduction, in order to manipulate the in vivo expression of genes of interest. It usually takes 6–7 weeks before significant amounts of human lymphocytes can be detected in the peripheral blood of the HIS (BALB-Rag/γ) mice. Blood samples and lymphoid organ suspensions are analyzed by flow cytometry to determine the level of human reconstitution, by measuring the frequency of hematopoiesis-derived ($CD45^+$) human cells and various immune cell populations. At the end of this protocol, we provide details on the outcome of lentiviral transduction and on the major dendritic cell populations found in HIS (BALB-Rag/γ) mice.

2. Materials

2.1. Production of Lentiviral Supernatants

1. Dulbecco's phosphate-buffered saline (DPBS) buffer without calcium/magnesium (20X stock solution): Dissolve 8 g KCl, 8 g KH_2PO_4, 57.6 g $Na_2HPO_4{\cdot}2H_2O$, and 320 g NaCl in 2 l of distilled water and adjust the pH to 7.2 with HCl. Dilute the 20X stock solution with distilled water. The 20×X and 1X solutions are kept at room temperature.
2. Antibiotic aliquots: PBS buffer as described in **Section 2.1**, Item 1, supplemented with 50×10^3 U/ml penicillin and 50 mg/ml streptomycin (powdered penicillin and streptomycin; Roche). Powdered antibiotics are kept at 4–8°C and dissolved aliquots are stored at −20°C.

3. Complete Iscove's modified Dulbecco's medium (IMDM) supplemented with 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), L-glutamine (Gibco-Invitrogen). Store at 4–8°C.
4. Complete IMDM as described in **Section 2.1**, Item 3, supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone). Store at 4–8°C.
5. Complete IMDM as described in **Section 2.1**, Item 3, supplemented with 10% heat-inactivated FCS and 0.2% (vol:vol) antibiotics (Roche). Antibiotics are prepared as described in **Section 2.1**, Item 2. Store at 4–8°C.
6. Opti-MEM medium (Gibco-Invitrogen). Store at 4–8°C.
7. Opti-MEM medium (Gibco-Invitrogen), supplemented with 0.2% (vol:vol) antibiotics (Roche). Antibiotics are prepared as described in **Section 2.1**, Item 2. Store at 4–8°C.
8. Human embryonic kidney (HEK) 293T cells, which are available at the American Tissue Type Culture Collection (ATCC, reference CRL-11268), are used for lentiviral production.
9. Polystyrene cell culture flasks of 25 cm^2 (T25) or 75 cm^2 (T75) if lentiviral supernatant concentration is required.
10. Lentiviral vector construct, e.g., pCDH1 (System Biosciences) for gene overexpression (*see* **Note 1**).
11. Conditional packaging system for third-generation lentivirus vector production is provided by plasmids encoding HIV products Gag and Pol (e.g., pMDLg/pRRE), Rev (e.g., pRSV-Rev), and components of the envelope for virions production (e.g., pVSV-g) (38) (*see* **Note 1**).
12. Lipofectamine-2000 (Invitrogen) as a transfection reagent. FuGENE-6 (Roche) can also be used in the same conditions.
13. Amicon Ultra-15 centrifugal filter units Ultracel 100 K (Millipore).
14. Human T-lymphocytic SupT1 cells (ATCC, reference CRL-1942) for titration of the lentiviral supernatant.

2.2. Preparation of Recipient Mice

1. Newborn BALB/c $Rag2^{-/-}\gamma_c^{-/-}$ mice (*see* **Note 2**).
2. Sterile laminar flow cabinet, and autoclaved individual ventilated cages, water bottles, and food pellets.
3. Sterile irradiation container in which newborn mice fit. If the device is hermetically closed, it should contain enough air for the complete duration of the irradiation process.
4. An irradiation source (*see* **Note 3**).

2.3. Isolation of Nucleated Cells from Human HSC Source

1. A source of human HSC. Umbilical cord blood (UCB) is withdrawn from the umbilical cord directly after delivery, and fetal liver is obtained from elective abortions with gestational age ranging from 12 to 20 weeks (*see* **Note 4**). The raw material can be maintained at 4–8°C overnight in a cold room or in a fridge.
2. PBS buffer as described in **Section 2.1**, Item 1.
3. Roswell Park Memorial Institute-1640 (RPMI) medium supplemented with 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), L-glutamine (Gibco-Invitrogen). Store at 4–8°C.
4. RPMI medium as described in **Section 2.3**, Item 3, supplemented with 2% heat-inactivated FCS and 0.2% (vol:vol) antibiotics. Antibiotics are prepared as described in **Section 2.1,** Item 2. Store at 4–8°C.
5. Ethanol 70% and scissors.
6. Stomacher®-80 Biomaster lab system (Seward), Stomacher® bags, and a bag-sealing device (e.g., Sealboy 236 Audion Elektro) (*see* **Note 5**).
7. Plastic disposables: 10- and 25-ml pipettes, 100 mm × 20 mm polystyrene Petri dishes, 50-ml polypropylene conical tubes.
8. Lymphoprep (Axis Shield). Store at 4–8°C.

2.4. Enrichment for $CD34^+$ Hematopoietic Stem Cells

1. RPMI medium as described in **Section 2.3**, Item 4.
2. CD34 progenitor cell isolation kit, human (Miltenyi Biotec) (*see* **Note 6**).
3. MACS buffer prepared as follows: PBS buffer as described in **Section 2.1,** Item 1, supplemented with 0.5% bovine serum albumin (BSA; Sigma) and 2 mM ethylene-diamine-tetra-acetic acid (EDTA; Sigma). Store at 4–8°C.
4. MACS separator, large-scale (LS) MACS separation columns, MACS pre-separation filters and recovery tubes (Miltenyi Biotec).

2.5. Cytometry Cell Sorting for $CD34^+CD38^-$ Hematopoietic Stem Cells

1. RPMI medium as described in **Section 2.3**, Item 4.
2. Any FCS-rich medium can be used to harvest the cells during the cell sorting. For instance, use IMDM medium as described in **Section 2.1**, Item 5.
3. Fluorescence-activated cell sorter, for isolation of the HSC-enriched population (*see* **Note 7**).
4. Fluorochrome-coupled monoclonal antibodies (*see* **Note 8**): anti-huCD38 (clone HB-7, BD Biosciences), anti-huCD34

(clone 581, BD Biosciences). Store the antibodies in the dark at 4–8°C.

5. 5-ml Polystyrene round-bottomed 12 mm × 75 mm tubes.
6. Sterile 50-μm Filcon filters (Becton Dickinson).
7. Sterile 1-ml Plastipak syringes (Becton Dickinson).

2.6. Lentiviral Transduction of Hematopoietic Stem Cells

1. Human cell culture medium: Complete IMDM as described in **Section 2.1**, Item 3, supplemented with 5% normal human serum (NHS; Invitrogen) and Yssel's supplement (50 ml in a 500-ml bottle of IMDM; Diaclone). Store at 4–8°C.
2. Aliquots (2 μg/ml) of the following recombinant cytokines in complete IMDM as described in **Section 2.1**, Item 3: human interleukin-7 (IL-7; Tebu-Peprotech), human stem cell factor (SCF; Tebu-Peprotech), and human thrombopoietin (TPO; Tebu-Peprotech).
3. Transduction medium: Human culture medium as described in **Section 2.6**, Item 1, supplemented with 20 ng/ml of human IL-7, SCF, and TPO (1/100 dilution of cytokine stocks described in **Section 2.6**, Item 2).
4. PBS buffer as described in **Section 2.1**, Item 1, supplemented with 2% heat-inactivated FCS. Store at 4–8°C.
5. Two series of 24-well plates, both non-tissue culture and tissue culture treated.
6. PBS buffer as described in **Section 2.1**, Item 1, supplemented with 30 μg/ml retronectin (Cambrex-TaKaRa). Store at 4–8°C.
7. PBS buffer as described in **Section 2.1,** Item 1, supplemented with 2% BSA. Store at 4–8°C.
8. RPMI medium as described in **Section 2.3**, Item 4.

2.7. Inoculation of Hematopoietic Stem Cells into Recipient Mice

1. RPMI medium as described in **Section 2.3**, Item 4.
2. BD Micro-Fine+ U-100 Insulin 0.5 ml 0.33 (29 ga) × 12.7 mm syringes (BD Biosciences).

2.8. Monitoring of "Human Immune System" (BALB-Rag/γ) Mice

1. Microvette® CB300 lithium heparin coated for capillary blood collection (Sarstedt), supplemented with one drop (5–10 μl) of heparin (5.10^3 IU/ml). Each Microvette® is composed of an outer tube and an inner tube embedded in it. The inner tube has a top cap and can receive a plug at the bottom part of the tube. Blood is collected by capillarity from the bottom and the bottom plug is put in position afterward.

2. 5-ml Polystyrene round-bottomed 12 mm × 75 mm tubes.
3. PBS buffer as described in **Section 2.1**, Item 1.
4. Lymphoprep (Axis Shield). Store at 4–8°C.
5. RPMI medium as described in **Section 2.3**, Item 4.
6. Fluorochrome-coupled monoclonal antibodies (*see* **Note 9**): anti-huCD45 (clone 2D1, BD Biosciences) for the detection of human hematopoiesis-derived cells and other antibodies according to the analyzed cell populations. Store the antibodies in the dark at 4–8°C.
7. FACS buffer: PBS buffer as described in **Section 2.1**, Item 1, supplemented with 2% heat-inactivated FCS and 0.02% sodium azide (NaN_3). Store at 4–8°C.
8. FACS buffer as described in **Section 2.8**, Item 7, supplemented with 0.2 mM 4′ , 6-diamidino-2-phenylindole (DAPI; Sigma) for dead cell exclusion. Store in the dark at 4–8°C. Store aliquots 100× concentrated (20 mM) at −20°C.
9. Round-bottomed 96-well plates.
10. 1.4-ml U-shaped FACS tubes.
11. Fluorescence-activated cell sorter, for analysis of the cell populations (*see* **Note 7**).

3. Methods

The production of HIS (BALB-Rag/γ) mice with engineered gene expression is strongly dependent on efficient planning and logistics. It can be summarized as follows: (a) the lentiviral vector supernatant – for enforced, knock-down, or inducible expression of one specific gene – is produced in 293T cells; (b) the human $CD34^+CD38^-$ HSC-enriched cell population is isolated by consecutive magnetic and fluorescence-activated cell sortings (MACS and FACS, respectively); (c) the HSC-enriched cell population is transduced in vitro with the lentiviral supernantant; and (d) newborn BALB/c Rag-$2^{-/-}\gamma_c^{-/-}$ mice are eventually inoculated with these genetically modified HSC (**Fig. 6.1**). Steps (a) and (c) are skipped when "unmanipulated" HIS (BALB-Rag/γ) mice are produced.

In this protocol, we describe the isolation of human HSC from fetal liver, but umbilical cord blood (UCB) can be easily used as well. Similarly, we focus here on the use of newborn BALB/c Rag-$2^{-/-}\gamma_c^{-/-}$ mice as recipients for humanized mice production, but alternative protocols can be considered for other

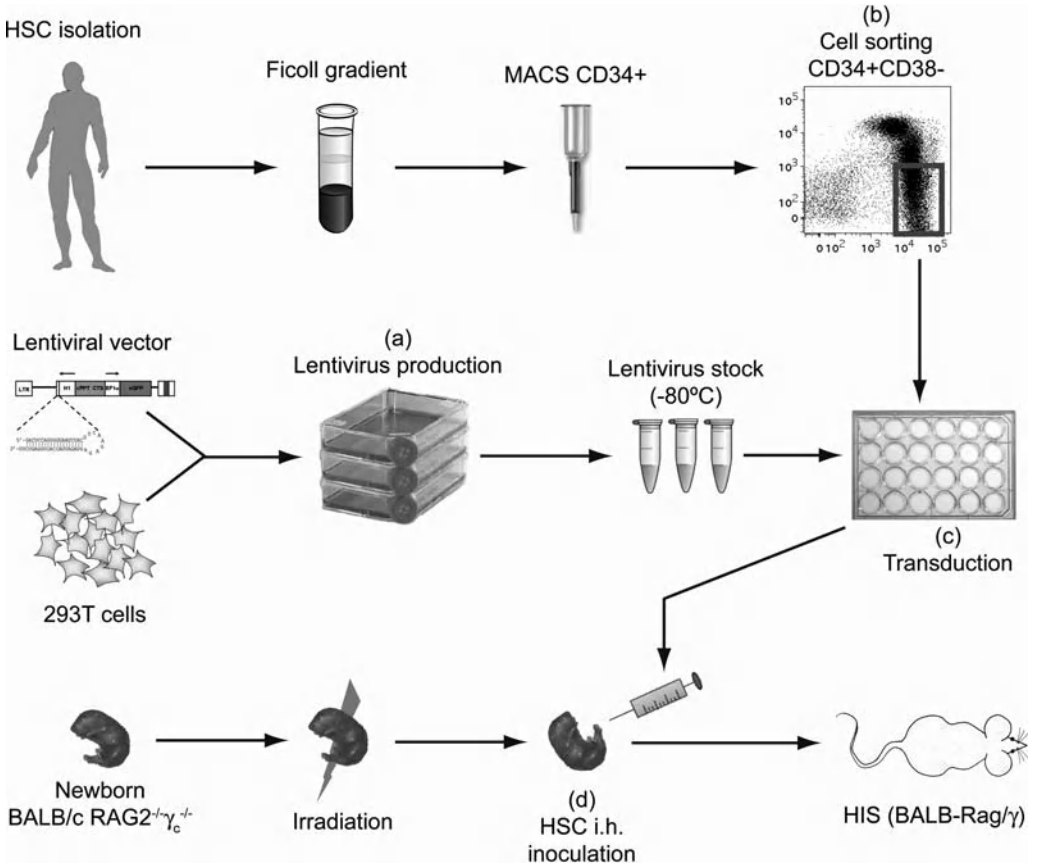

Fig. 6.1. Schematic protocol for the production of HIS (BALB-Rag/γ) mice with lentiviral-mediated gene transfer. Lentiviral supernatant is produced on HEK 293T cells and frozen until used. Human hematopoietic progenitors are prepared from fetal, perinatal, or adult sources. Live nucleated cells are isolated on a density gradient, $CD34^+$ are then magnetically isolated and further purified for the $CD34^+CD38^-$ fraction using fluorescence-activated cell sorting. Lentivirus-mediated gene transduction of sorted HSC is performed immediately after cell sorting. Sublethally irradiated newborn BALB/c Rag-$2^{-/-}\gamma_c^{-/-}$ mice are injected intra-hepatically with the cell preparation, which contains a fraction of effectively transduced cells. The HIS (BALB-Rag/γ) mice are tested after 7–8 weeks for the presence of human (transduced) cells in the blood.

mouse strains and specific applications (39). All isolation steps have to be performed in a sterile manner under laminar flow. Both $CD34^+$ (**Section 3.4**) or $CD34^+CD38^-$ cells (**Section 3.5**) can be used to engraft recipient mice, depending on the available amount and the desired degree of purity. Still, $CD34^+CD38^-$ cells are more reliable and more potent, and tenfold less cells are required to obtain good human engraftment, as compared to non-sorted $CD34^+$ cells. Since $CD34^+CD38^-$ cells yield from UCB is usually limited, we advise to inject $CD34^+$ cells from UCB, whereas $CD34^+CD38^-$ cells can be routinely isolated from fetal liver. Concerning the number of cells to be injected into the newborn recipients, we have experienced reliable and high

reconstitution level when at least 5×10^5 $CD34^+$ cells and 5×10^4 $CD34^+CD38^-$ cells are inoculated per pup, lower numbers result in higher variability in the outcome. In the case of ex vivo HSC manipulation by lentiviral transduction, it is of course wise to produce the lentivirus far before use, since virus aliquots can be kept frozen for years (**Section 3.1**). Although both fetal liver $CD34^+CD38^-$ and total $CD34^+$ cell populations are susceptible to lentivirus-mediated transduction, the use of $CD34^+CD38^-$ cells is preferred in order to spare manipulation time, expensive reagents, and lentiviral supernatants. Alternative lentiviral supernatant production and HSC transduction protocols can be used to adapt to particular conditions and targeted cell type (40).

3.1. Production of Lentiviral Supernatants

1. Maintain a stock of HEK 293T cells in an incubator at 37°C with 5% CO_2, in IMDM 10% FCS medium with antibiotics. From the stock, prepare one flask of HEK 293T cells per lentiviral supernatant to be produced. Seed $1–2 \times 10^6$ cells and $4–6 \times 10^6$ cells per T25 and T75 flasks, respectively, in an appropriate volume of IMDM 10% FCS medium without antibiotics. Within 1 day, the cells should reach 60–70% confluence in the flask and are then ready to use.
2. For each lentiviral vector, prepare one tube with a mixture of DNA plasmids in Opti-MEM medium without antibiotics, as follows: for a T25 flask, mix 2.4 μg of the lentiviral vector, 1.5 μg of pMDLg/pRRE plasmid, 0.8 μg of pVSV-g plasmid, and 0.6 μg of pRSV-Rev plasmid, in 0.6 ml of Opti-MEM medium; for a T75 flask, mix 7.1 μg of the lentiviral vector, 4.6 μg of pMDLg/pRRE plasmid, 2.5 μg of pVSV-g plasmid, and 1.8 μg of pRSV-Rev plasmid, in 2 ml of Opti-MEM medium. Incubate for 5 min at room temperature.
3. For each lentiviral vector, prepare one tube containing 16 μl of lipofectamine-2000 in 0.6 ml of Opti-MEM medium (T25 flask) or 48 μl of lipofectamine-2000 in 2 ml of Opti-MEM medium (T75 flask). Incubate for 5 min at room temperature.
4. Add the lipofectamine-containing medium into the DNA-containing medium and mix gently. The final volume of the lipofectamine–DNA mixture is 1.2 ml (T25 flask) or 4 ml (T75 flask). Incubate for 20 min at room temperature.
5. Replace the medium in the flask, to reach a final volume of 2.2 ml (T25 flask) or 6.6 ml (T75 flask) IMDM 10% FCS culture medium without antibiotics. Perform the transfection by adding the lipofectamine–DNA mixture to

the culture medium in the flask. Incubate overnight at 37°C with 5% CO_2.

6. At day 1 after transfection, replace the medium as follows: 3.5 ml (T25 flask) or 10.5 ml (T75 flask) of Opti-MEM medium with antibiotics. Incubate overnight at 37°C with 5% CO_2.
7. At day 2 after transfection, harvest the supernatant from the flask and store it at 4–8°C. Replace the medium in the flask: 3.5 ml (T25 flask) or 10.5 ml (T75 flask) of Opti-MEM medium with antibiotics. Incubate overnight at 37°C with 5% CO_2.
8. At day 3 after transfection, harvest the supernatant from the flask, and pool it with the stored supernatant of the previous day. Centrifuge the supernatant to pellet detached HEK 293T cells (450 × *g*, 5 min). Filter the centrifuged supernatant with a 0.45-μm filter.
9. (*Optional*) If necessary, concentrate the lentivirus on Amicon column. Apply 15 ml of supernatant on top of the column and centrifuge (1,000 × g, 10 min). Most of the supernatant is going through and the lentiviral virions are retained on top of the column. Harvest the remaining supernatant on top of the column and adjust the volume if necessary, by adding the desired volume of Opti-MEM medium.
10. Aliquot the lentiviral supernatant (0.3–0.5 ml per tube) and store at −80°C.
11. Titrate the transduction units per ml in the lentiviral supernatant on SupT1 cells. In brief, seed a fixed amount of SupT1 cells (e.g., 2×10^5) in a 24-well plate, in IMDM 10% FCS medium with antibiotics. To each well, add a decreasing amount of the lentiviral supernatant, for instance 100, 30, 10, 3, and 1 μl, in a final volume of 100 μl. Wash the SupT1 cells after 6 h of transduction. Three days after transduction, harvest the SupT1 cells and measure the frequency of GFP^+ cells by flow cytometry. The titer of the viral supernatant is calculated for each well as follows: transduction units (TU)/ml = [%GFP^+] × [number of SupT1 cells] × 10/[used volume of lentiviral supernatant in μl]. Example: if 1 μl of virus on 2×10^5 SupT1 cells gives 15% GFP^+ cells, the titer is 3×10^7 TU/ml. The most accurate titer evaluations are obtained in wells with a GFP^+ frequency in a 10–30% range (*see* **Note 10**).

3.2. Preparation of Recipient Mice

1. BALB/c Rag-$2^{-/-}\gamma_c{}^{-/-}$ mice exhibit profound immunodeficiency and the colony has to be maintained in strict health

conditions, e.g., in isolators or individual ventilated cages. To hinder the risk of infection, always work in sterile conditions, e.g., under laminar flow, wearing lab coat, disinfected gloves, and clean material. After irradiation or inoculation of the human cells, the mice are transferred back to cages. Therefore, the necessary cage space, sterile food, water, and material have to be prepared accordingly.

2. Production of newborn mice has to be planned depending on the frequency and amount of available human HSC. Since engraftment efficiency is age-dependent (18), we recommend the use of newborn mice as early as possible, and always before a week of age (*see* **Note 11**). Once pregnant, female mice are removed from the breeding cage and male mice are kept in the breeding colony. The female and her nest are taken out of the breeding colony and manipulated under laminar flow during all subsequent manipulations.
3. Isolate the newborn mice from the breeding cage and transfer them to the sterile irradiation container. Transport the container to the irradiation apparatus and apply sublethal total body irradiation to the newborn mice (~3–4 Gy) (*see* **Note 3**). Bring the newborns back to the cage containing the mother and let them rest 4–24 h before xenograft transplantation.

3.3. Isolation of Nucleated Cells from Human HSC Source (Fetal Liver)

1. Prepare material under laminar flow as follows (per fetal liver): one Stomacher® bag; one 100 mm × 20 mm dish; six 50-ml tubes; one bottle (500 ml) of RPMI 2% FCS medium (*see* **Note 12** for alternative procedure using UCB as a source of HSC).
2. Pieces of fetal liver are collected in 15-ml plastic tubes containing 2–4 ml of RPMI 2% FCS medium and have to be processed into cell suspension. Transfer the content of one tube into one Stomacher® bag, add 15–20 ml of RPMI 2% FCS medium and seal the bag twice to avoid any liquid loss. Install the bag inside the Stomacher®-80 Biomaster lab system and let it mechanically process the liver pieces for 1 min at high speed. The bag now contains a cell suspension mixed with non-dissociated liver stroma.
3. Wash the bag with ethanol 70% and open it with sterile scissors. Transfer the contents of the bag into a 100 mm × 20 mm Petri dish.
4. Pour RPMI 2% FCS medium directly from the bottle to the dish in order to dilute the cell suspension. Let the big pieces sediment for 1–2 min. Using a 25-ml pipette, recover the supernatant from the dish and distribute equally among the six 50-ml tubes. Always avoid the pieces of stroma at the

bottom of the dish. When it becomes difficult to avoid stroma, pour new RPMI 2% FCS medium into the plate and repeat this procedure until the recovered medium is clear. Usually, pouring fresh medium three times is enough.

5. Add RPMI 2% FCS medium to the tubes until you reach approximately 20 ml. Wash the cell suspensions by centrifugation (450 × *g*, 5 min). Aspirate the fat-containing supernatant with a pipette or a vacuum system.
6. Resuspend the cell pellet with a 10-ml pipette by adding 13 ml RPMI 2% FCS medium into each tube. Once a homogeneous cell suspension is obtained by repeated pipetting, bring 10 ml Lymphoprep underneath the cell suspension with another 10 ml pipette, carefully avoiding air bubbles that could disturb the formation of a clearly distinguishable interface (*see* **Note 13**).
7. Carry the tubes to the centrifuge, carefully avoiding disturbing the interface. Centrifuge the tubes at 1, 100 × g for 15 min with low acceleration and no brake (the centrifugation lasts for ~25 min). After centrifugation, the pellet mostly contains erythrocytes and dead cells, whereas live nucleated cells are concentrated as a cell ring at the interface. Recover the top supernatant and the cell ring from each tube and pool into new tubes (three tubes into one).
8. Wash the cell suspensions by centrifugation (450 × *g*, 5 min) and pool pellets in a total of 10 ml of RPMI 2% FCS medium. Count the amount of nucleated cells in the suspension (*see* **Note 14**).

3.4. Enrichment for CD34+ Hematopoietic Stem Cells

1. Pellet the nucleated cells by centrifugation (450 × *g*, 5 min).
2. Enrichment for the CD34-expressing fraction is done by magnetic sorting, using a two-step strategy: (a) anti-CD34 hapten-coupled antibody; (b) anti-hapten antibody conjugated with colloidal paramagnetic beads (*see* **Note 6**). Perform the first step of indirect MACS labeling: for 10^8 nucleated cells in the pellet, use 75 µl of RPMI 2% FCS medium to resuspend the pellet and add 25 µl of each "A" reagent (FcR blocking human immunoglobulins; monoclonal hapten-conjugated anti-huCD34 antibody). Keep the tube at 6–12°C for 15 min (in the fridge, as recommended by the MACS kit's manufacturer).
3. Add 10 ml of RPMI 2% FCS medium into the tube and wash the cell suspensions by centrifugation (450 × *g*, 5 min).
4. Perform the second step of indirect MACS labeling: for 10^8 nucleated cells in the pellet, use 100 µl of RPMI 2%

FCS medium to resuspend the pellet and add 25 μl of the "B" reagent (colloidal super-magnetic MACS MicroBeads conjugated to an anti-hapten antibody). Keep the tube at 6–12°C for 15 min (in the fridge, as recommended by the kit's manufacturer).

5. During the incubation, install the magnetic separation column and a pre-separation filter on top of it. Prepare the collection tube for the column flow-through. Proceed to filter/column washing with MACS buffer, following the manufacturer's instructions (*see* **Note 15**). For an LS separation column, apply 3 ml of MACS buffer and let it run through.
6. Add 10 ml of RPMI 2% FCS medium into the tube and wash the cell suspensions by centrifugation (450 × *g*, 5 min).
7. Resuspend the cell pellet in MACS buffer, using 500 μl of buffer per 10^8 cells.
8. Apply cell suspension through pre-separation column to remove clumps and let the cells pass through the column. Perform column washings with MACS buffer, as indicated in the manufacturer's hand-guide. For an LS column, apply three times 3 ml of MACS buffer on the filter/column assemblage, adding buffer only when the column reservoir is completely empty.
9. Harvest the magnetically labeled cells from the column. Place the column on top of a new 15-ml tube containing 5 ml of RPMI 2% FCS medium. For an LS column, pipette 5 ml of MACS buffer onto the column and apply the plunger on the column. Immediately flush out the CD34-enriched fraction (*see* **Note 16**).
10. Wash the cell suspension by centrifugation (450 × *g*, 5 min). Resuspend the pellet in a volume of 1 ml of RPMI 2% FCS medium and count the amount of nucleated cells in the suspension (*see* **Note 17**).

3.5. Cytometry Cell Sorting for $CD34^+CD38^-$ Hematopoietic Stem Cells

1. Pellet the nucleated cells by centrifugation (450 × *g*, 5 min).
2. Meanwhile, prepare the monoclonal antibody mixture for cell sorting: mix 1 μl of the anti-CD34 antibody and 1 μl of the anti-CD38 antibody per 10^6 cells (with a minimal final volume of 20 μl).
3. Remove as much supernatant as possible after centrifugation of the cells. Apply the antibody mixture on the dry pellet and resuspend the cells by repeated pipetting. Incubate for 10 min on ice (to avoid antibody capping) and in the dark (to avoid fluorochrome bleaching).

4. Add 5 ml of RPMI 2% FCS medium into the tube and wash the cell suspensions by centrifugation (450 × *g*, 5 min).
5. During centrifugation, prepare a set of 5-ml polypropylene tubes: one sorting tube that will contain the sample to be sorted; one recovery tube with 1 ml of FCS-rich medium (e.g., IMDM 10% FCS). For each sample to be sorted, prepare one syringe attached to a 50-μm Filcon filter.
6. After centrifugation, discard the supernatant and resuspend the pellet in RPMI 2% FCS medium to obtain a cell concentration of 5–10 × 10^6 cells/ml (minimum 0.5 ml) (*see* **Note 18**). Transfer the cells to the syringe and flush them through the filter to remove potential sources of clogs for the cell sorter.
7. Sort the human HSC-enriched $CD34^+CD38^-$ population. If possible, maintain the sorted sample and the recovery tube at 4°C, if possible on the used cell sorter.
8. After the cell sorting, centrifuge the recovery tubes (450 × *g*, 5 min). Resuspend the cells in an accurate volume of RPMI 2% FCS medium and count the amount of nucleated cells in the suspension (*see* **Note 19**).

3.6. Lentiviral Transduction of Hematopoietic Stem Cells

1. In a tissue culture treated 24-well plate, seed a maximum of 5 × 10^5 sorted $CD34^+CD38^-$ HSC per well in 1 ml of the human cytokine-supplemented transduction medium. Incubate the cells overnight at 37°C.
2. Prepare the transduction plate for the next day. For this purpose, coat wells of a non-tissue culture treated 24-well plate with 30 μg/ml retronectin, using 0.5 ml per well. Use one well per lentiviral construct to be used, for a maximum of 5 × 10^5 cells per well, and determine the number of wells that will be required during lentiviral transduction. Incubate overnight at 4–8°C (or for 1 h at 37°C if you prepare this transduction plate the next morning).
3. The next day, recover the retronectin from the transduction plate (it can be re-used at least twice afterward) and replace it by 0.5 ml of PBS 2% BSA. Incubate for 15–30 min at 37°C.
4. From the culture plate containing the HSC, remove 0.4 ml of culture medium from each well and spare in an unused well of the plate. Resuspend the HSC with the remaining 0.6 ml of culture medium and transfer into a 15-ml tube. Wash each well with 0.4 ml of spared culture medium. At this stage, all wells can be pooled into one single tube.
5. Centrifuge the cells (450 × *g*, 5 min) and harvest the supernatant in a separate tube. This culture medium supernatant is used to resuspend the cells in the desired final volume

(0.3 ml per transduction well). Add cytokines to compensate for the volume of the viral supernatant (0.3 ml), i.e., temporarily spare the cells in culture medium with a 2X cytokine concentration.

6. Prepare a plate for the verification of transduction efficiency. The left-over of culture medium supernatant is distributed in a tissue culture treated 24-well plate (1 well per transduction condition). Add fresh human cell culture medium to reach a final volume of 1 ml per well. It is not necessary to compensate with fresh cytokines, unless their concentration goes below 5 ng/ml. To avoid excessive evaporation in the culture well, distribute 0.5 ml of PBS into the surrounding wells.
7. Remove the PBS 2% BSA from the wells of the transduction plate and wash with 1 ml of PBS. Distribute 0.3 ml of HSC per well in culture medium with 2X cytokines (maximum 5×10^5 cells). Add 0.3 ml of lentiviral supernantant per well (*see* **Note 20**). Incubate for 6 h at 37°C.
8. After incubation, harvest the cells from the wells into separate 15-ml tubes, and wash the wells with 1 ml RPMI 2% FCS medium. Centrifuge the cells (450 × *g*, 5 min), resuspend in an accurate volume of RPMI 2% FCS medium, and count the cells.
9. In the plate for the verification of transduction efficiency, seed a small aliquot of each set of transduced HSC in the prepared wells ($1–5 \times 10^4$ cells per well is sufficient). Incubate the plate for 3 days at 37°C. After 3 days, harvest the cells and measure the transduction efficiency by checking GFP expression by flow cytometry.

3.7. Inoculation of Hematopoietic Stem Cells into Recipient Mice

1. For optimal conditions, plan to inoculate 10^5 $CD34^+CD38^-$ cells per newborn. Pellet the desired amount of cells by centrifugation (450 × *g*, 5 min). Resuspend the pellet in the desired volume of RPMI 2% FCS medium (35 µl per newborn mouse to be injected) (*see* **Note 21**).
2. Transfer the cells into a 1.5-ml eppendorf tube, to ensure full access of the needle to the cell suspension.
3. In a laminar air flow cabinet, isolate the newborn mice on a piece of absorbent paper on the bench. For each newborn mouse repeat the following sequence: hold the mouse between thumb and index fingers, with the head down; inject 30–35 µl of cell suspension by intra-hepatic route, i.e., between the thoracic cage and the milk-filled stomach, which appears white through the skin (**Fig. 6.2**);

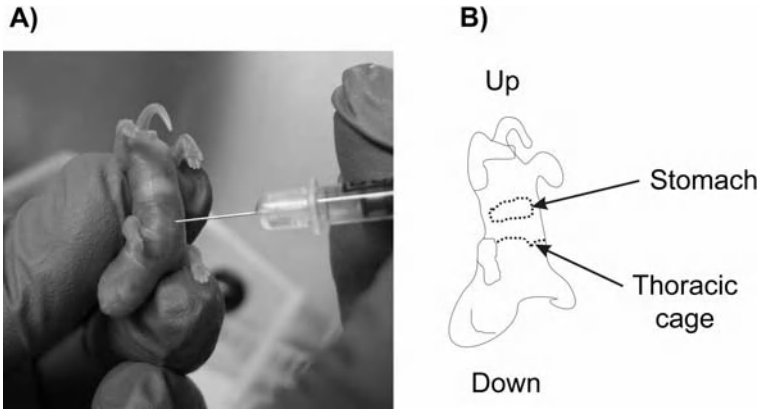

Fig. 6.2. Intra-hepatic inoculation of human progenitor cells in newborn mice. (**A**) The newborn animals are maintained upside down between two fingers. Using a microsyringe, the cell mixture is injected into the liver, between the stomach and the thoracic cage. (**B**) Schematic positions of the stomach and thoracic cage are indicated. Reproduced from Ref. (49) with permission from Springer.

place the mouse back to its nest in the cage containing the mother. If necessary, toe mark the newborns with sterile scissors.

4. The cage containing the nest and the mother does not require special food diet or water (no antibiotics required). The animals can be weaned normally at 3–4 weeks of age and kept until use.

3.8. Monitoring of "Human Immune System" (BALB-Rag/γ) Mice

1. Peripheral reconstitution of the inoculated mice by human HSC is checked by flow cytometry 7–8 weeks after injection. The fraction of human hematopoiesis-derived is determined by anti-CD45 staining. For each HIS (Rag/γ) mouse, shave one of the hind legs between knee and ankle (lateral side) with a scalpel and make blood arise from the saphenous vein using a needle for limited puncture (**Fig. 6.3**). Collect the blood drops (~50 μl) by capillarity with the Microvette® inner tubes. Mark the mice for numbering and label the Microvette® accordingly.
2. The blood samples have to be enriched for nucleated cells, using "small scale density gradient" purification. Prepare two series of numbered 5-ml round-bottomed tubes. Distribute 1.5 ml of PBS buffer into the group of dilution tubes, and 1.5 ml of Lymphoprep into the group of gradient tubes.
3. Pipette 1 ml of PBS buffer from the first 5-ml round-bottomed tube. Get the inner tube from the Microvette®, open the top cap, horizontally maintain the tube, and

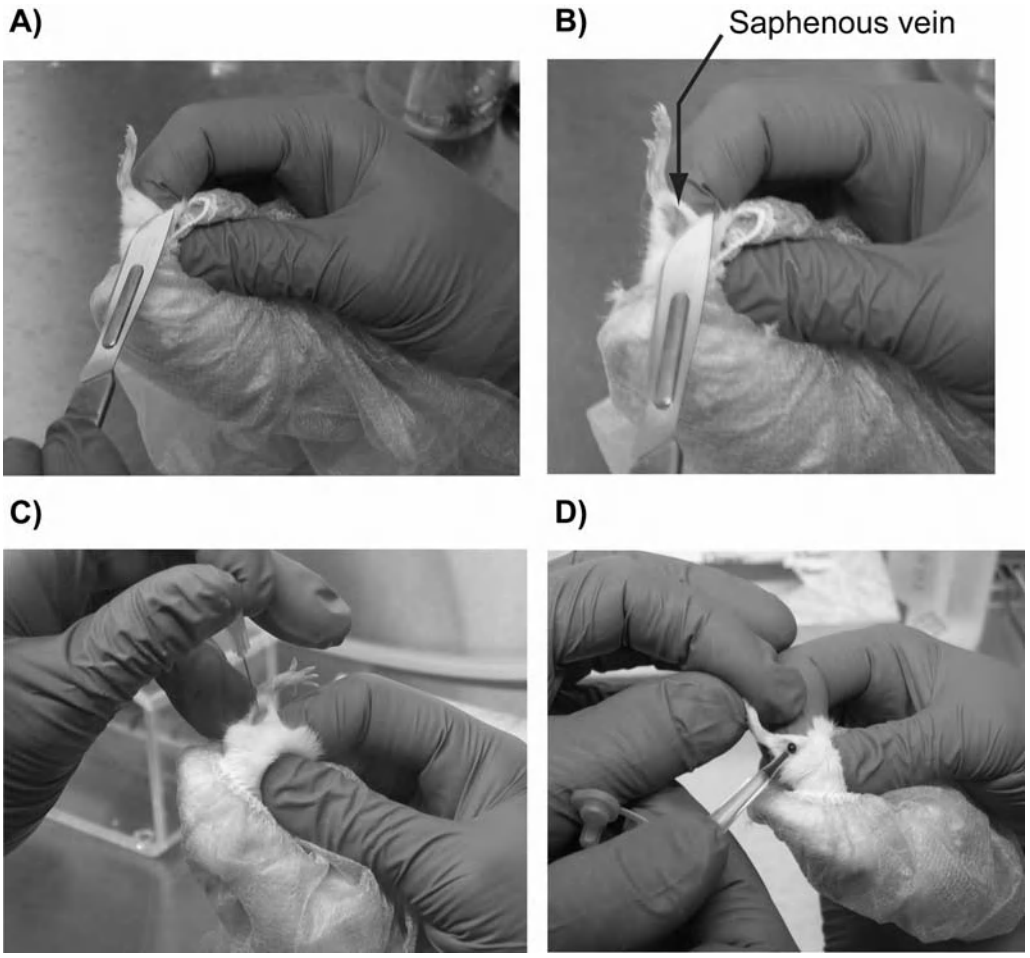

Fig. 6.3. Puncture for blood at the saphenous vein. (**A**) The mice are restrained with the head and part of the body inside a cap. The hand should firmly hold the hind leg outside the cap, so that it can be shaved on the external lateral side, between the knee and the ankle. (**B**) The saphenous vein is exposed and (**C**) a needle is used to make a small puncture. (**D**) Blood drops are collected in the heparin-containing tubes by capillarity. Reproduced from Ref. (49) with permission from Springer.

remove the bottom plug. Put the bottom end of the open Microvette® inner tube against the wall of the corresponding dilution tube. Pipette out the 1 ml of PBS buffer through the inner Microvette® tube: all the blood that was contained will be washed away. Check inside the plug for remaining blood. Repeat this step for every sample.

4. Transfer ~1.5 ml of PBS-diluted blood to the gradient tube, on top of the Lymphoprep layer, by careful pipetting. For instance, use a plastic 2-ml pipette and flush drop wise. Repeat this step for every sample.

5. Carry the tubes to the centrifuge, carefully avoiding disturbing the interface. Centrifuge the tubes at 1,100 × *g* for 15 min with low acceleration and no brake (the centrifugation lasts for ~25 min). After centrifugation, the pellet mostly contains erythrocytes and dead cells, whereas live nucleated cells are concentrated as a cell ring at the interface. Recover the supernatant and the cell ring into series of new numbered 15-ml tubes.
6. Add 5 ml of RPMI 2% FCS medium into the tube and wash the cell suspensions by centrifugation (450 × *g*, 5 min).
7. Meanwhile, prepare the monoclonal antibody mixture for human cell staining: per sample, mix 4 μl of the FITC-coupled and PerCP-Cy5.5 antibodies and 2 μl of the R-PE-coupled, PE-Cy7, APC, and APC-Cy7 antibodies (that is to say 16 μl total per sample in this example).
8. Remove the supernatant after centrifugation of the cells. Resuspend each pellet with 200 μl of FACS buffer and immediately transfer the suspension to individual contiguous wells in a round-bottomed 96-well plate.
9. Pellet the cell suspensions by centrifugation (450 × *g*, 2 min) and remove the supernatant by quick inversion over the sink. Maintain the plate in this position and dry it against a piece of absorbent paper on the bench. Vortex the plate briefly and place it on ice.
10. Apply the antibody mixture on the dry pellet by distributing 15.5 μl per well and briefly vortex the plate. Incubate for 10 min on ice (to avoid antibody capping) and in the dark (to avoid fluorochrome bleaching).
11. During incubation, prepare one FACS tube per sample on the appropriate rack.
12. At the end of the incubation, distribute 100 μl of FACS buffer per well and wash the plate by centrifugation (450 × *g*, 2 min). Remove the supernatant by quick inversion over the sink (*see* **Step 3.6.9**). Bring the plate back on ice and distribute 50 μl of the DAPI-containing FACS buffer per well. Transfer the content of the wells to their respective FACS tube (e.g., with multichannel 200 μl pipette) and perform cytometry analysis.
13. We show here an example of transduction efficiency after transduction (**Fig. 6.4A**) and the recovery of GFP^+ human cells in the corresponding HIS (Rag/γ) mice (**Fig. 6.4B**). Human hematopoiesis is similar to the transduced (GFP^+) and non-transduced (GFP^-) cell populations, as shown by frequency of HSC in bone marrow or

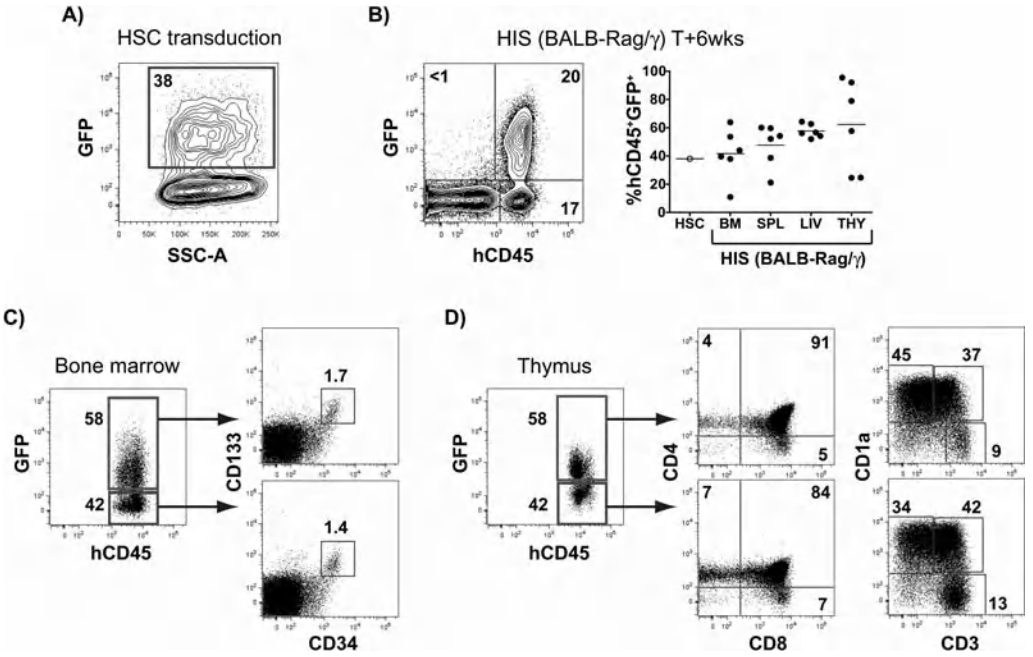

Fig. 6.4. Monitoring of human reconstitution and cell transduction in HIS (BALB-Rag/γ) mice. The blood collected at 6–7 weeks after HSC inoculation is analyzed by flow cytometry. (**A**) At the time of HSC inoculation, an aliquot of the cells is kept in culture for 3 days. Eventually, GFP expression is analyzed by flow cytometry to evaluate the transduction efficiency. (**B**) Few weeks after reconstitution, human hematopoiesis-derived cells are detected with a CD45-specific antibody. GFP expression is restricted to the human cells (*left plot*) and the frequency of GFP is on average at least as good as the initial transduction efficiency (*right graph*). (**C**) Frequency of human HSC ($CD34^+CD133/2^+$) that have colonized the HIS (BALB-Rag/γ) bone marrow 6 weeks after reconstitution, in the transduced (GFP^+, *top plot*) and non-transduced (GFP^-, *bottom plot*) populations. (**D**) A similar analysis was performed of T-cell development in the thymus of HIS (BALB-Rag/γ) mice. All pictures are obtained from 6-week-old HIS (BALB-Rag/γ) mice produced with HSC transduced with a GFP-expressing pCDH1 vector.

T-cell development in the thymus (**Fig. 6.4C/D**). The large majority of human cells found in the blood and lymphoid organs of HIS (Rag/γ) mice is composed by B and T lymphocytes, but several populations of dendritic cells are also detected. The two major dendritic cell populations are $BDCA2^+CD11c^-HLA\text{-}DR^+$ plasmacytoid dendritic cells (pDCs) and $BDCA2^-CD11c^+HLA\text{-}DR^+$ conventional dendritic cells (cDCs) (**Fig. 6.5A**). Most of dendritic cells are found in the bone marrow (especially cDCs), the spleen, and the liver, where pDCs can represent up to 30–40% of total human cells. The pDCs also express IL-3Rα/CD123 on their surface and cDCs express CD40 and B7, as expected from studies in human individuals (**Fig. 6.5B** and not shown).

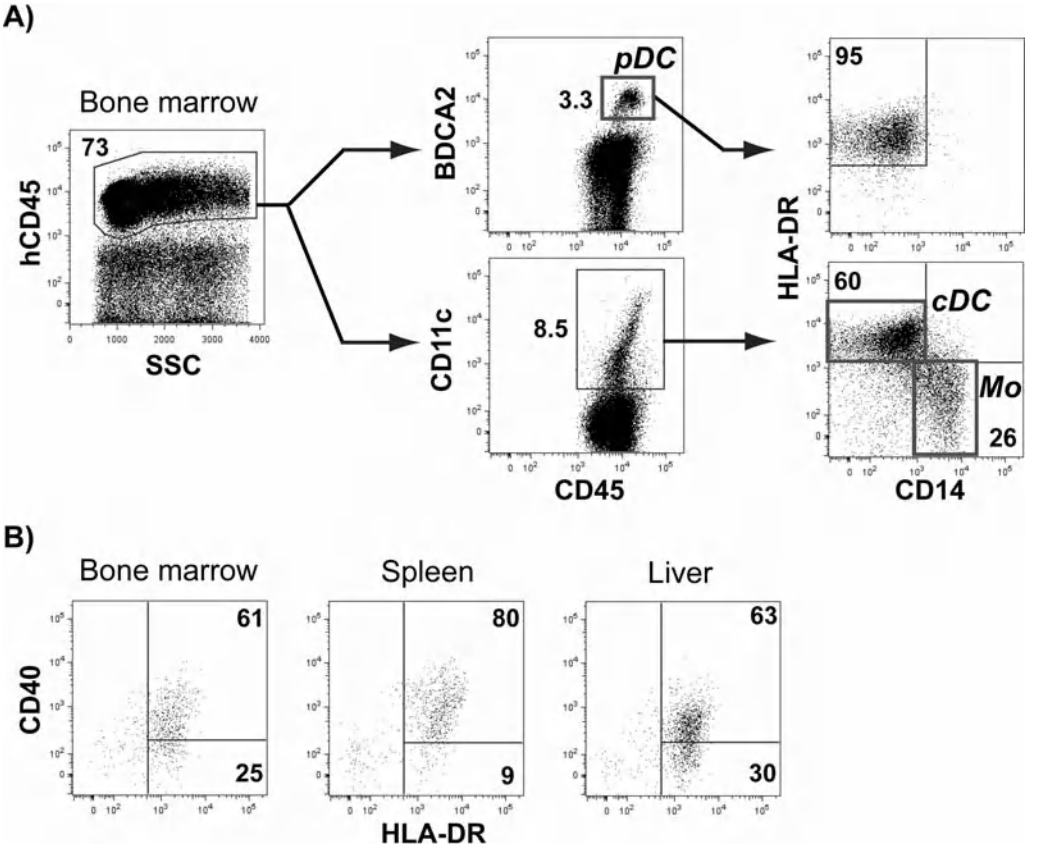

Fig. 6.5. Dendritic cell development in HIS (BALB-Rag/γ) mice. (**A**) The bone marrow of adult HIS (BALB-Rag/γ) mice was analyzed for the presence of plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs, also known as myeloid DCs), based on the expression of BDCA2 and CD11c, respectively. Of note, BDCA2 and CD11c expression is mutually exclusive, and the $CD11c^{+}CD14^{+}$ fraction is described as belonging to the myelo-monocytic (Mo) lineage. (**B**) The majority of the cDC population found in the bone marrow, spleen, and liver of HIS (BALB-Rag/γ) mice expresses the co-stimulatory molecule CD40. All pictures are obtained from 8 to 10-week-old HIS (BALB-Rag/γ) mice produced with HSC transduced with a GFP-expressing pCDH1 vector, and no difference was observed between GFP^{+} and GFP^{-} populations.

4. Notes

1. Gene transfer in immune cell has made extensive use of HIV-derived lentiviral vectors over the last decade, with incremental optimizations and modifications (40, 41). The choice of the vector has to be carefully done, according to specific needs and desired features. For instance, vectors efficiently used for gene overexpression experiments are not necessarily appropriate for RNA interference-mediated knock-down experiments. For bio-safety concerns, the

production of the third-generation HIV-based virions depends on *trans*-elements that are provided by several packaging plasmids. The HIV products Gag and Pol are required for the formation of the viral capsid, the maturation of the virion, the reverse transcription of the lentiviral vector RNA genome into double-stranded DNA, and the subsequent integration of the lentiviral vector DNA into the genome of the transduced cells. Rev binds to the Rev responsive element (RRE) contained in the HIV-derived lentiviral vector and the pMDLg/pRRE packaging plasmid and drives export of the viral RNA to the cytoplasm before potential splicing events occur in the nucleus. Some plasmids encode these three products, such as pCMV.R8.91 (42). The envelope of the produced virions is often based on the vesicular stomatitis virus glycoprotein (VSV-g), but this might be modified, for instance when specific cell types are targeted (40). Simply replace the pVSV-g plasmid by the desired envelope plasmid of your choice when necessary.

2. At least two Rag-deficient (43, 44) and four γ_c-deficient mouse strains (45–48) have been independently generated so far. Two inbred strains of Rag2$^{-/-}\gamma_c^{-/-}$ mice are currently available, respectively, in the C57Bl/6 (black) and BALB/c (white) genetic background. Newborn C57Bl/6 Rag2$^{-/-}\gamma_c^{-/-}$ mice are not efficiently reconstituted by human HSC. Therefore, the use of BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ mice is strongly recommended, although we have been using Rag2$^{-/-}\gamma_c^{-/-}$ mice in a mixed BALB/c × 129/OLA background with identical results.
3. We have used both Roentgen X-ray and ^{137}Cs sources, and we have observed similar outcome when applying 3–4 Gy total body irradiation on BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ mice. The dose has to be adapted to the mouse strain used. For instance, all SCID mouse strains, such as NOD/SCID/$\gamma_c^{-/-}$ mice, are known to be extremely irradiation-sensitive and lower dose is required to ensure survival of the animals.
4. Fetal liver is the richest source of human HSC but, depending on local legislation and necessity of informed consent, it may not be easily available for research purposes. Umbilical cord blood is easier to obtain, but the yield of HSC is much lower. Considering the inter-donor variability, decisions have to be to determine how experimental groups of HIS (Rag/γ) mice are produced. There are several alternatives: (a) produce HIS (Rag/γ) mice each time that a source of human HSC is available; (b) freeze

each individual source of human HSC and make series of age-synchronized HIS (Rag/γ) mice with the individual sources; (c) pool several source donors and make series of age-synchronized HIS (Rag/γ) mice with the same "normalized" pool. The last possibility saves a lot of time and reagents, especially in the situation of recurrent availability of HSC source from numerous (≥ 3) donors. In **Section 3**, we describe the protocol used for processing of one individual fetal liver, and it should be up-scaled accordingly for multiple donors.

5. We list here the materials required for automated mechanical preparation of cell suspension. The preparation can also be made manually, simply using a metallic mesh on which the pieces of fetal liver are mechanically processed.
6. Be aware that the "Indirect CD34 MicroBead kit" is used at this step and not the direct kit (anti-CD34 bead-conjugated antibody). We have observed that the direct kit is not optimally appropriate on fetal liver and UCB cell suspensions, in contrast to post-natal thymocyte suspensions. Furthermore, manufacturer's recommendations are to use 1 ml of each reagent per 10^9 cells in the suspension, but we are routinely using 25% of the recommended volumes without observing yield loss. These adapted volumes are indicated in **Section 3**.
7. We use an FACSAria™ machine (BD Biosciences) for cell populations sorting and a BD LSR II™ machine (BD Biosciences) for cell population analysis, both with 9-parameters (size, scatter, and seven fluorescence colors including DAPI). It has to be noted that any 5-parameter (size, scatter, and three fluorescence colors) cell sorter is sufficient to isolate HSC-enriched populations.
8. The choice of fluorochromes has to be adapted to the available light filters in the cell sorter. We routinely use anti-huCD38 antibody coupled to R-phycoerythrin (R-PE) and anti-huCD34 antibody coupled to phycoerythrin-cyanine-7 (PE-Cy7). Other markers can be used for exclusion of contaminating lineage-positive cell populations, such as CD3 (T cells), CD19 (B cells), CD56 (NK cells), CD11c (cDC), and BDCA2 (pDC).
9. Similar to **Note 8**, the fluorochromes have to be chosen in accordance to the available light filters in the FACS analyzer. As an example, we routinely use fluorescein-isothiocyanate (FITC) anti-huCD19, R-PE anti-huCD8, peridinin chlorophyll protein-cyanin-5.5 (PerCP-Cy5.5) anti-huCD4, PE-Cy7 anti-huCD3, allophycocyanin (APC) anti-BDCA2, allophycocyanin-cyanin-7 (APC-Cy7)

anti-huCD45. The proposed staining mixture for reconstitution analysis is adapted to a 9-parameter cytometer, e.g., the BD LSR II™ machine (BD Biosciences) (*see* **Note 7**), and is designed for the determination of frequency of B lymphocytes (CD19), pDC (BDCA2), and T-lymphocyte subpopulations (CD3, CD4, CD8) within the human hematopoiesis-derived cells (CD45). In the case of a 5-parameter cytometer, we advise to use only anti-CD45, anti-CD3, and anti-CD19 antibodies with the proper fluorochrome combination. The choice of the antibodies has to be adapted to the cell populations of interest, but should always include a CD45-specific antibody to measure to what extent human reconstitution took place and exclude aspecific staining on mouse cells. Cell surface antigens expressed on human cells in HSC-engrafted mice are described in several publications (17, 18, 39). In case of transduction, one channel has to be reserved for analysis of GFP expression (usually the FITC channel), and the antibody mixture has to be redesigned accordingly.

10. SupT1 T cells are used for titration of lentiviral supernatant and exhibit high susceptibility to lentiviral transduction. Be aware that hematopoietic progenitors (or any other human cell manipulated ex vivo) do not necessarily exhibit the same sensitivity to lentiviral transduction. Furthermore, the required titer for optimal transduction of fetal liver HSC might differ between lentiviral vectors and applications. It is, therefore, highly advised to always perform an in vitro transduction test on the desired cell population (for instance here, human fetal liver HSC) before starting in vivo mouse experiments with transduced cells. In our experience, we always try to reach a minimum titer around $5–10 \times 10^6$ TU/mL to ensure good transduction (20–50% GFP^+) of fetal liver HSC, but higher titers can be required in some cases, e.g., with vectors expressing short-hairpin RNA.
11. There is no strict need for "time-pregnant" female mice, especially in the case of a recurrent human HSC source. Reconstitution is optimal between 1 and 4 days of age, and efficiency usually drops severely after 5 days of age. We therefore recommend using BALB/c $Rag2^{-/-}\gamma_c^{-/-}$ newborn mice that are not older than 5 days of age. Still, we have already observed good reconstitution using 7-day-old newborns.
12. Alternatively, UCB can be used instead of fetal liver. In brief, transfer ~60 ml of UCB into a 250-ml flask.

Dilute UCB with ~120 ml of PBS buffer, reaching a total volume of ~180 ml. Prepare 12 50-ml tubes and distribute ~15 ml of diluted UCB to each tube. Similar to fetal liver, carefully bring 10 ml Lymphoprep underneath the cell suspension in order to get a clear interface. Centrifuge the tubes at 1,100 × *g* for 15 min with low acceleration and no brake. Recover the supernatant and the cell ring from each tube, pool into new tubes. Wash the cell suspensions by centrifugation (450 × g, 5 min) and pool pellets in a total of 15 ml of RPMI 2% FCS medium. After Lymphoprep gradient, the yield from 60 ml UCB is usually around 50–100 × 10^6 nucleated cells. In contrast to the suspension obtained from fetal liver, contamination by erythrocytes is usually still clearly noticeable by eye (cell suspension is red) but is not a problem for the next steps.

13. In order to avoid waste of plastic disposables, prepare two 50-ml tubes, which contain RPMI 2% FCS medium and Lymphoprep, respectively. Resuspend the cell pellet with the same pipette in the whole six-tube series, change your 15-ml pipette and next bring Lymphoprep with the same pipette in the whole series of tubes. Simply pour new medium or Lymphoprep directly from the stock bottle into the 50-ml tube when needed.
14. After Lymphoprep gradient, the yield from one medium-sized fetal liver is usually around 100–400 × 10^6 nucleated cells. Important variations are observed, depending on the size of the material, age of the donor, and care during tube manipulation.
15. The protocol is described for manual MACS separation. We advise to use LS separation columns, which can hold 10^8 magnetically labeled cells from up to 2 × 10^9 total cells. In theory, smaller columns (e.g., MS separation columns) should fit for UCB samples, but we have frequently observed column clog with such samples. Alternatively, one can consider using an automatic device like AutoMACS™ (Miltenyi Biotec).
16. According to the manufacturer, one can expect a degree of purification of 85–98% $CD34^+$ cells. We routinely reach purification of >90–95% $CD34^+$ cells after the manual MACS separation.
17. After MACS separation, the yield of $CD34^+$ cells is around 0.5–5% of the initial amount of nucleated cells. One can expect 1–10 × 10^6 $CD34^+$ cells per liver and 0.1–1 × 10^6 $CD34^+$ cells per UCB.

18. Volumes and cell concentrations for the cell sorting are convenient for the FACSAria^{TM} sorter (BD Biosciences) and should be adapted to other machines according to manufacturer's instructions.
19. Be aware that the sorter cell counts are rarely fully accurate. It is therefore reasonable to expect after sorting of $CD34^+CD38^-$ cells a yield around 10% of the initial $CD34^+$ cell counts before cell sorting, despite the fact that $CD34^+CD38^-$ cells usually represent 20–40% of $CD34^+$ fetal liver cells.
20. We describe here an ideal situation where the lentivirus has been titrated so that 0.3 ml of lentiviral supernatant mixed with 0.3 ml of HSC in culture would result in 50% transduction efficiency. This has to be of course adapted to the lentiviral supernatant titer and may require dilution of the virus if necessary. The number of cultured HSC in the well has also an influence on the final transduction efficiency, since the multiplicity of infection is dependent on the number of viral particles and the number of target cells.
21. Variability in reconstitution efficiency increases when lower numbers of progenitors are injected. Still, we have routinely obtained similar levels of reconstitution by inoculating 5×10^5 $CD34^+$ or 5×10^4 $CD34^+CD38^-$ cells per mouse.

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Chapter 7

Generation of Stable Th1/CTL-, Th2-, and Th17-Inducing Human Dendritic Cells

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Abstract

Dendritic cells (DC) are the most potent inducers and regulators of immune responses, responsible for communication within immune system. The ability of DC to act both as the inducers of immune responses and as regulatory/suppressive cells led to the interest in their immunotherapeutic use in different disease types, ranging from cancer to autoimmunity, and as a tool to prevent the rejection of transplanted tissues and organs. Over the last years, several groups including ours have demonstrated the feasibility of obtaining monocyte-derived DC with different functions, by modulating the conditions and the duration of DC maturation. The current chapter provides a detailed protocol of generating type-1-, type-2-, and type-17-polarized DC for testing the cytokine-producing abilities of these cells and their effectiveness in inducing Th1, Th2, and Th17 responses of $CD4^+$ T cells and CTL responses of naïve and memory $CD8^+$ T cells.

Key words: Dendritic cells, Th cells, CTLs, vaccines, cancer.

1. Introduction

Dendritic cells (DC) are the most potent inducers and regulators of immune responses, responsible for intercellular communication between other immune cells. They act as sentinel cells in the peripheral tissues, being key to the development of effective immune responses to the pathogens residing in different cellular compartments and susceptible to different immune mechanisms (1–8). In line with their central role in pathogen control, DC dysfunction has been implicated in the pathogenesis and progression

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of a wide range of disease conditions, ranging from autoimmunity to chronic infections and cancer, with multiple pathogens developing ways to interfere with DC functions as a mean to avoid eradication by the immune system (3, 9–15).

Both the efficiency of DC as an effective element of immune system and their susceptibility to pathogen-induced dysfunction result from an enormous plasticity of the DC system (1, 7, 8). Distinct DC subsets or DC developing or maturing in different conditions show striking functional differences (1, 2, 5–8, 16–19). One aspect of DC function that is a subject to strict regulation is their ability to induce such effector immune cells as Th1-, Th2-, or Th17-type $CD4^+$ Th cells or cytotoxic $CD8^+$ T cells (CTLs) (1, 7, 8) as opposed to regulatory T(reg) cells (20–25).

In contrast to the inhibitory Tregs, all the above effector T-cell types have been shown to provide essential elements of protection against different classes of pathogens and have been implicated in different forms of autoimmunity. Th1-type $CD4^+$ T cells (key producers of IFN-γ and lymphotoxin) and $CD8^+$ CTLs (main type of antigen-specific killer cells) are generally considered as the effector cells key to our ability to effectively fight intracellular bacteria and viruses, as well as to eliminate tumor cells. In addition, Th1 cells provide support for the production of several immunoglobulin classes by B cells. Th2 cells, producing mainly IL-4 and IL-5, are an essential component of our defenses against intestinal parasites and contribute to the majority of antibody production. The more recently discovered IL-17-producing Th cells (Th17 cells) are required for the protection against certain bacteria. Moreover, Th17 cells have been implicated to play a role in the development and/or maintenance of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and colitis (26–40).

The ability of DC to act both as the inducers of immune responses and as regulatory/suppressive cells led to the interest in their immunotherapeutic use in different disease types, ranging from cancer to autoimmunity, and as a tool to prevent the rejection of transplanted tissues and organs (24, 41–50). Taking into account the plasticity of DC and their ability to adopt different functions, it is important to match the desired type of the DC to the type of their clinical or laboratory application.

Over the last years, we and multiple other groups demonstrated the feasibility of obtaining monocyte-derived DC with different functions, by modulating the conditions of their early development (51, 52), the conditions of their maturation (53–60), or the length of DC maturation period (54, 61). The current chapter provides a detailed protocol of generating type-1-, type-2-, and type-17-polarized DC, the protocols used to test the cytokine-producing capacity of these cells, and their ability to induce Th1, Th2, and Th17 responses of $CD4^+$ Th cells as well as the CTL responses of naïve and memory $CD8^+$ T cells.

2. Materials

2.1. Isolation of Peripheral Blood Monocytes and CD45RA$^+$ Naïve CD4$^+$ and CD8$^+$ T cells

1. Vacutainer blood collection tubes (sodium heparin; Becton-Dickinson, Franklin Lakes, NJ, USA).
2. 50-ml Polypropylene tubes.
3. 10-ml Polypropylene tubes.
4. Lymphocyte separation medium (CellGro/Mediatech, Manassas, VA, USA) ($d = 1.077$).
5. Percoll (Sigma) is aliquoted (30 ml) and stored at 4°C.
6. 10 X Concentrated "acidic" (pH 4.6, 1.051 g/ml) PBS: 13.5 g NaCl, 0.1 g Na_2HPO_4 (corresponding to 0.125 g of $Na_2HPO_4 \cdot 2H_2O$), 2.1 g KH_2PO_4, 200 ml distilled water. This PBS solution is sterilized by 0.22-μm filtration and stored at 4°C in 4-ml aliquots.
7. Medium for Percoll separation: IMDM (Gibco/Invitrogen, Grand Island, NY, USA) with 10% FCS (Hyclone, Logan, UT, USA) or serum-free media: AIM-V (Gibco) or CellGenix DC medium (CellGenix, Germany).
8. Medium for washing the cells: RPMI (Gibco/Invitrogen) with 2% FCS (Hyclone).
9. Isolation columns for human CD4$^+$CD45RA$^+$ naïve Th cells and CD8$^+$CD45RA$^+$ naïve Th cells and CTL precursors. We have been successfully using any of the three methods (a) CD4 (8)$^+$CD45R0$^-$ negative isolation columns from R&D, (b) customized StemSep system for the negative isolation of CD4 (8)$^+$CD45R0$^-$ cells (StemCell Technologies), or (c) positive selection using magnetic isolation columns from Miltenyi Biotech Gmbh. In this last method, naïve cells are isolated by the inclusion of additional CD45RO-depletion step applied prior to CD4$^+$ T-cell (or CD8$^+$ T cell) isolation.

2.2. Generation of Immature DC and Their Maturation in DC1, DC2, and DC17-Polarizing Conditions

1. Media for DC culture: (a) IMDM (BioWhittaker) with 10% FCS (Hyclone); (b) serum-free AIM-V medium (Gibco); (c) serum-free CellGenix DC medium (CellGenix, Germany).
2. Medium for washing the cells: 2% FCS/RPMI.
3. rhu GM-CSF (Schering-Plough; Kenilworth, NJ, USA).
4. rhuIL-4 (Strathmann Biotech/Miltenyi Gmbh, Germany).
5. rhuTNF-α (Strathmann/Miltenyi).
6. rhuIL-1β (Strathmann/Miltenyi).
7. IL-6 (Endogen, Woburn, MA, USA).
8. LPS (from *Escherichia coli* 011:B4; Sigma, St. Louis, MO, USA).

9. rhuIFN-γ (Strathmann/Miltenyi).
10. PGE_2 (Sigma, St. Louis, MO, USA).
11. Poly-I:C (Sigma).
12. IFN-α (IFN-α2b; Intron A; Schering-Plough).
13. Peptidoglycan (PGN; Invivogen, San Diego, CA, USA).

2.3. Analysis of Cytokine Production by Differentially Polarized DC

1. CD40L-transfected J558 cells were a kind gift from Dr Peter Lane (University of Birmingham, Birmingham, UK). They express high levels of mouse CD40L that binds both mouse and human CD40.
2. sCD40L (Alexis Biochemicals, San Diego, CA, USA).
3. Human $CD4^+$ Th cells (bulk population) used as IL-12 inducers are isolated as described in **Section 2.1, Item 9**.
4. SEB (Staphylococcal Enterotoxin B; Sigma or Toxin Technologies) is used as an Ag surrogate.

2.4. In Vitro Priming of $CD4^+CD45RA^+$ Naïve Th Cells with Polarized DC Subsets

1. SEB (Sigma or Toxin Technologies).
2. rhuIL-2 (10 U/ml; a gift of Cetus Corporation, Emeryville, CA, USA).
3. CD3 mAb (CLB-T3/3; CLB, Amsterdam, The Netherlands) plus CD28 mAb (CLB-CD28/1; CLB) or alternatively CD3/CD28 T-cell expander beads (Dynal AS, Oslo, Norway) were used to induce the cytokine production in differentially primed populations of Th cells.

2.5. In Vitro Priming of $CD8^+CD45RA^+$ CTL Precursors with Polarized DC Subsets

1. SEB (Sigma or Toxin Technologies).
2. rhuIL-2 (50 U/ml; a gift of Cetus Corporation, Emeryville, CA, USA).
3. IL-7 (10 ng/ml; PeproTech).

3. Methods

3.1. Isolation of Peripheral Blood Monocytes and $CD4^+CD45RA^+$ Naïve Th Cells

1. Collect blood in heparinized tubes and dilute 1:1 with RPMI.

3.1.1. Collection of Peripheral Blood

3.1.2. Isolation of PBMC

1. Overlay 30 ml of diluted blood over 15 ml of lymphocyte isolation medium in each 50-ml tube.
2. Centrifuge at 1,000 × *g* for 30 min, at room temperature (RT; 21°C). Acceleration: 1–1,000 × *g* should take 60 s. Deceleration: 5 min. Wash the cells twice at RT.

3.1.3. Isolation of the Light Fraction of PBMC on Percoll Gradient

1. Prepare standard isotonic Percoll solution (SIP) by mixing nine parts of Percoll with one part of 10X concentrated "acidic" PBS.
2. Prepare three dilutions of SIP (v/v) in 10% FCS/IMDM (*see* **Notes 1–4**):
   a. 60% SIP (9 ml)$^+$ 40% FCS/IMDM (6 ml)
   b. 48% SIP (9.6 ml)$^+$ 52% FCS/IMDM (10.4 ml)
   c. 34% SIP (3.4 ml)$^+$ 66% FCS/IMDM (6.6 ml)
3. Suspend PBMC (maximum 3 × 10^7 cells per ml) in 60% SIP. Layer 2–2.5 ml of cell suspension at the bottom of each of the 15-ml tube (maximum 7.5 × 10^7 cells/tube), overlay with 48% SIP (5 ml), and next with 34% SIP (2 ml).
4. Centrifugation: 2,400 × *g*, 45 min, at RT (21°C). Acceleration: 60 s. Deceleration: 5 min.
5. Harvest monocytes from the upper interphase (the interphase corresponding to 48% (or 45%) SIP and 34% SIP) and lymphocytes from the lower interphase (60% SIP and 48% (or 45%) SIP).
6. Wash the monocyte fraction three times and count the cells (*see* **Note 5**).

3.1.4. Adherence and Depletion of Non-adherent Cells

1. Seed the cells at 0.5 × 10^6 per ml per well in 24-well plate (or 2 × 10^6 in 4 ml in 6-well plate) and let them adhere for 45 min, 37°C, 5% CO_2 (*see* **Note 6**).
2. Remove non-adherent cells by washing the wells —two to three times with a gentle stream of medium. This step requires eye-control of the washing to assure high purity of monocytes and to prevent an excessive loss of the attached cells. Use washing medium at room temperature.

3.1.5. Isolation of $CD4^+CD45RA^+$ Naïve Th and $CD8^+$ $CD45RA^+$ Naïve T cells from Peripheral Blood

1. Harvest the lymphocytes from the heavy fraction of PBMC (*see* **Section 3.1.3**) and wash two times.
2. Isolate naïve Th cells ($CD4^+CD45RA^+$ cells) or naïve CTL precursors ($CD8^+CD45RA^+$ cells), by one of the negative selection systems (*see* **Section 2.1**) according to the manufacturers' instructions. Although rare subsets of pathogen-specific $CD8^+CD45RA^+$ T cells can contain effector cells, the overall polyclonal population of "bulk" periph-

eral blood $CD8^+CD45RA^+$ T cells displays a uniform $CD62L^{high}/CCR7^+$ phenotype and functions characteristic of naïve $CD8^+$ T cells (58, 62). Please note that the optimal generation of Th17 cells benefits from the use of "bulk" $CD4^+$ T cells or memory-enriched $CD4^+CD45R0^+$ T cells as the starting population (27) (*see* **Note 24**). The use of memory T-cell fraction or the use of bulk, unseparated $CD4^+$ or $CD8^+$ T cells is also recommended when inducing antigen-specific responses (60, 63, 64), since Ag-specific precursor cells are enriched in the memory cell population.

3. Freeze the isolated T cells until use.

3.2. DC Culture and Maturation

1. After the last wash of the monocytes, add fresh culture medium (IMDM/FCS; CellGenix or AIM-V), containing at least 500 U/ml GM-CSF and 250 U/ml IL-4 (1 ml per well; currently we use 1,000 U/ml of each of these cytokines).
2. On day 3 of the culture, remove 1/2 of medium and add the same amount of fresh medium with the double-concentrated growth factors. At this time-point, a portion of the cells are already non-adherent, so it is necessary to let them sediment for 10 min, resting the plate at a certain angle, supported at one side. Gently, to avoid taking up the cells, take up 0.5 ml of medium with a 1-ml pipette, from the lower side of each well. Add the new medium with double-concentrated GM-CSF and IL-4 (pre-warmed to room temperature) at the same spot, releasing the volume gently to reduce stirring up the cultures.
3. At day 6 (*see* **Note 7**), take out 1/2 of the spent medium and add new medium containing GM-CSF, double-concentrated maturation-inducing factors without or with a polarizing factor (see below). Within 2 days the expression of CD80, CD86, and CCR7, will increase, and the cells will lose the ability to re-adhere, after moving to another well (*see* **Note 9**). At the very early stage of maturation (6–12 h) the cells become $CD83^+$ and lose the expression of CD115.
   a. DC1-inducing cocktail applicable for serum-supplemented media (57):
      LPS (final conc. 250 ng/ml) plus IFN-γ (final conc. 1,000 U/ml).
      Maturation time: 42–48 h.
   b. αDC1-inducing cocktail (clinical-grade DC1-inducing cocktail effective both in serum-supplemented media and in serum-free CellGenix DC medium and in AIM-V) (60):

Poly-I:C (final conc. 20 μg/ml), TNF-α (final conc. 50 ng/ml), IL-1β (final conc. 25 ng/ml), IFN-α (final conc. 3,000 U/ml), and IFN-γ (final conc. 1,000 U/ml).
Maturation time: 42–48 h.

c. DC2/standard(s) DC-inducing cytokine cocktail (clinical grade; all media) (60, 65):
TNF-α (final conc. 50 ng/ml), IL-1β (final conc. 25 ng/ml), IL-6 (final conc. 1,000 U/ml, and PGE_2(final conc. 1 μM).

d. DC17-inducing conditions (26):
PGN (final conc. 10 μg/ml).
For the optimal induction of Th17 cells, DC should be matured *only for 16 h*, rather than 42–48 h (26).

3.3. Analysis of Cytokine Production by Differentially Polarized DC

1. Harvest DC to polypropylene tubes and wash thoroughly to remove all the cytokines.
2. Plate the cells at 2×10^4 cells/well in flat-bottomed 96-well plates.
3. Add the cytokine-inducing stimulus. We normally use three types of CD40L-based stimuli: J558-CD40L (5×10^4 cells/well), soluble CD40L, and cross-linking kit (Alexis Biochemicals, San Diego, CA, USA), either alone or in combination with rhuIFN-γ (1,000 U/ml) or $CD4^+$ T cells (1×10^5 cells/well) in the presence of superantigen (SEB; 1 ng/ml). The induction of cytokine production is routinely performed in a final volume of 200 μl/well (*see* **Note 10**).
4. Following either of the first two modes of stimulation, we harvest 24-h supernatants, while the T-cell-dependent IL-12p70 induction requires a longer, 48 h stimulation (to allow T cells to elevate CD40L expression).

3.4. In Vitro Priming of $CD4^+CD45RA$ Naïve Th Cells with Polarized DC Subsets

1. Harvest DC to polypropylene tubes and wash thoroughly to remove all the cytokines.
2. Plate the DCs in flat-bottomed 96-well or 48-well plates. Add SEB (1 ng/ml) and (after 1 h) T cells at 10:1 ratio (e.g., 2×10^3 DC and 2×10^4 T cells in 200 μl or 5×10^4 DC and 5×10^5 T cells in 500 μl). For the optimal induction of Th17 cells, the concentration of SEB may be reduced to 100 pg/ml (26).
3. At day 5, add rhuIL-2 (final conc. 10 U/ml).
4. Starting from this point onward the cells proliferate rapidly over the period of next 4–6 days. The next day after the IL-2 addition, the cells usually need to be transferred to 1 ml wells. Subsequently, every 1–3 days, each well needs to be

divided into —two to three wells. At this point, the optimal culture density for the expansion of Th cells is 1.5–3 × 10^6 cells per well (1 ml). The cultures reach quiescence about days 9–12 and need to be restimulated (*see* **Note 11**).

5. At 10–14 days after priming, induce the cytokine production in Th cells by their restimulation for 24 h with CD3 mAb (1 μg/ml; CLB-T3/3; CLB) plus CD28 mAb (1 μg/ml; CLB-CD28/1; CLB), or CD3/28-coated T-cell-activating beads. The levels of IFN-γ, IL-4, and IL-5 in 24 h supernatants can be then analyzed by specific ELISAs. Alternatively, the differentially primed Th cells can be restimulated with PMA (100 ng/ml) and ionomycin (1 μg/ml) for 6 h, the last 4 h in the presence of Brefeldin A (10 μg/ml) and the intracellular expression of IFN-γ, IL-4, and IL-17 is determined following cell permeabilization using saponin and cytokine-specific staining using αIL-17 abs (R&D), αIFN-γ Abs (Pharmingen), and αIL-4 Abs (Pharmingen).

3.5. In Vitro Induction of Peptide-Specific CTLs

1. Harvest DCs (e.g., αDC1 or sDC) to polypropylene tubes (to limit adherence) and wash thoroughly to remove all the cytokines.
2. Plate DC at 5 × 10^4 cells/well in flat-bottomed 48-well plates in 10% HS/IMDM. Add SEB (1 ng/ml) or antigenic peptide(s) (at 1–10 μM), $CD8^+$ T cells (5 × 10^5/well; *see* **Section 3.1** for the isolation procedure; depending on application naïve or bulk CD8+ T cells may be used). As an option, 3000R-irradiated J558-CD40L cells (5 × 10^4/well, as a surrogate of CD40L-expressing Th cells *may* be added; *see* **Comment 23**). The addition of CD40L was originally used in our protocols (60) to assure that the differences in the magnitude and quality of the CTL responses induced by polarized DC1 and non-polarizing sDC cannot be overcome by the presence of Th cell-related signals. However, our recent studies demonstrated that similar differences can be observed in the absence or presence of CD40L (62, 64).
3. At days 3–4, add rhuIL-2 (final conc. 50 U/ml) and IL-7 (10 ng/ml).
4. The proliferation of cells is significantly less pronounced than in the SEB model ($CD4^+$ T cells). The cells usually need to be fed with 50% of fresh IL-2-containing medium every 3 days and transferred to 1-ml wells at about day 7. The cultures reach quiescence about days 12–14 and need to be restimulated.
5. At days 12–14 after priming, the cells are restimulated with peptide-pulsed autologous PBMC (at 1:1 ratio) or with peptide-loaded Th2 cells (at 2:1 ratio) *(peptide-pulsing is*

important at this stage: do not add peptide directly to CTL cultures to prevent CTL fratricide) and expanded for another 12–14 days. This restimulation step allows to demonstrate the stability of the DC-induced differences in CTL activity and facilitates ELISPOT analysis of Ag-specific responses, by reducing the non-specific background (LAK activity; significant in $CD8^+$ T cells recently stimulated by αDC1s; especially in the presence of CD40L). At days 24–28 (10–14 days after secondary stimulation), the frequency of Ag-specific T cells is analyzed by IFN-γ ELISPOT. This secondary stimulation step can be omitted, allowing to compare the CTL induction already at days 12–14.

4. Notes

Our serum-supplemented conditions of DC culture involve FCS, rather than human serum, since DC obtained in the presence of human serum do not express CD1a and show a relative resistance to maturation. FCS/IMDM-based media allow the generation of type-1-polarized DC (DC1), using a combination of TNF-α and IFN-γ (or LPS and IFN-γ). In contrast, the generation of fully mature DC1 in serum-free media (such as AIM-V or CellGenix) requires the addition of IFN-α and poly-I:C (αDC1, Ref. (60).

The SEB-based model of naïve Th cell priming was first described in Ref. (51). It is based on the ability of SEB to activate a substantial proportion of naïve T cells (66, 67). This allows to use it as a substitute of the TCR-transgenic models that are not available in the human system. In contrast, the traditional allogeneic MLR model does not allow to induce any detectable amounts of IL-12 within the first 3 days of DC–Th cell interaction, most likely due to 100–1,000-fold lower frequency of responsive T cells. The possible applications and the typical results obtained with use of the described protocols can be found in our previous publications (51, 53, 54, 57–59, 62, 64, 68–72).

Based on the past experiences on introducing the described protocol in other labs, we would like to draw your attention to the following issues critical for its outcome.

1. Monocytes isolated from fresh blood give better results than monocytes isolated from buffy coats that often yield a lower percentage of $CD1a^+$ cells. In addition, DC generated from buffy coat-isolated monocytes frequently show signs of partial maturation (loss of CD115) and tend to produce lower amounts of IL-12p70. They are also less susceptible to polarization. The reason(s) for these differences is not completely clear to us, but the quality of DC appears

to inversely correlate with the level of platelet contamination that is substantially higher in case of the monocytes isolated from buffy coat, compared to fresh blood.

2. Isolation of monocytes should be performed at room temperature. Rapid changes of temperature increase the risk of monocyte activation and clumping. We advise the use of polypropylene tubes to reduce cell attachment.
3. We also recommend the use of heparin as anticoagulant to avoid activation of monocytes in the course of decalcification/recalcification. Use Ca^{++}/Mg^{++}-containing media at all stages of the monocyte isolation.
4. 48% layer of SIP is designed for freshly drawn blood. A lower-density layer of SIP (45%) should be used for the isolation of monocytes from buffy coats.
5. At this stage, the monocytes should be 80–90% pure (judged by CD14 expression). Higher contamination with $CD14^-$ cells indicates the need to reduce the concentration of SIP in the middle layer.
6. Do not exceed the starting cell density of 0.5×10^6 cells per ml of culture medium. Consider reducing it to 0.4×10^6 if the CD1a expression is poor. Generally, the lower the starting density of the monocytes, the higher the purity of resulting $CD1a^+$ DC, although very low-density cultures result in a poor recovery of DC (as a percentage of the plated monocytes).
7. Relevant for FCS-supplemented cultures: At day 6, the cultures contain up to 90% $CD1a^+CD115^+$ immature DC. They are expressing low-to-intermediate levels of CD80 and CD86 and lack CD83 expression. Poor CD1a expression may indicate (a) too high initial density of monocytes at the onset of cultures; (b) poor batch of serum/medium (*see* **Notes 12–14**); (c) poor mAb (in our hands, OKT6 proved superior to several other CD1a mAbs). It may also suggest poor activity of the IL-4 used and the need to increase its concentration.
8. Optimal type-1 polarization of DC requires complete DC maturation and is impaired in DC that do not undergo full CD83/CCR7 conversion. IFN-γ and the maturation-inducing factor should be administered simultaneously. Pre-treatment of DC, with either of the factors alone, reduces the ability of DC to produce IL-12p70 after subsequent stimulation.
9. While our standard protocol of generation of polarized effector DC involves a 48 h maturation stage, a shorter

maturation/polarization time may be considered depending on the DC application.

10. Bacterial products, such as LPS or SAC (alone or in combination with IFN-γ), are effective inducers of IL-12p70 production in immature ($CD83^-$) DC, but not in mature DC. CD40L stimulation remains effective in mature DC, although mature DC show impaired responsiveness to the IL-12p70 enhancing action of IFN-γ (54).
11. The proliferation of Th cells is very susceptible to the temperature changes, especially within the first 5 days of culture. To optimize the yield of the differentially primed Th cells, try to minimize the length of time when the cells are outside the incubator and use pre-warmed medium to dilute the cultures.
12. A batch of FCS is important. We observed strong differences between several different batches of FCS in their ability to support the DC1.
13. The source of medium can make a difference as well.
14. We advocate using disposable plastic tubes, media flasks, and pipettes to reduce the chance of endotoxin contamination at the onset of cultures.
15. Although difficult to avoid for some applications, gamma irradiation impairs the ability of DC to produce IL-12. Typically, the IL-12p70 production by 2500R-irradiated DC is only 15–25% compared to non-irradiated DC.
16. We routinely observe that the addition of even 0.5% of human serum or plasma, particularly not only from cancer patients but also banked human AB serum, inhibits DC maturation with negative effects on the expression of CCR7, migratory properties and the ability to produce IL-12p70.
17. We advocate a thorough testing of the applicability of each media, rather than assuming that the suggested concentrations of cytokines will be optimal for any media. For example, our collaborators observed that cultures performed in X-VIVO medium may require up to 10,000 U of IFN-α (instead of our 3,000 U) and up to 100 ng/ml of TNF-α for the optimal activity of αDC1s. We are not sure if these differences reflect the differential impact of medium or different specific activity of the cytokines used in the "alpha-type-1" maturation cocktail. *Excessive cell adherence* seems to be the most sensitive indicator of an incomplete DC maturation. Our recent back-to-back comparison of different serum-free media demonstrated that the cells generated

in CellGro DC medium from CellGenix yield DC1s with the highest quality and yield (64).

18. In any of the functional assays or for the preparation of the vaccine, we *do not harvest the adherent cells (whenever present*). These macrophage-like cells are not stimulatory and may be suppressive. We have seen that scraping the cell or using Ca/Mg-free medium to wash the cells reduces their IL-12-producing capacity. EDTA is even worse.
19. In a limited number of experiments using our LPS/IFN-γ-based protocol of inducing DC1s, we have attempted to obtain polarized DC1 in Teflon bags. These attempts were met with a limited success, raising the possibility that cell adherence may be important for the generation of DC1s. While this issue needs to be readdressed using currently available culture bags, our clinically applied αDC1s are currently grown in T25 and T75 culture flasks.
20. Although αDC1 can be frozen without any significant reduction of their viability (compared to standard, PGE2-matured DC; sDC), freezing reduces their subsequent ability of both cell types to produce IL-12 by about 60–70%. Although freezing of αDC1 and sDC preserves the ratio of their IL-12-producing capacities, if you have a choice between freezing patients' monocytes (and vaccinating with freshly-generated DCs) or freezing the ready to use vaccine, the first option (less convenient) may allow to fully benefit from DC1 biology. We cannot say at this moment if freezing makes any difference for the final performance of DCs, but we will use fresh DCs in our first protocol.
21. Although we currently generate DC, using 1,000 U/ml of both GM-CSF and IL-4, it is possible to reduce the levels of these cytokines to at least 500 U/ml (GM-CSF) and 250 U/ml (IL-4). The cells need to be monitored for the signs of decreased yield (insufficient GM-CSF) and excessive cell adherence and persistence of CD14 (signs of insufficient IL-4). The exact minimum level of IL-4 needed highly depends on the amount of activation of monocytes during their isolation (quality of reagents, de/re-calcification, duration of cell adherence) and the cell density (affecting the concentration of endogenous monocyte-derived factors affecting their differentiation, such as prostanoids or CSF-1).
22. Please note that DC1 produces only limited amounts of IL-12 spontaneously after removing them from the maturation cultures (low pg concentrations can be detected) but produces a "second wave" of IL-12p70 following interac-

tion with T cells, particularly not only $CD4^+$ T cells, but also $CD8^+$ T cells. While our early work with isolated $CD8^+$ T cells involved CD40L-transduced J58 cells as a surrogate of Th cells (60), we have recently observed, using the systems of in vitro CTL induction using polyclonal stimuli (SEB) and Ag-specific stimulation of $CD8^+$ T cells that the inclusion of CD40L in these assays (during the DC-mediated sensitization of tumor-specific $CD8^+$ T cells) may be counterproductive and induce LAK activity in $CD8^+$ T cells (increasing non-specific background in ELISPOT observed after 2 weeks of priming). No CD40L has been used in our recent work demonstrating the advantage of using αDC1s in inducing tumor-specific CTLs (62, 64).

23. Recently, it was suggested that type-1 DC polarization is suboptimal in X-VIVO medium suggested (73). In our experience *(see* **Note 17**) the maturation of alpha-DC1s cultured in some batches of X-VIVO medium is associated with excessive cell adherence and low cell recovery, but we did not see such effects with all batches of that medium, so such deficit may be batch-dependent. While we occasionally observe differences in the DC1 generation in different batches of the same medium, the CellGenix DC is our current medium of choice. The comparison of different media was performed in our recent paper (64).
24. In the mouse system, the development of Th17 cells from naïve precursors is well-documented: the activation of naïve $CD4^+$ Th cells in the presence of IL-6 and TGF-β will readily induce the development of high numbers of RORγT expressing Th17 cells (29, 35, 38–40, 74, 75). In contrast, it is less clear as to how effective is the direct pathway of development of Th17 from human naïve precursors. In contrast, human Th17 cells can be efficiently and reproducibly induced from the population of $CD45RO^+$ memory Th cells by the DC activated by bacteria or by peptidoglycan (PGN) (26).
25. In addition to their superior ability to induce Th1 and CTL responses αDC1 shows also preferential ability to attract with these T cell types (76), which may contribute to their previously documented elevated activity in promoting tumor-specific Th1 and CTL responses (60, 63, 64). In contrast to αDC1s which mainly produce Th1- and CTL-attracting CXCR3-ligands and CCR5 ligands (MIG, IP10, RANTES and similar chemokines), standard(s) DC mainly produce Treg-attracting CCL22 (76). As a result, DC1 attracts overall higher numbers of T cells, but significantly lower numbers of Tregs, compared to sDC (76).

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Chapter 8

Pharmacological Modification of Dendritic Cells to Promote Their Tolerogenicity in Transplantation

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Abstract

Dendritic cells (DCs) are uniquely specialized antigen-presenting cells (APC) that play critical roles in both the stimulation and regulation of immune responses, including T-cell responses to transplanted organs. The inherent tolerogenicity of non-activated or "immature" DCs is well documented. Importantly, the infusion of DCs that are made resistant to activating inflammatory stimuli by "conditioning" through exposure to clinically approved immunosuppressants, such as corticosteroids, deoxyspergualin, and recently, rapamycin (RAPA), has produced encouraging outcomes in experimental models. Indeed, the infusion of RAPA-conditioned, host-derived DCs, pulsed with alloantigen, prolongs allograft survival. In particular, when the RAPA-conditioned DCs are delivered repeatedly or in combination with a short course of immunosuppression indefinite allograft survival is observed, typically associated with increased Foxp3$^+$ T-regulatory cells (Treg). Herein, we detail the procedures to generate and characterize RAPA-conditioned murine DCs (RAPA-DCs) ex vivo and in vivo. RAPA-DCs represent a pharmacologically conditioned DC population that promotes allograft survival and enriches for antigen-specific T-regulatory cells (Treg). DCs conditioned with immunosuppressive agents, like RAPA, represent novel and clinically applicable vectors or "negative" cellular vaccines, which can be loaded with donor antigen, and potentially used to promote/maintain organ transplant tolerance.

Key words: Dendritic cells, immunosuppression, rapamycin, vaccines, cell therapy, regulatory T cells, transplantation tolerance.

1. Introduction

DCs are rare bone marrow (BM)-derived APC and potent endogenous initiators of inflammatory effector T-cell responses to viral and bacterial pathogens (1). However, they are also critically involved in central and peripheral tolerance, acting as

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proficient inducers of T-cell apoptosis and anergy, in addition to being inducers/stimulators of Treg (1–6). This diversity in DC function arises from an inherent functional plasticity that is directly tied to the degree of DC activation. In the quiescent state, DCs exist as "immature" cells, having the ability to efficiently interact with T cells, but with a low potential to stimulate inflammatory T-cell responses. Instead, immature DCs (iDCs) generally induce T-cell anergy and apoptosis in the T cells with which they interact, presumably through a limited capacity to provide co-stimulation. iDCs also have the potential to stimulate/induce Treg (7, 8) or Treg type-1 (Tr1) cells that make IL-10 (9).

The stimulatory function of DCs is promoted when iDCs, acting as immunological sentinels, sense and become activated by local pathogen-derived products and endogenous donor signals through various DC-expressed receptors. These receptors include the Toll-like receptors (TLR), nucleotide oligomerization domain (NOD) receptors, and receptor for advanced glycation end products (RAGE) (10, 11). Triggering of these receptors initiates DC activation or "maturation," a process through which cell surface expression of major histocompatibility complex (MHC), accessory, and co-stimulatory molecules is greatly increased, as is the inflammatory potential of the DC. Thus, activated T-effector cells are generated when mature DCs present antigenic peptides bound to MHC classes I and II and simultaneously display adequate co-stimulatory molecules (especially CD86) and secrete cytokines, such as TNF-α, IFN-γ, and IL-12 (12). The ability of iDCs to interface with naïve T cells and quell effector T-cell responses, but facilitate regulatory activity, renders them promising therapeutic targets for immune modulation in transplant medicine (6). Indeed, there is promising experimental evidence that the inherent tolerogenic potential of DCs can be targeted directly in vivo or harnessed as "negative" cellular vaccines when ex vivo-generated DCs are infused before and/or after transplant surgery (6, 13).

Distinct subsets of DCs have been characterized in the circulation, lymphoid and non-lymphoid tissues of humans, primates, and rodents (10, 14). To date, however, most experimental studies and therapeutic tolerance protocols have utilized either myeloid (m)DCs or plasmacytoid (p)DCs (6, 10). Different approaches to "customize" DCs for tolerance induction include genetic (transgene insertion), biologic (specific culture conditions; anti-inflammatory cytokine exposure), and pharmacologic manipulation (5, 6). Rapamycin (RAPA) is a macrolide antibiotic pro-drug with potent immunosuppressant properties, approved as anti-rejection therapy in clinical renal transplantation (15, 16). There is now considerable evidence that RAPA profoundly impacts rodent and human DC generation, maturation, and T-cell stimulatory function (17–25).

Below are detailed methods for the ex vivo propagation of murine BM-derived RAPA-conditioned mDCs (RAPA-DCs). Also, methods are provided for the in vivo generation of RAPA-DCs through DC expansion/mobilization with the DC growth factor, fms-like tyrosine kinase 3 ligand (Flt3L), in combination with RAPA administration. Both in vivo (17) and ex vivo, RAPA-DCs typically have reduced cell surface levels of MHC antigens and significantly reduced surface expression of co-stimulatory molecules (especially CD86; **Fig. 8.1**) (25). Decreased CD86 expression and reduced allostimulatory capacity persist even when RAPA-DCs are exposed to potent inflammatory stimuli, such as bacterial lipopolysaccharide (LPS), cytosine guanine dinucleotide (CpG), and CD40 ligation [**Fig. 8.2**(25) and (17, 20, 21)]. RAPA-DCs are weak stimulators of alloreactive T cells [**Fig. 8.2**; (25)] and induce antigen-specific T-cell hyporesponsiveness (17, 20) and apoptosis (21). However, they retain the capacity to stimulate and enrich for Treg (21). Also, important for the function of any tolerogenic cellular DC vaccine, although of an immature phenotype, RAPA-DCs maintain the expression and regulation of CCR7 typical of "mature" DCs, and exhibit migration to CCL19, CC21, and secondary lymphoid tissue (20, 21, 26). As "negative" DC vaccines, alloantigen (alloAg)-pulsed, recipient-derived RAPA-DCs subvert anti-allograft immune responses in rodent transplant models.

We have found that when infused systemically into mice before transplantation of a fully MHC-mismatched heart, recipient-derived, donor alloAg-pulsed RAPA-DCs prolong allograft survival significantly. When delivered repeatedly, or combined with short-term, low dose RAPA, indefinite graft survival is observed (20, 21). More recently, alloAg-pulsed, recipient-derived RAPA-DCs, combined with transient immunosuppression (anti-lymphocyte serum + cyclosporine), promoted long-term graft survival in Lewis rats across a full MHC barrier when

Fig. 8.1. (continued) RAPA-conditioned DCs are a homogenous population of small $CD11^+$ cells expressing decreased CD86. (**A**) BM-derived B10 myeloid DCs were generated in the absence (control [CTR]-DCs) or presence of 10 ng/ml RAPA (RAPA-DCs) and purified to >97% purity by CD11c immunobead-positive selection. Compared to CTR-DCs (*upper panels*), RAPA-DCs (*lower panels*) constituted a homogenous population of $CD11c^+$ cells of reduced size, which (**B**) displayed reduced surface expression of MHC class II ($I\text{-}A^b$) and CD86. (**B**) However, the expression of CD40, TLR2, and TLR4 by RAPA-DCs was not altered significantly. RAPA-DCs were also positive for intracellular TLR9. Histograms represent $CD11c^+$-gated cells and numbers indicate mean fluorescence intensity (MFI) for each condition. Shaded area = isotype control. RAPA-DCs are represented by thick black lines. The data shown are representative of more than six experiments performed. Figure and legend reproduced with permission from *The Journal of Immunology* (25).

delivered systemically, pre-transplant (23), or post-transplant (22). In total, the study of pharmacologically modified DCs, including RARA-DCs, is both moving DC therapy toward clinical assessment in transplant medicine, while also yielding new insights into DC immunobiology.

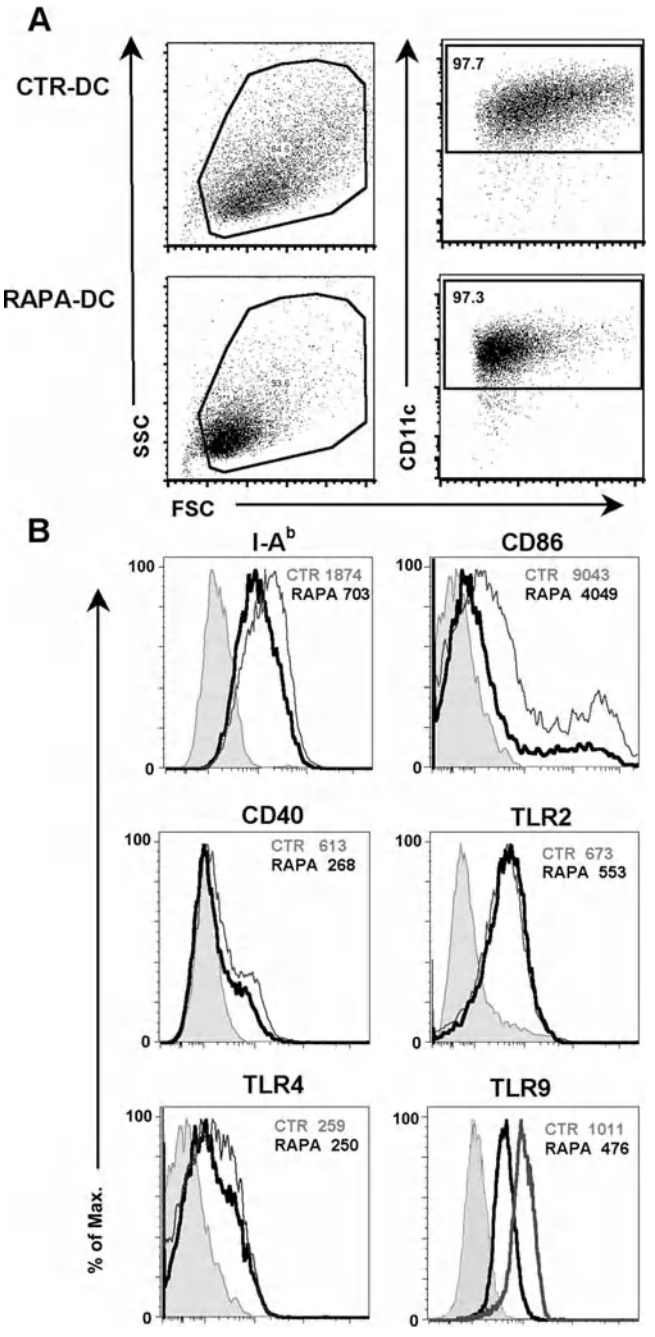

Fig. 8.1. (continued)

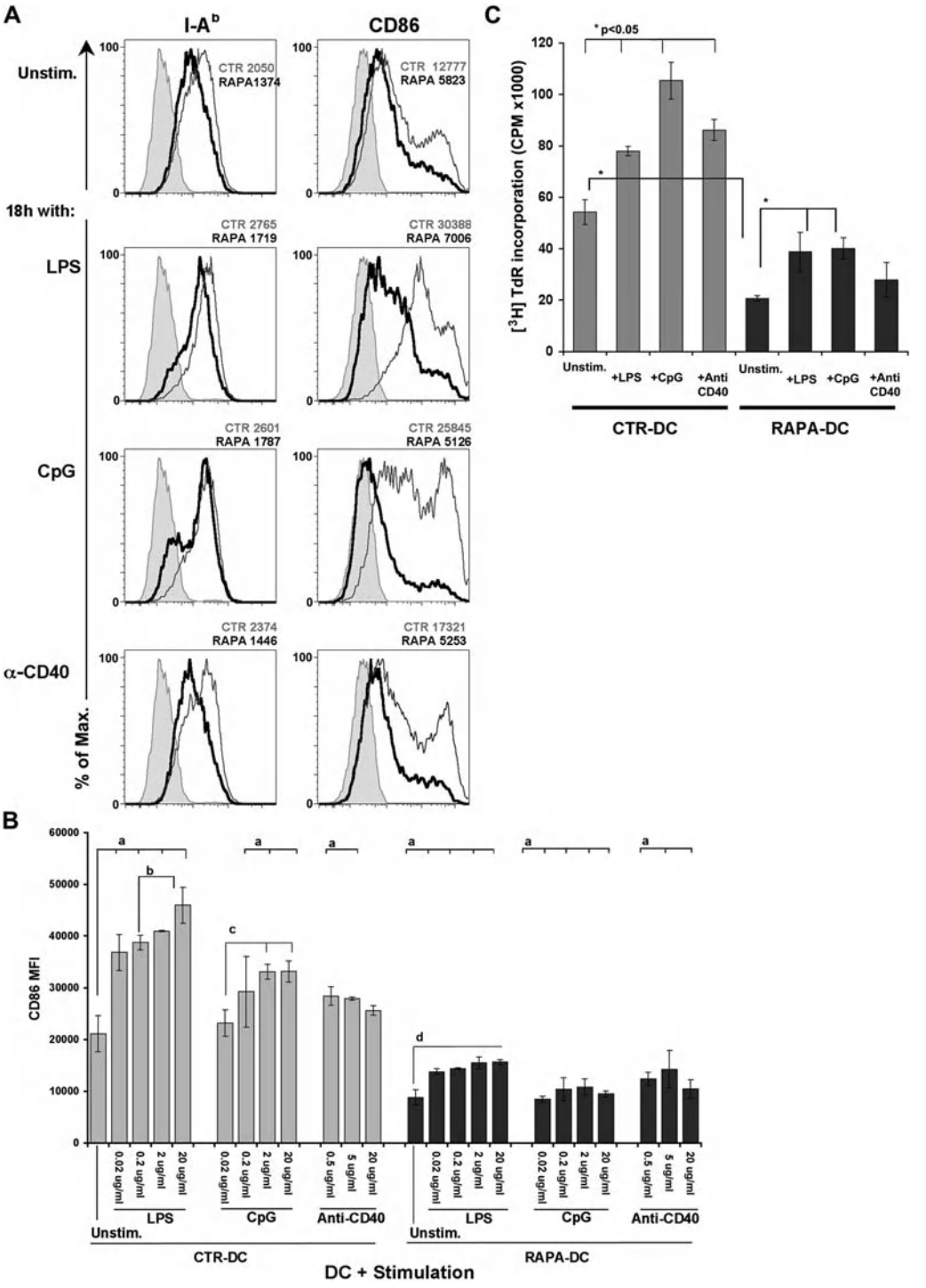

Fig. 8.2. (continued)

2. Materials

2.1. Mouse Bone Marrow (BM) Harvest and Myeloid (m)DC Propagation

1. Phosphate-buffered saline (PBS): 10 mM phosphate buffer, pH 7.4, 2.7 mM KCl, and 150 mM NaCl.
2. Fetal bovine serum (FBS)/EDTA solution: 500 ml PBS, 0.5% heat-inactivated FBS, 2 mM EDTA.
3. Complete RPMI-1640 media: RPMI-1640 (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS, 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 1 M MEM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid).
4. Red blood cell (RBC) lysis buffer: 10 mM Tris–HCl, 1 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton X-100. Adjust pH to 7.2–7.5 with NaOH.
5. Sterile needles, 27-ga, 1/2-inch.
6. Sterile syringes (5 ml).
7. Sterile transfer pipettes.
8. Nylon mesh cell strainers, 70 μm (BD Biosciences, Franklin Lakes, NJ, USA).
9. Recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF) (Schering-Plough, Kenilworth, NJ, USA).

Fig. 8.2. (continued) RAPA-conditioning of DCs confers resistance to maturation following exposure to pro-inflammatory ligands. (**A**) Following overnight incubation with 1 μg/ml LPS, 2 μg/ml CpG, or 5 μg/ml agonistic anti(α)-CD40 mAb, RAPA-DCs did not increase MHC class II (I-A^b) to levels found on CTR-DCs and failed to upregulate CD86. Histograms represent $CD11c^+$-gated cells and MFI is indicated for each condition. Unstim. = unstimulated DC. Shaded area = isotype control. RAPA-DCs are represented by *thick black lines*. (**B**) Upregulation of CD86 on RAPA-DCs was inhibited over a range of concentrations of LPS, CpG, and α-CD40. MFI = mean fluorescence intensity. The data are means ± 1SD from two independent experiments where $CD11c^+$ cells were assessed. Lower case alphabetic characters symbolize $p < 0.05$ by Student's "*t*"-test and are as follows: a = vs. CTR-DCs; b = CTR-DCs + 0.2 μg/ml LPS vs. CTR-DCs 20 μg/ml LPS; c = CTR-DCs + 0.02 μg/ml CpG vs. CTR-DCs 2 and 20 μg/ml CpG; d = vs. unstimulated RAPA-DCs. (**C**) Furthermore, RAPA-DCs exposed to LPS (1 μg/ml), CpG (2 μg/ml), or α-CD40 mAb (5 μg/ml) displayed significantly reduced T-cell allostimulatory capacity (1 DC: 20 $CD3^+$ T cells) compared to similarly stimulated CTR-DCs. The data are means ± 1SD from sample replicates of one experiment representative of more than four performed. In panel C, $* = p < 0.05$ by Student's "*t*"-test; the data shown are representative of more than three experiments performed. Figure and legend reproduced with permission from *The Journal of Immunology* (25). (Copyright 2008. The American Association of Immunologists, Inc.)

10. Recombinant murine IL-4 (R&D Systems, Minneapolis, MN, USA).
11. Rapamycin (Sigma–Aldrich, St. Louis, MO, USA): Resuspend 1 mg of powder in 500 μl EtOH, then aliquot 50 μl of suspension into 1,950 μl of complete media, for a final concentration of 50 μg/ml (*see* **Note 1**).
12. Anti-CD11c immunomagnetic beads (Miltenyi Biotec, Auburn, CA, USA).
13. MACS® separation columns (Miltenyi Biotec, Auburn, CA, USA).
14. Bead buffer: 25 ml complete RPMI-1640, 25 ml PBS, 2 mM EDTA.
15. Bright-Line™ Hemacytometer (Fisher Scientific, Pittsburgh, PA, USA).
16. Cell culture dish, 100 mm tissue culture treated polystyrene (BD Biosciences).
17. 0.4% (w/v) Trypan Blue in 0.81% sodium chloride and 0.06% potassium phosphate, dibasic (Sigma–Aldrich, St. Louis, MO, USA).

2.2. Phenotypic Analysis of RAPA-DCs

1. 10% v/v normal goat serum in PBS.
2. Stain buffer: 500 ml PBS, 5% heat-activated FBS, 0.1% (w/v) sodium azide, pH 7.4–7.6.
3. Appropriate monoclonal antibodies (mAbs).
4. 4% paraformaldehyde (PFA): 4 g of PFA per 100 ml of PBS.
5. BD GolgiPlug™ (BD Biosciences, Franklin Lakes, NJ, USA) for intracellular staining.
6. Permeabilization (Perm) buffer: 500 ml PBS, 1% heat-inactivated FBS, 0.1% sodium azide, 0.1% saponin.
7. Data are typically acquired with an LSR II flow cytometer (BD ImmunoCytometry Systems; San Jose, CA, USA) and analyzed using the FlowJo 8.1.1 (Tree Star, Inc., Ashland, OR, USA).

2.2.1. DC Activators: Inducers of Maturation (mAb and TLR Ligands)

1. Anti-mouse CD40 mAb (clone HM40-3, No Azide/Low Endotoxin Format™, 5 μg/ml; BD Biosciences, Franklin Lake, NJ, USA).
2. CpG-ODN (ODN 1826: TCCATGACGTTCCTGACGTT, 2 μg/ml; Invivogen, San Diego, CA, USA).
3. LPS (serotype 111:B4, 1 μg/ml; Sigma, St. Louis, MO, USA).

2.3. Functional Testing of RAPA-DCs in a Mixed Leukocyte Reaction (MLR)

1. To obtain negatively depleted T cells, purified rat-α-mouse mAbs: α-CD11b (clone # M1/70), α-TER-119 (clone # Ter-119), α-Gr-1 (clone # RB6-BC5), α-I-A/I-E (clone # M5/114.15.2), α-B220 (clone # RA3-6B2) (BD Biosciences, Franklin Lakes, NJ, USA) are needed.
2. Depletion Dynabeads® (Dynal Biotech, Oslo, Norway).
3. DynaMag™-15 magnet (Dynal Biotech).
4. BD Falcon™ 96-well microplate, tissue culture treated, round-bottomed with lid (BD Biosciences).

2.4. In Vivo Expansion and Isolation of RAPA-DCs

1. Fms-like tyrosine kinase 3 ligand (Flt3L; Abcam, Cambridge, MA, USA): 10 μg suspended in 100 μl of PBS delivered i.p. once daily for 10 days.
2. RAPA dissolved in 50% polyethylene glycol, 5% TWEEN® 80, 5% EtOH (vehicle, all reagents from Sigma–Aldrich, St. Louis, MO, USA).
3. FBS/EDTA – see above.
4. Complete RPMI-1640 media (see above).
5. 92-kDa Type IV collagenase (Sigma–Aldrich, St. Louis, MO, USA): 100 and 400 U/ml in complete media.
6. HistoDenz (Sigma–Aldrich, St. Louis, MO, USA): 16% w/v solution in PBS.

3. Methods

3.1. Generation of BM-Derived RAPA-DCs

1. From 8 to 12-week-old mice, dissect the femur, tibia, humerus, and ilium, removing as much muscle as possible, before placing in a 15-ml tube containing a few ml of EDTA/FBS on ice.
2. In a laminar flow hood, using aseptic technique, place the bones in a Petri dish containing 10–15 ml of EDTA/FBS and use scissors and forceps to remove any remaining muscle. Place the clean bones in a second Petri dish with 10–15 ml of EDTA/FBS.
3. Carefully cut all bones in the middle with scissors and flush out the BM, using a 27-ga, 1/2 inch needle, a 5-ml syringe, and FBS/EDTA as your flush solution.
4. Collect cells with syringe and transfer through a 70 μm nylon filter into to a 50-ml tube.
5. Centrifuge at 500 × *g* for 5 min at room temperature (RT).
6. Gently aspirate the supernatant and resuspend the cell pellet in 2.5–5 ml RBC lysis buffer. Use a sterile transfer

pipette to mix for 3–4 min. Resuspend to 40 ml with cold PBS.

7. Centrifuge at 500 × *g* for 5 min at 4°C.
8. Resuspend in 10 ml complete RPMI-1640 and spin as before (*see* **Note 2**).
9. Resuspend in complete RPMI-1640 and count viable cells using Trypan Blue and a hemacytometer.
10. In a 100-mm dish, plate 0.2–0.3 × 10^6 cells/ml in 20 ml of complete medium supplemented with GM-CSF and IL-4 at 1,000 U/ml.
11. Culture the cells for 7 days at 37°C, in a humidified 5% CO_2 incubator.
12. On day 2 of culture, 15 ml of culture media is pipetted off, collected, and spun down. Cells are then resuspended in 15 ml of fresh media, with GM-CSF and IL-4. Treatment with RAPA (10 ng/ml) is initiated and maintained for the remainder of the culture period. The media will contain a negligible amount of EtOH (0.03%; *see* **Note 3**).
13. On day 4, gently swirl plates and then remove 15 ml of culture media by pipette. Discard the media containing the floating cells and add back fresh media (15 ml) with cytokines and RAPA to the culture in a final volume of 20 ml.
14. On day 6, like day 2, again remove 15 ml of culture media and spin down the cells, resuspending them in fresh media with cytokines and RAPA.
15. On day 7, non-adherent cells are harvested by collecting all culture medium in a 50-ml tube. Wash the plate lightly with FBS/EDTA and collect these cells as well.
16. $CD11c^+$ RAPA-DCs may then be positively selected to enrich from typically 75–80% $CD11c^+$ DCs in culture to >95% DCs positive for CD11c. This is completed on the cultured cells using anti-CD11c immunomagnetic beads following the standard Miltenyi protocol.
17. To demonstrate/verify resistance to maturation, RAPA-DCs are compared to CTR-DCs, following their overnight (18–22 h) incubation with anti-mouse CD40 mAb or TLR ligands such as CpG-ODN or LPS. When assessed for phenotypic or functional maturation, RAPA-DCs should display markedly decreased CD86 expression by flow cytometry (see below) and decreased T-cell allostimulatory capacity in a direct MLR, also described below.
18. When these DCs are to be used as recipient-derived negative cellular vaccines in transplantation, donor (allogeneic)

splenocyte lysates are generated in PBS by three rapid freeze–thaw cycles (in liquid N_2). Clumps can be removed by passage through a 22-μM nylon filter into to a 50-ml tube. The purified $CD11c^+$ DCs are typically incubated with the splenocyte lysate at an equivalent ratio of ten lysed cells per DC. This incubation is performed overnight at 37°C in 6- or 24-well plates (20).

19. Following overnight incubation, the DCs are harvested by light scraping and washing of the plates with FBS/EDTA. Following washing, the cells are washed in PBS alone, before final suspension in PBS prior to lateral tail vein infusion ($1–10 \times 10^6$ cells in 200 μl of PBS) or functional analysis.

3.2. Phenotypic Analysis of DCs by Flow Cytometry

3.2.1. Detection of Surface and Intracellular Staining

1. DC surface Ag expression can be analyzed by flow cytometry on day 7 of culture.
2. Specifically, fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, CyChrome-conjugated or biotinylated mAbs followed by labeling with streptavidin-conjugated PE-Cy7 are used to stain DC.
3. Typically, 5×10^5 cells are blocked with 10% v/v normal goat serum (10 min; 4 °C), then stained with appropriate mAbs (30 min; 4 °C). Appropriate isotype-matched IgGs should be included as negative controls.
4. The mAbs (clones) often used to phenotype murine myeloid DC are CD11b (M1/70), CD11c (HL3), CD40 (HM40-3), CD54 (ICAM-1; 3E2), CD80 (16-10A1), CD86 (GL1), TLR2 (6C2), TLR4 (MTS510), TLR9 (M9.D6), CCR7 (4B12), MHC classes I and II mAbs specific to strain.
5. Cells are washed well (two times) in stain buffer. Data can then be acquired immediately. Alternatively, the DCs can be fixed in 1–4% PFA in stain buffer and analyzed at a later time-point.
6. Data are acquired and then analyzed.

3.2.2. Intracellular Cytokine Staining

1. To measure intracellular cytokine production by unstimulated or inflammatory ligand-exposed DCs, approximately 10^6 cells are treated with GolgiPlug (1 μl/ml) for 4–12 h at 37°C in 1 ml of complete media.
2. Following the incubation period, the DCs should undergo appropriate surface staining, as outlined in **Section 3.2.1**.
3. After washing the DCs in stain buffer, the cells are fixed in 4% PFA for 30 min at 4°C.

4. The cells are then washed in Perm buffer and incubated with appropriate Ab against the desired internal protein for 30 min at 4°C.
5. Wash twice in perm buffer, followed by resuspension in stain buffer and analysis.

3.3. Testing T Cell Allostimulatory Activity in MLR

1. Immunomagnetic bead-sorted $CD11c^+$ DCs are γ-irradiated (2,000–4,000 rad) and placed in round-bottomed, 96-well plates. In a "direct" MLR, the DCs are used as stimulator cells and purified, allogeneic splenic T cells are used as responders. In an "indirect" MLR, DCs pulsed with donor Ag are used to stimulate syngeneic T cells.
2. The spleen is harvested using sterile technique and placed in FBS/EDTA solution on ice. The spleen is then placed in a Petri dish with 15–20 ml of fresh FBS/EDTA solution for dissection.
3. Following mechanical dissection into small pieces no larger than 2–3 mm, the dissected spleen should be forced through a 70-μm nylon mesh cell strainer into a 50-ml conical tube using a sterile 5-ml syringe.
4. The splenocytes are then centrifuged at $500 \times g$ for 5 min at RT, and the cell pellet is resuspended in 2.5–5 ml of RBC lysis buffer for approximately 3–4 min.
5. Following lysis, add enough PBS to the tube to bring the total volume to 40 ml and spin again under the same conditions. The cells are then ready for counting/T-cell isolation.
6. Splenic T cells are isolated by negative selection of non-T cells using α-CD11b (M1/70), α-TER-119, α-Gr-1(RB6-8C5), α-I-A/I-E (M5/114.15.2), and α-B220 (RA3-6B2) purified rat-anti-mouse mAbs (BD Biosciences, Franklin Lakes, NJ, USA). Removal of non-T cells is accomplished via Depletion Dynabeads following the manufacturer's protocol, with minor modifications. Specifically, resuspend T cells at 2.5×10^8 cells/ml and add the above depleting antibodies at 1:100. Following 30 min incubation on ice, wash once in required buffer and add to Dynabeads® at a 1:1 ratio. Incubate for 45 min at 4°C with rocking and purified according to manufacturer's protocol.
7. Typically, the T cells are plated at $1–2 \times 10^5$ cells in 100 μl of complete media in a graded ratio versus γ-irradiated (20 Gy) DC.
8. After 72 h, the T cells are collected and analyzed by flow cytometry or assessed for proliferation.

9. To assess T-cell proliferation, individual wells can be pulse-labeled with 1 μCi ^{3}[H] thymidine for the final 16–18 h of the MLR. Radioisotope incorporation is determined using β-scintillatography.

3.4. In Vivo DC Expansion and RAPA Administration

1. The in vivo effects of RAPA can be investigated in normal animals and in mice in which DCs are expanded by the administration of rhuman Flt3L (10 μg/day, intraperitoneally days 1–10).
2. Mice are injected with RAPA (0.5 mg/kg per day; i.p.) or vehicle for 7–10 days (days 3–10 or days 1–10). Due to the long elimination half-life, mice are given a loading dose on day 1 (1.5 mg/kg) (17).

3.4.1. Splenic RAPA-DC Isolation and Purification

1. Spleens should be harvested from treated mice in a sterile fashion and placed in FBS/EDTA on ice until ready for digestion.
2. Following the harvest, inject the spleens with 100 U/ml of type IV collagenase in complete media, then disrupt and chop the organs with fine scissors. Keep the resulting cell suspension at 4°C.
3. Digest the remaining tissue fragments in 400 U/ml of type IV collagenase in complete media for 45 min at 37°C.
4. Finally, pool the cells and pass them through a 70-μm nylon mesh strainer. Wash the splenocytes in sterile, ice-cold PBS and spin at 500 × *g* for 5 min.
5. Next, suspend the cells in RBC lysis buffer, as outlined above. Wash with PBS and spin.
6. DCs are then enriched by density gradient centrifugation using 16% Histodenz in PBS at 1,200×g for 20 min at 4°C.
7. To obtain highly purified DC populations for the analysis of their allostimulatory activity or cytokine production, collect the non-parencymal cell layer following centrifugation. Proceed with magnetic bead-conjugated anti-CD11c mAb labeling and selection, as outlined above.

4. Notes

1. RAPA is insoluble in water, however, it is readily soluble in ethanol; 2–10 mg/ml stock solution of RAPA is typically made in 100% EtOH and stored at −80°C for up to 4 months. For in vitro work, 50 μg/ml stocks are made in

RPMI-1640 without additives and stored at −80°C for up to 4 months.

2. Prior to plating BM cells for culture, contaminating non-BM cells can be removed with purified Abs followed by rabbit complement treatment (20).
3. It is our experience that the day on which RAPA is added to the culture greatly impacts the level of DC maturity and robustness of maturation resistance upon harvest on day 7. We observe that adding RAPA on day 2 of culture results in the greatest inhibition of CD86 expression and responses to TLR and CD40 ligation, while still producing an acceptable DC yield. When RAPA is added on day 4 or day 5 of culture, an inhibition of DC maturation is still observed, but not to the extent observed when RAPA is added earlier (17). However, the timing of DC treatment with RAPA varies widely in the reported literature and may account for the divergent descriptions of the inhibitory effects of RAPA on human and mouse DCs.

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Chapter 9

Towards a Standardized Protocol for the Generation of Monocyte-Derived Dendritic Cell Vaccines

Michael Erdmann and Beatrice Schuler-Thurner

Abstract

For more than one decade patients have been treated with dendritic cell (DC) immunotherapy against malignancies and infectious diseases. Proof of principle studies demonstrated immunogenicity and clinical responses were observed in a fraction of patients. Overlooking more than 200 publications one realizes, however, that it is almost impossible to compare many of these trials even in a given clinical setting or disease. This is primarily due to the fact that dendritic cell generation procedures are highly variable. There is a requirement for a standardized DC generation protocol which provides 'reference dendritic cells' to which other dendritic cells (e.g. differently matured ones) can be compared to in order to further optimize this promising vaccination approach.

In this chapter, we describe in detail our standard DC generation protocols established during the last decade with over 200 melanoma patients treated and over 2,000 vaccinations applied in clinical studies at our hospital. We do not claim that these dendritic cells are the best ones, but the generation procedure is highly reliable and reproducible and provides a standardized reference DC vaccine.

Key words: Dendritic cell, monocyte-derived dendritic cell, monocyte, counterflow elutriation, GMP.

1. Introduction

During the last decade in addition to surgery, radiotherapy and chemotherapy – the traditional oncologic treatment modalities – much insight and experience with immunotherapy in cancer have been gained. Scientists and physicians treated patients suffering from immunogenic tumours such as melanoma, prostate cancer renal cell carcinoma and multiple myeloma with

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immunomodulatory agents in order to induce or enhance anti-tumour-immune responses. In contrast to antigen-unspecific immunotherapies such as interferon-alpha (1), IL-2 (2) and CTLA-4-antibodies (3) tumour-antigen-specific therapies have been performed by delivering antigens as peptides (4), proteins, DNA or RNA (5) with or without antigen-presenting cells. Alternatively, antigen-loaded DCs were adoptively transferred into the patient to induce or expand antigen-specific T cells in the patient by DC vaccination. The adoptive T-cell transfer (6) as another approach has proven successful in inducing regression of even large tumour masses, albeit this has been possible so far only in the setting of melanoma and modulation of the host (by non-myeloablative lymphodepletion and delivery of high doses of IL-2).

As dendritic cells (DCs) are the most potent antigen-presenting cells (7) several aspects make them attractive for targeted immunotherapy in cancer: (i) during ex vivo generation several aspects of DC physiology, e.g. DC type and maturation status can be manipulated, which are pertinent to the control of immune responses, (ii) tumour-antigens can be loaded in a controlled and pharmaceutically feasible fashion using peptides, proteins or RNA, (iii) autologous tumour strategies (dying tumour cells or whole tumour RNA) aim to include also the patient-specific, notably individually mutated tumour antigens. One major regulatory obstacle of this innovative and versatile immunotherapy is the requirement of good manufacturing production (GMP) modalities. Therefore GMP-grade ingredients (medium, cytokine, peptide/RNA) as well as legally approved clean rooms and well-trained and GMP staff working under standard operating procedures are required. There exists a broad heterogeneity of DC generation and application protocols as reviewed for DC therapy in metastatic melanoma (8).

We describe here our standard protocol to generate clinical-grade monocyte-derived DCs under standard operating procedures (SOP) established during the last decade with over 200 patients treated and over 2,000 vaccinations applied in clinical studies (9–12).

Figure 9.1 depicts the process of DC differentiation, maturation, loading and application. In summary patients receive a leukapheresis at the local Department of Transfusion Medicine. The leukapheresis is processed via gradient/adhesion technique or counterflow elutriation (CE) in order to enrich monocytes. Adhesion- or CE-enriched monocytes are cultured for 6 days to generate immature DCs. On day 6 cells are matured by a maturation cocktail (TNF-alpha, IL-6, PGE-2, IL-1beta). On day 7 mature DCs are loaded by peptides or RNA electroporation and subsequently frozen for subsequent intracutaneous or intravenous application.

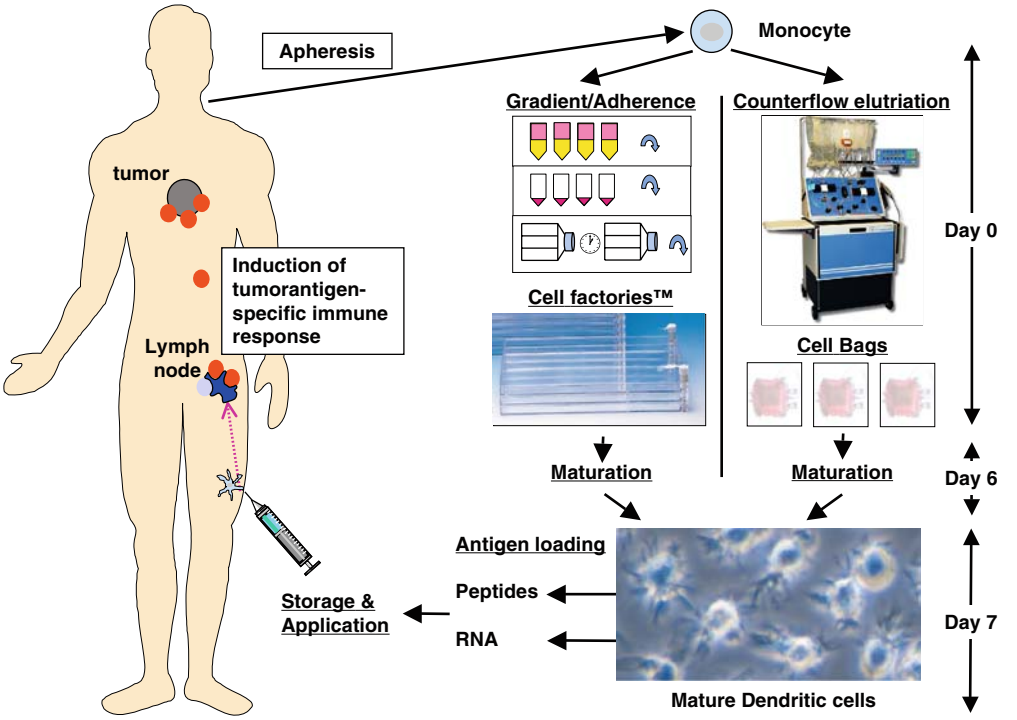

Fig. 9.1. Overview of human dendritic cell differentiation, maturation, antigen loading and administration for clinical application.

2. Materials

2.1. Leukapheresis and Autologous Plasma

As performed by local Department of Transfusion Medicine.

2.2. Generation of Complete Medium

1. Heat-inactivate autologous plasma (collected by Department of Transfusion Medicine) (56°C for 30 min) in water bath.
2. Spin heat-inactivated plasma (22°C, 20 min, 1,300 × *g*).
3. Filter supernatant with Stericup-GP Filter (Millipore, Schwalbach, Germany).
4. Add into each 500-ml bottle of RPMI-1640 5.0 ml L-glutamine and 5.1 ml heat-inactivated filtered plasma (8 bottles for **Section 3.2.1**, 18 bottles for **Section 3.2.2**).

2.3. Monocyte Enrichment

2.3.1. Gradient/Adhesion at Day 0

1. PBS (500 ml) supplemented with ACD-A (50 ml) (Fresenius, Schweinfurt Germany).
2. PBS/EDTA.
3. Lymphoprep (Progen, Heidelberg, Germany).
4. Cell factory with Nunclon®-surface (Nunc, Wiesbaden, Germany).

2.3.2. Counterflow Elutriation (CE) by Elutra® at Day 0

1. Plasma pooling set (3 l) (Cell Max, Beldico SA).
2. Elutra cell separation system (Gambro BCT, USA).
3. Elutra set (Gambro BCT, USA).
4. CD14-FITC.
5. Cell culture bag (O_2 + CO_2 permeable – 3 l) (Cell Max, Beldico SA).

2.4. Dendritic Cell Differentiation and Maturation

1. GM-CSF (Berlex, Sargramostim, Montville, NJ, USA).
2. IL-4 (Cell Genix, Freiburg, Germany).
3. Maturation cocktail: TNF-alpha 1 μg/ml, IL-1b 20,000 IU/ml, IL-6 100,000 IU/ml, PGE_2 100 μg/ml.

2.5. Dendritic Cell Loading

1. Tumour-associated antigen peptides may be obtained from various sources.
2. Culture flask (Nunc, Wiesbaden, Germany).
3. OptiMem (Invitrogen–Gibco, USA).
4. mRNA coding for tumour antigens may be purchased or produced.
5. Cuvettes for electroporation (peqlab, Erlangen, Germany).
6. Gene Pulser Xcell (Bio-Rad, Hercules, USA).

2.6. Dendritic Cell Harvest and Storage

1. Cryotubes.
2. Freezing medium: autologous serum (provided by local Department of Transfusion Medicine) or HSA 20% (55%), glucose 40% (25%) and DMSO (20%).

2.7. Dendritic Cell Application

1. Tissue culture dish with Nunclon®-surface (Nunc, Wiesbaden, Germany).

2.8. Flow Cytometry Analysis of DCs and Release Criteria for Immunotherapy

1. FITC-coupled antibodies against CD14, CD80, CD83, CD86, HLA-DR, CD25, CD1a and CD3, CD19 and CD56.
2. Sterility tests are performed at the local Department of Microbiology or respective company.
3. Tryptan blue.
4. Antibodies against respective peptides may be obtained from various sources.

3. Methods

All procedures are performed in a biological safety cabinet (clean room class A) unless otherwise specified.

As the majority of clinical trials as well as our group apply monocyte-derived dendritic cells we will focus on their generation and application. For information on haematopoietic stem cells (CD34+)-derived (13) and Flt3 (14)-mobilized DCs we refer to the respective groups.

The traditional robust methods for generation of monocyte-derived DCs is gradient centrifugation followed by an adhesion step for monocyte enrichment with subsequent DC differentiation and maturation in culture vessels such as cell factories (15). In order to enhance monocyte yield during the enrichment step as well as semi-automation with the aim of reducing open steps (**Fig. 9.2**) during generation two techniques for clinical scale monocyte enrichment were developed. One system applies anti-CD14-magnetic bead-coupled antibodies to specifically enrich CD14+ monocytes (16–18). Counterflow elutriation (which we favour and describe below) separates blood cells via their volume without need for any antibodies (19, 20). After

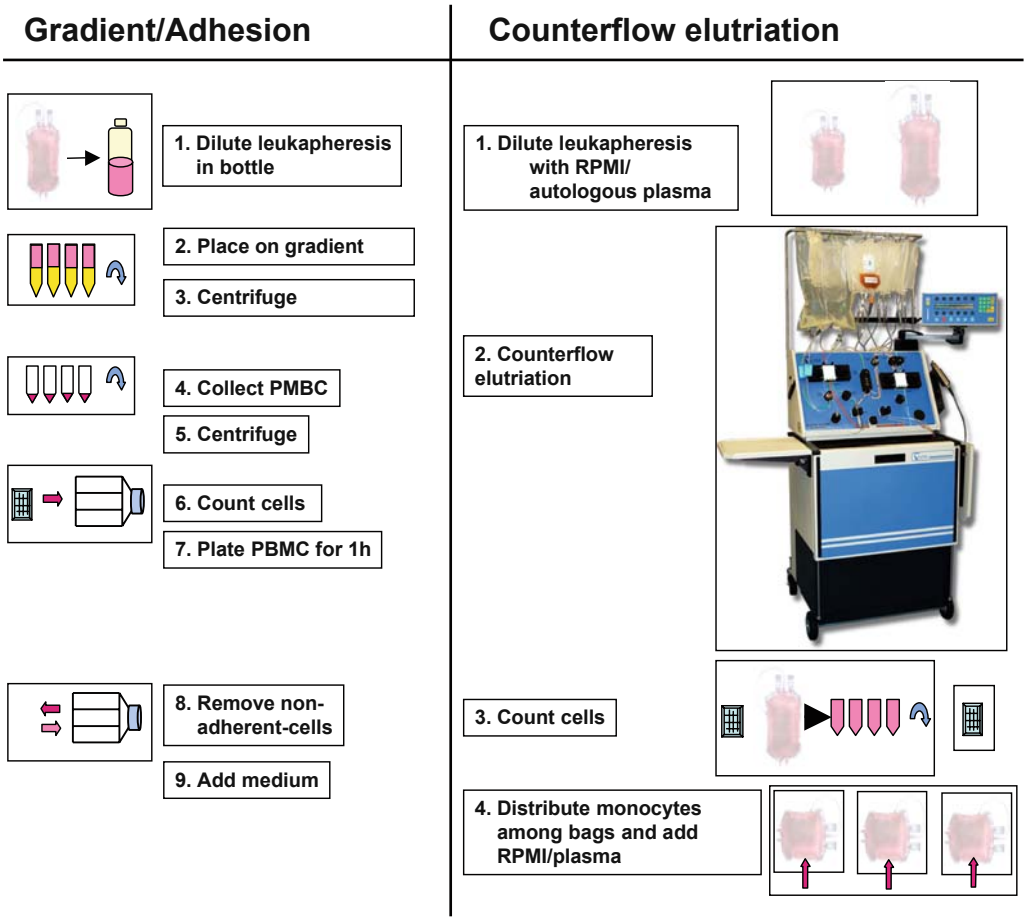

Fig. 9.2. Open steps during monocyte enrichment in gradient/adherence versus counterflow elutriation procedures.

counterflow elutriation in culture medium enriched monocytes are differentiated and matured in culture bags. We describe our currently applied antigen-loading techniques, namely, peptide and RNA. There exists a huge heterogeneity of protocols for DC differentiation from monocytes and subsequent maturation of immature in vitro-generated DCs. The following protocols reflect our current standard DC differentiation and DC maturation SOPs applied during our recent peptide and RNA-loaded DC trials.

3.1. Leukapheresis and Autologous Plasma

The local Department of Transfusion Medicine performs the leukapheresis. In order to generate sufficient DCs for therapeutic purposes at least 200 ml containing 20,000 white blood cells (WBC)/μl is required. If monocyte enrichment by CE is planned low red blood cell (RBC) counts (<0.6 mio/μl) will be essential (*see* **Note 1**).

3.2. Monocyte Enrichment

3.2.1. Gradient/Adhesion at Day 0

1. Dilute leukapheresis with warm PBS/ACD-A to a final volume of 726 ml.
2. Overlay carefully preloaded (15 ml) Lymphoprep tubes with 30 ml diluted leukapheresate.
3. Spin in warm centrifuge (22°C, 30 min, 400 × *g*).
4. Harvest Lymphoprep interphase and spin each tube for 10 min and wash twice with PBS/EDTA (4°C) (250 × *g*, subsequently 175 × *g*).
5. Finally spin cells for 10 min at 110 × *g*, collect and resuspend all pellets with RPMI-1640 (4°C) to a total volume of 300 ml.
6. Count PBMC.
7. Calculate the number of cell factories required (1,200 million PBMC per cell factory).
8. Spin appropriate amount of cells to wash (4°C, 10 min, 110 × *g*) in 50-ml tubes.
9. Transfer 1,200 million cells resuspended in 10 ml complete medium into each cell factory preload with 190 ml complete medium.
10. Incubate for 1 h (37°C, 5% CO_2, humidity > 70%).
11. Decant non-adherent fraction (NAF) and freeze separately.
12. Rinse cell factories twice with 100 ml warm RPMI-1640.
13. Add 240 ml warm complete medium into each cell factory.
14. Document percentage of adherent cells and make photograph for documentation.
15. Incubate for 13–25 h (37°C, 5% CO_2, humidity > 70%).

Continue with **Section 3.3.1**.

3.2.2. Counterflow Elutriation (CE) by Elutra® at Day 0

1. Place 2.5 l of complete medium (**Section 3.2**) into each plasma pooling set for counterflow elutriation.
2. Perform counterflow elutriation of the leukapheresis according to the manufacturer's manual (*see* **Notes 2** and **3**).
3. Count cells in monocyte-enriched fraction 5 and calculate the total amount of cells.
4. Distribute cells of fraction 5 into cell culture bags, 400 million cells each and add complete medium till the bag contains a total volume of 400 ml.
5. Perform flow cytometry analysis to measure percentage of CD14+ monocytes in fraction 5.

Continue with **Section 3.3.2**.

3.2.3. Magnetic CD14-Positive Selection of Monocytes

As our experience with the clinical application of CD14-positive selection of monocytes for DC vaccination is very limited we propose to address the groups (16–18) applying this technique for further information.

3.3. Dendritic Cell Differentiation and Maturation (see Note 4)

3.3.1. Monocytes Enriched by Gradient/Adhesion Cultured in Cell Factories

Day 1

1. Add 20 ml of complete medium, 5.2 ml of GM-CSF (40 IU/μl) (final concentration 800 IU/ml in cell culture) and 625 μl of IL-4 (100 IU/μl) (final concentration 250 IU/ml in cell culture) into each cell factory.
2. Culture for 36–60 h in incubator (37°C, 5% CO_2, humidity > 70%).

Day 3

1. Add 40 ml of complete medium, 6.0 ml of GM-CSF (40 IU/μl) and 750 μl of IL-4 (100 IU/μl) into each cell factory.
2. Culture for 36–60 h in incubator (37°C, 5% CO_2, humidity > 70%).

Day 5

1. Add 40 ml of complete medium, 6.8 ml of GM-CSF (40 IU/μl) and 850 μl of IL-4 (100 IU/μl) into each cell factory.
2. Culture for 12–27 h in incubator (37°C, 5% CO_2, humidity > 70%).

Day 6

1. Check if medium exchange is required (if medium turned yellow).

If no: Add 3.6 ml of maturation cocktail into each cell culture bag.

If yes: 1. Decant and collect 150 ml of cell suspension of cell factory.

2. Spin to wash (22°C, 10 min, 110 × *g*) and resuspend these cells in 150 ml feeding medium [150 ml of complete medium supplemented with 3 ml GM-CSF (40 IU/μl) and 375 μl IL-4 (100 μg/ml)].
3. Add washed cells in fresh medium into cell factory.
4. Add 3.6 ml of maturation cocktail into each cell culture bag.

2. Culture for 18–26 h in incubator (37°C, 5% CO_2, humidity > 70%).

3.3.1.1. Day 7

1. Wash and resuspend cells from cell culture bags into 200 ml RPMI-1640
2. Count cells.

3.3.2. Monocytes Enriched by Counterflow Elutriation Cultured in Cell Culture Bags

Day 0

1. Add 8.0 ml of GM-CSF (40 IU/μl) (final concentration 800 IU/ml in cell culture) and 1,000 μl of IL-4 (100 IU/μl) (final concentration 250 IU/ml in cell culture) into each cell culture bag.
2. Culture for 3 days in incubator (37°C, 5% CO_2, humidity > 70%).

Day 3

1. Add 60 ml complete medium, 9.2 ml GM-CSF (40 IU/μl) and 1150 μl IL-4 (100 IU/μl) into each cell culture bag.
2. Culture for 2 days in incubator (37°C, 5% CO_2, humidity > 70%).

Day 5

1. Add 60 ml complete medium, 10.4 ml GM-CSF (40 IU/μl) and 1,300 μl IL-4 (100 IU/μl) into each cell culture bag.
2. Culture for 2 days in incubator (37°C, 5% CO_2, humidity > 70%).

Day 6

1. Check if medium exchange is required (if medium appears yellow).

If no: Add 5.5 ml of maturation cocktail into each cell culture bag.

If yes:

1. Decant and collect 250 ml of cells of cell culture bag.
2. Wash and resuspend these cells in 240 ml feeding medium [250 ml of complete medium supplemented with 5 ml GM-CSF (40 IU/μl) and 625 μl IL-4 (100 μg/ml)].
3. Add washed cells in fresh feeding medium into cell culture bag.
4. Add 5.5 ml of maturation cocktail into each cell culture bag.

2. Culture for 18–26 h in incubator (37°C, 5% CO_2, humidity > 70%).

3.3.2.1. Day 7

1. Wash and resuspend cells from cell culture bags into 200 ml RPMI-1640.
2. Count cells.

3.4. Dendritic Cell Loading

1. Place up to 60 million DCs into each culture dish (2 million DCs/ml in complete medium). If multiple peptides are applied use one culture dish for each peptide.

3.4.1. DC Loading by Peptides

2. Pulse cells with target peptides at a concentration of 10 μM for MHC class I, 20 μM for MHC class II (concentrated stock solution) (*see* **Note 5**).
3. Swing slightly and incubate for 3–4 h. Move dishes slightly every 30 min.
4. Harvest cells into a 50-ml tube and rinse culture dish twice with RPMI-1640.
5. Count cells.

Continue with **Section 3.5.1**.

3.4.2. DC Loading by RNA

Prepare culture flasks containing 30 ml complete medium, GM-CSF (800 IU/ml) and IL-4 (250 IU/ml) for each electroporation aliquot.

1. Centrifuge and resuspend DCs in OptiMem at a concentration of 40 million DCs/ml.
2. Place 600 μl of DC-containing OptiMem into RNA-containing (30–45 μg) cuvettes.
3. Electroporate (Gene Pulser Xcell – square wave – 500 V, 1 ms) each cuvette according to manufacturer's manual.
4. Transfer cells with transfer pipette into 30 ml complete medium.
5. Repeat thrice to empty cuvette – rinse cuvette.
6. Repeat Steps 3–5 for each cuvette.
7. Transfer cells into incubator for 4 h.
8. Pool cells and resuspend cells in 40–100 ml RPMI.
9. Count cells.

Continue with **Section 3.5.2**.

3.5. Dendritic Cell Storage

Perform Steps 1–8 for each peptide batch.

1. Spin cells (4°C, 12 min, 110 × *g*).

3.5.1. Freezing of Peptide-Loaded DC: In 1-ml Aliquots (Containing 12 Million DCs)

2. Prepare labelled cryotubes and store at 2–8°C.
3. Freezing medium required: number of vaccines × 0.5 ml + 7 ml.
4. Resuspend cells with cold autologous serum at a concentration of 24 million DCs/ml.

5. Pipette 0.5 ml of DCs in autologous serum into each cryotube and add 1 ml of freezing medium.
6. Mix carefully and transfer immediately to freezer container.
7. Transfer freezer container to refrigerator (−80°C) for at least 2 h or overnight.
8. Store vaccines in gas phase of liquid nitrogen.

Continue with **Section 3.6.1**.

3.5.2. Freezing of RNA-Loaded DCs: In 4.5-ml Aliquots (Containing 45 Million DCs)

1. Calculate the number of vaccines (45 million DCs each round appropriately).
2. Prepare labelled cryovials and store at 2–8°C.
3. Freezing medium required: number of vaccines × 2.25 ml + 7 ml (buffer).
4. Wash DCs, resuspend in autologous serum (2.25 ml × number of vaccines).
5. Pipette 2.25 ml of DCs in autologous serum into cryotube and add 2.25 ml of freezing medium.
6. Mix carefully and transfer immediately to freezer container.
7. Transfer freezer container to refrigerator (−80°C) for at least 2 h or overnight.
8. Store vaccines in gas phase of liquid nitrogen.

Continue with **Section 3.6.2**.

3.6. Dendritic Cell Application

3.6.1. Thaw and Inject Peptide-Loaded DCs

1. Prepare water bath with distilled water and heat up to 56°C.
2. Preload 15-ml tubes for each cell batch with 3 ml PBS 1x.
3. Thaw cryotubes briefly (approximately 20 s) in warm water.
4. Transfer cells from cryotubes into 15-ml tubes. Rinse cryotube once with 1 ml PBS 1X and add to 15-ml tube.
5. Count cells. Perform photograph for documentation.
6. Take up each cell batch into a 5-ml syringe.
7. Inject each syringe (containing approximately 10 million DCs in 5 ml) in 1.5-ml portions intracutaneously in proximity of inguinal or axillary lymph nodes.

3.6.2. Thaw RNA-Loaded DCs

1. Prepare water bath with distilled water and heat up to 56°C.
2. Place 14 ml of cold complete medium containing 800 IU/ml GM-CSF (resting medium) into one tissue culture dish.
3. Thaw cryotubes briefly (approximately 20 s) in warm water.
4. Transfer cells from cryotubes into culture dish. Rinse cryotube twice.
5. Incubate cells for 1–2 h.
8. Inspect cells and harvest into 50-ml tube. Rinse culture dish twice with warm complete medium.

9. Centrifuge DCs (140 × *g*, 10 min, 18°C).
10. Count cells. Perform photograph for documentation.
11. Resuspend pellet with 5 ml of warm PBS. Transfer into perfusor syringe preloaded with 15 ml PBS. Rinse 50-ml tube once with 5 ml warm PBS and transfer into perfusor syringe.
12. Infuse 25 ml containing approximately 30 million DCs in 30 min intravenously.

3.7. Flow Cytometry Analysis of DCs and Release Criteria for Immunotherapy

One cell batch is required for flow cytometry and sterility analysis.

1. Perform flow cytometry of mature DCs.
   1. Stain thawed cells separately with FITC-coupled antibodies against CD14,

Table 9.1
Flow cytometry nomial values and GMP cell release thresholds of DC vaccine

RBC (EG/pi)	Leukapharesis volume
0.6	131.55
0.58	136.09
0.56	140.95
0.54	146.17
0.52	151.79
0.51	154.76
0.5	157.86
0.48	164.44
0.46	171.59
0.44	179.39
0.42	187.93
0.4	197.32
0.38	207.71
0.36	219.25
0.34	232.15
0.32	246.66
0.3	263.10
0.28	281.89
0.26	303.58
0.24	328.87
0.22	358.77
0.2	394.65

CD80, CD83, CD86, HLA-DR, CD25, CD1a and CD3, CD19 and CD56.

2. In the FSC/SSC dot blot place region 1 on large, high granular cells (DCs) and region 2 on small low granular cells (containing lymphocytes).
3. Nominal values and GMP cell release thresholds are summarized in **Table 9.1**.

2. Sterility: no aerobe or anaerobe bacterial growth after 14 days. No detection of endotoxin in frozen cell product. No mykoplasma growth after 7 days.
3. Living cells after thawing as counted by Tryptan blue exclusion must exceed 41% of DCs frozen on day 7.
4. For defined RNA-loaded DCs perform intracellular flow cytometry analysis with antibodies against respective proteins.

4. Notes

1. Clinical-scale generation of DCs for immunotherapy requires close collaboration with the local Department of Transfusion Medicine which will perform the leukapheresis (**Section 3.1**). Especially, if you apply counterflow elutriation (**Section 3.2.2**) in order to enhance monocyte yield continuous communication with this department to optimize leukapheresis modalities (low erythrocyte and granulocyte count in leukapheresis product) is required.
2. During counterflow elutriation (**Section 3.2.2**) avoid the debulking step whenever possible as this procedure results in a significant loss of monocytes for further DC cultivation. Therefore, calculate the maximum of volume which can be processed by counterflow elutriation (**Table 9.2**) and remove additional volume from the leukapheresis which can either be stored or used for a subsequent counterflow elutriation procedure.
3. Counterflow elutriation (**Section 3.2.2**) is a semi-automatic procedure which may leave this mode into manual mode if a problem occurs. To prevent loss of the leukapheresis product it is essential to be able to run counterflow elutriation in manual mode.
4. Check regularly if medium is exhausted (turned yellow) and on days 3, 5, 6 and 7 photograph the development of cells in culture for documentation.

Table 9.2
Maximum processable leukapheresis volume by counterflow elutriation depended on red blood cell count of leukapheresis without entering debulking mode

Surface marker	Nominal value(%)	GMP release threshold(%)
CDI4[a]	<5	
CD80[a]	>70	>50
CD83[a]	>75	>50
CD86[a]	>85	>75
HLADR[a]	>90	>75
CD25[a]	>50	
CDIa[a]	>50	
CD3+CDI9+CD5G[b]	<5	<30

[a]Region 1 – gated cell or regions 1 and 2 – gated cells.
[b]Region 1 and 2 – gated cells.

5. If you apply tumour-associated antigen peptides make sure that the donors HLA-type matches those of the peptides.
6. Clinical-scale DC generation for therapeutic immunotherapy requires GMP-trained staff and clean room conditions. Therefore systems which reduce open steps during DC generation or even the development of a complete closed system are required to enable broad application of this therapy

The authors state no conflict of interest.

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Section III

Murine Dendritic Cell Methods

Chapter 10

CD8$^+$, CD8$^-$, and Plasmacytoid Dendritic Cell Generation In Vitro Using flt3 Ligand

Shalin H. Naik, Meredith O'Keeffe, Anna Proietto, Hubertus Hochrein Ken Shortman, and Li Wu

Abstract

The generation of dendritic cells (DCs) from monocytes and early progenitors in GM-CSF cultures has been the gold standard for in vitro generation of DCs for three decades. However, the most recent evidence suggests that these cultures represent the migratory and inflammatory DC subtypes and not the DC subtypes found in the steady state. By contrast a different culture method was described where mouse bone marrow is cultured with flt3 ligand for 9 days. Here, we describe this method in detail for the generation of the phenotypic, functional, and developmental equivalents of CD8$^+$, CD8$^-$, and plasmacytoid DCs. This includes growth and purification of recombinant flt3 ligand from Chinese hamster ovary cells, isolation of bone marrow cells, and phenotypic characterization of the subsets. This simple method allows generation of large numbers of DCs (60–100 million from one mouse) compared to splenic DC isolation (5 million per mouse).

Key words: Dendritic cell, in vitro, steady state, subsets, flt3 ligand.

1. Introduction

Three functionally distinct DC subsets can be defined in a steady-state mouse spleen. These include the plasmacytoid pre-DC (pDC), CD8$^+$ conventional DC (cDC), and CD8$^-$ cDC subsets, which appear to be distinct and are not precursor-product related in the steady state (1). However, the diminutive numbers of DC in the mouse, and difficulty in their isolation, have often precluded study of splenic DC development and function. For example, at most 1×10^6 CD8$^+$ cDCs can be recovered with

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high purity from one mouse spleen after an elaborate purification protocol (2). A culture method for generating higher yields of these subtypes would make them more accessible.

There are several well-established procedures for generating DCs in culture from BM precursors or from blood monocytes using GM-CSF with or without IL-4 (GM-DCs) (3–8). However, GM-DCs do not seem to show the heterogeneity in DC phenotype and function found with splenic DC. In fact, it is apparent that GM-CSF-derived DCs are counterparts of inflammatory DCs but not those of the steady state (1, 9, 10).

Culture of BM with fms-like tyrosine kinase 3 ligand (FL) is a more recent method that allows the generation of both cDC and pDC in large numbers (now referred to as FL-DCs) (11–13). We have shown previously that, despite the absence of surface CD4 and CD8 expression, FL-DC can be clearly segregated by surface markers into the equivalents of steady-state splenic pDC, $CD8^+$ cDC, and $CD8^-$ cDC (14). The shared properties between the FL-DC subsets and the splenic DC subset counterparts included surface marker expression, transcription factor expression and dependence for development, ability to cross-present cellular antigen to CD8 T cells, expression of toll-like receptors (TLR) and chemokine receptors, and production of cytokines and chemokines in response to TLR stimulation. This system allows access to large numbers of the DC subsets for further study. In particular, up to 25×10^6 of the $CD8^+$ cDC equivalents can be generated from culturing the BM of one mouse with FL.

2. Materials

2.1. Flt3 Ligand Generation

1. Medium: RPMI 1640 medium with 5% fetal calf serum.
2. Chinese hamster ovary cell line expressing murine flt3 ligand conjugated to FLAG (generated by Prof N. Nicola, WEHI).
3. T25 and T75 tissue culture flasks.
4. 2 Liter Roller bottles (BD Falcon™ TufRol™ EZ Roller Bottles or similar).
5. 37°C incubator or room with rolling rack for roller bottles.
6. Trypsin: 0.05% w/v trypsin, 0.02% w/v EDTA.
7. 250-mL Disposable conical bottom tubes.
8. 0.22-μM Low protein binding filter, Millipore.

2.2. Flt3 Ligand Isolation

1. EZview™ Red ANTI-FLAG® M2 Affinity Gel, Sigma.
2. TBS (Tris-buffered saline): 50 mM Tris, 150 mM NaCl, adjust to pH 7.4 with HCl.

3. P-10 polyacrylamide size exclusion columns.
4. FLAG® peptide: 100 μg/mL lyophilized powder made in TBS.
5. 0.22-μM Low protein binding filter, Millipore.

2.3. Bone Marrow Cell Culture

1. C57BL/6 or similar mouse strain.
2. Scissors and forceps.
3. 21-ga Needle and 2-mL syringe.
4. Complete medium: RPMI 1640 medium (Gibco/BRL) with 5% fetal calf serum and penicillin/streptomycin.
5. Red cell removal buffer: 0.168 M NH_4Cl in deionized and sterile water at pH 7.2.
6. 70-μM Cell strainer.
7. 50-mL Falcon tube.
8. Desktop centrifuge with swinging buckets and speed capability of 1,100 × *g*.
9. Culture vessel, e.g., 96-well, 24-well, 6-well plates, or T25, T75, or T175 flasks.
10. Antibodies to CD11c (N418), SIRP-α (p84, BD Biosciences), CD24 (M1/69), CD45RA (14.8), or CD45R (B220, RA3-6B2), MHC Class II.
11. Four-color flow cytometer (FACSCalibur or the like).

3. Methods

3.1. Flt3 Ligand Generation

1. Pre-warm 1 L complete RPMI 1640 medium to 37°C.
2. Thaw the vial of CHO flt3 ligand expressing cells at room temperature with the lid slightly open to avoid explosion (*see* **Note 1**).
3. Add the cells with a pipette to 25 mL of warm medium, mix gently, and spin immediately at 1,000 × *g* for 6 min for all procedures (unless otherwise stated).
4. Remove supernatant, gently resuspend cells using a 10-mL pipette in another 50 mL medium, and spin again.
5. Resuspend cells in 10 mL medium and split into 2 × T25 tissue culture flasks (*see* **Note 2**).
6. The next day, gently remove the supernatant to eliminate any residual DMSO and replace with fresh medium.
7. The cells should be adherent and in a monolayer when ready to split.

8. At this time, add 1–2 mL trypsin to the flasks, let sit at room temperature for 1 min, then strike the side of the flask hard in order to dislodge the cells.
9. Wash and remove the cell suspension with 50 mL complete medium and spin.
10. Resuspend in 15 mL and transfer to T75 flasks and culture until confluent.
11. When confluent, harvest again with trypsin (2–4 mL), wash, and spin.
12. Resuspend the cells in 20 mL medium and seed a 2-L roller bottle that contains 500 mL of complete medium (*see* **Note 3**).
13. Let stand upright in the incubator with the lid unscrewed slightly for 4 h to allow the CO_2 in and to equilibrate.
14. Close the lid tight and place onto a rolling rack in a 37°C incubator (~20 rpm).
15. Monitor the cell growth over the coming days with an inverted microscope.
16. When confluent (usually after 3–4 days) add another 500 mL of RPMI 1640 medium without FCS to each roller bottle and culture further. This in effect reduces FCS concentration to 2.5%.
17. After a further few days, add another 1 L of medium without FCS. Now the concentration is effectively 1.25%.
18. After 2 weeks, the color of the medium should become "yellow." At this time, the supernatant is ready to harvest. Do not leave longer as this can release proteases from dying cells and affect flt3 ligand recovery (*see* **Note 4**).
19. Pour the supernatant into disposable 250-mL conical centrifuge bottles and spin at 1,600 × *g* for 5 min to pellet the CHO cells.
20. Tip off but keep the supernatant (which contains the flt3 ligand) in a fresh roller bottle for storage.
21. Continue until all of the culture supernatant has been separated from the cells.
22. Pass the supernatant through a 0.22- or 0.45-μm filter column to remove any remaining debris. At this stage, 0.02% sodium azide can be added for longer-term storage.
23. Use dialysis or another methodology to concentrate the supernatant approximately 100-fold (*see* **Note 5**). We use a Hemoflow F40S from Fresenius Medical Care.

3.2. Flt3 Ligand Isolation

1. Wash 10 mL of M2 beads twice in TBS by spinning, removing the supernatant, and resuspending.
2. Resuspend the beads using 100–200 mL of concentrated flt3 ligand-containing supernatant and place into a cylindrical-shaped sterile container and mix on a benchtop shaker on low speed at 4°C overnight (*see* **Note 6**).
3. The following morning, harvest the mixture and spin down the beads at 1,000 × *g* for 10 min.
4. Remove the supernatant and keep as a control (*see* **Note 7**).
5. Resuspend the beads in 10 mL of TBS and load 5 mL onto 2 × P-10 columns.
6. Allow the fluid to drain from the bottom of the P-10 columns but ensure the beads are always in contact with liquid.
7. Wash with 4 × 5 mL column volumes of TBS. This will remove the FCS and other components.
8. Elute the bound flt3 ligand with 20 mL of 100 ng/mL FLAG peptide in TBS per P-10 column and collect 2.5 mL fractions into polypropylene FACS tubes.
9. Check the 280 nm absorbance spectra of each fraction to determine which fractions contain the flt3 ligand and thus which ones to pool and which to discard.
10. Calculate the protein concentration using the best available method (Bradford, BCA, or similar method).
11. Check the purity of the fraction by running a small amount on a 12.5% polyacrylamide protein gel with a known MW ladder. Flt3 ligand runs at approximately 16 kDa.
12. Dialyze the protein against a large volume (2 L) of PBS overnight.
13. Filter sterilize using a 0.22-μM filter and, working sterile, store aliquots of the protein at −70°C at a concentration of no less than 1 mg/mL (*see* **Note 8**).

3.3. Bone Marrow Isolation

1. Keep a large tube of complete medium on ice for the collection of bones.
2. Sacrifice mouse by cervical dislocation or CO_2 asphyxiation.
3. Place mouse on back and liberally add 70% ethanol.
4. Make an incision horizontally across the stomach of the mouse.
5. Separate by clasping the skin either side of the incision with a thumb and forefinger until the skin is completely torn apart laterally around the entire torso of the mouse.

6. Remove the legs, including feet, from out of the skin – as though the skin with fur was a pair of trousers.
7. Using the scissors make a single incision at the rear of the knee and cut toward the knee itself.
8. Then cut flat along the thigh muscle toward the abdomen on both sides of the femur to expose the femur.
9. Hold the scissors with the open cutting edges against the femur toward the abdomen but do not cut through. This prevents the bone from dislocating from the hip in the next step.
10. With forceps, separate the tibia from the femur in a vertical motion. This will expose the femur, but remove the kneecap along with the tibia (*see* **Note 9**).
11. Using scissors cut off the foot and the kneecap (*see* **Note 10**).
12. Using two sets of tweezers, hold the foot-end of the tibia with one and remove the calf muscle from the tibia with the other. With practice, this can be done in one swift motion.
13. Then, cut the femur at the base toward the abdomen, ensuring the "ball" of the femur that joins the socket of the hip is removed.
14. To isolate the hip bone (ilium), turn the mouse on its front and cut along one side of the spine (*see* **Note 11**).
15. Insert your scissors underneath the hip bone and cut toward the belly of the mouse.
16. Cut the underside of the hip separating it from the body.
17. Then cut the flesh on the top of the hip to remove the flesh.
18. The hip should now be ready to remove with forceps with some clearing of flesh with scissors or forceps along the way.
19. Use a 21-ga needle and a 2-mL syringe for harvesting the bone marrow (*see* **Note 12**).
20. Fill the needle and syringe with 2 mL medium and insert by a gentle twisting motion into the bone.
21. For tibias, the needle should be inserted into the epiphysis at the "knee-end."
22. For femurs, the needle should be inserted into the epiphysis at the "hip end."
23. For the hips, the needle should be inserted into the thin end of the "fan"-shaped hip bone.
24. Eject the contents of the syringe, flushing the bone marrow into the cold medium.
25. While the needle is still inside the bone, rotate it 180°, then aspirate medium through the bone. This ensures that

maximum amount of marrow is isolated considering the bevel of the needle.

26. When all the bone marrow is harvested (the bone should be white), ensure a single-cell resuspension by aspirating with the needle and syringe prior to centrifugation.
27. Spin the cells, then remove the supernatant and dislodge the pellet by striking the bottom of the tube with your fingers or by dragging it across the grill in the laminar flow cabinet.
28. Add 1 mL of red cell removal buffer per 8 mice, and gently flick the pellet until it is well resuspended. Do not pipette as this reduces cell viability.
29. Leave the cells with the RCRB for no more than 60 s.
30. Immediately dilute the cells with a large volume of medium and spin. The pellet should be off-white in color due to the lysis of red cells (*see* **Note 13**).
31. Remove the supernatant and repeat the wash step twice.
32. Resuspend the cells in approximately 10 mL medium per 8 mice and pass through a 70-μM filter to remove bone, muscle, dead cell clumps, and other debris.
33. Count the cells and test their viability using Trypan Blue exclusion or the like. This should be around 85–95%. Numbers are important to gauge at this point. For "Australian" C57BL/6 mice, we typically obtain
    a. $\sim 55 \times 10^6$ cells for both tibias and femurs.
    b. $\sim 90 \times 10^6$ cells for tibias, femurs, and ilium.

    For "European" C57BL/6 mice, we typically obtain
    c. $\sim 70 \times 10^6$ cells for both tibias and femurs.
    d. $\sim 120 \times 10^6$ cells for tibias, femurs, and ilium (*see* **Note 14**).

3.4. Determining the Concentration of flt3 Ligand and Batch of FCS

1. The batch of FCS has a profound effect on the development of the DC subtypes and their numbers.
2. The concentration of flt3 ligand must also be determined to minimize use but maximize numbers.
3. Gather several aliquots of FCS from suppliers or colleagues.
4. Create a matrix of conditions in a 24-well plate covering the different flt3 ligand concentrations and the different batches of FCS (*see* **Note 15**).
5. Culture the cells at 1.5– 3 $\times 10^6$ total BM cells per mL of complete medium with flt3 ligand for 9 days at 37°C with 5% (or 10%) CO_2 in air. One can also harvest as early as day 7, and as late as day 11, but these may contain a higher proportion of precursors or less DCs, respectively.

6. Determine the cell number at the end of the culture period. Typically, we generate as many $CD11c^+$ DCs as the number of total BM cells initially cultured. Numbers rise and fall according to death of non-progenitors (max death at day 3) followed by increase in cell numbers with proliferation.
7. Examine the cells under a microscope. There should not be too many adherent macrophages. The majority of DCs are semi- or non-adherent (*see* **Note 16**).
8. Stain an aliquot for CD11c, B220, and SIRP-α. In the absence of SIRP-α, CD11b can also be used. CD24 and CD103 can also assist in discrimination of the $CD8^+$ cDC. A typical profile of the subtypes can be seen in Fig. **10.1**. Note, however, that from lab to lab there can be great variation with composition of subtypes, which can be related to FCS batch, different types of FL, etc.
9. Depending on the purpose, DCs can be grown in as small or as large a culture vessel as is necessary. They can be used as they are or enriched either using MACS or flow sorting.

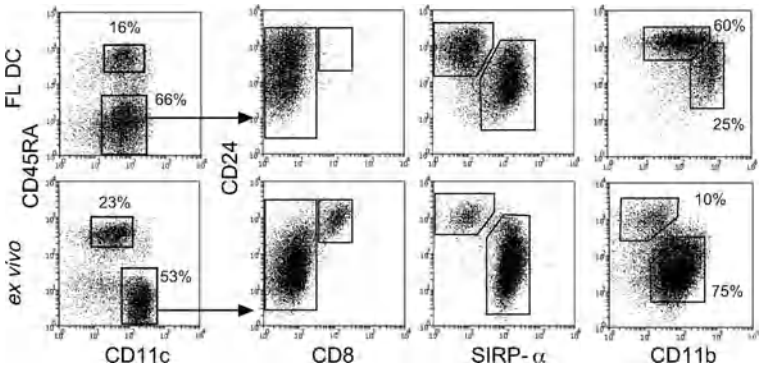

Fig. 10.1. Surface marker discrimination of FL and spleen DC subsets. (**a**) Both FL-DC and splenic DC from WT mice were stained with indicated combinations of surface markers and analyzed by flow cytometry.

4. Notes

1. This cell line is available upon request.
2. Steps 3–6 are important to remove residual DMSO, which can affect cell viability.
3. You will ultimately need one flask to seed one roller bottle, so seed the appropriate number of flasks.
4. We have found that leaving the cells longer increases the recovery of FL. However, one can harvest the supernatant earlier (e.g., at 10–14 days).

5. This will enrich the supernatant to, essentially, pure FCS. Be very careful to avoid bubble formation when working with this fluid.
6. A cylindrical-shaped container maximizes binding of the free FLAG-tagged flt3 ligand to the anti-FLAG beads.
7. The supernatant should be free of flt3 ligand. If not, this may indicate overloading of the resin or poor binding to the resin.
8. Flt3 ligand is stable at 4°C for a long time. We have seen activity even after months of storage. However, we still recommend thawing small aliquots such that no longer than 8 weeks at 4°C of a working stock is required. Refreezing is discouraged.
9. This requires a bit of strength, but be careful not to snap the bone with the forceps.
10. The kneecap is white and can be differentiated from the rest of the tibia. However, be careful not to cut too much of the kneecap off. It is documented, and also our experience, that the bulk of progenitors is at the epiphysis ends (joints) of the bone. Cutting too much off will result in reduced DC generation.
11. The ilium is particularly difficult to isolate without a prior demonstration and practice.
12. A 21-ga needle has a wide bore but this ensures that a maximum amount of the marrow is actually recovered.
13. If the pellet is not white, this may be due to several reasons. The red cell lysis buffer may not be effective. Remake the buffer or opt for a commercial source. An incomplete resuspension of bone marrow into single cells with the needle prior to centrifugation or an incomplete red cell lysis after dislodging of the pellet can reduce access to the RCRB.
14. These numbers do not appear to be related to cleanliness of the mouse house. We are not sure what accounts for this discrepancy. Numbers less than these may indicate a substandard BM isolation procedure.
15. Typically, we have found maximum activity of flt3 ligand ranging between 30 and 400 ng/mL. This depends on the purity and activity of the FL that can vary batch to batch.
16. An excess of macrophages generally indicates that the culture conditions are substandard, usually due to not enough flt3 ligand or a "poor" batch of FCS. At about day 4–5, you will start seeing clusters of cells like a bunch of grapes – this is a good sign that the cultures are working.

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Chapter 11

Generation of Large Numbers of Pro-DCs and Pre-DCs In Vitro

Shalin H. Naik

Abstract

The $CD8^+$, $CD8^-$, and plasmacytoid dendritic cell (DC) subtypes develop from progenitors that express surface *fms*-like tyrosine kinase 3 (Flt3). Recently, two developmentally sequential progenitors have been identified that give rise to these subtypes. This includes a transition from an earlier $CD11c^-MHC\text{-}II^-$ "pro-DC," which divides and differentiates to give rise to $CD11c^+MHC\text{-}II^-$ "pre-DC," en route to generating the three $CD11c^+MHC\text{-}II^+$ DC subtypes – plasmacytoid DCs, $CD8^+$ DCs, and $CD8^-$ DCs. In this chapter, we describe the very simple method of generating large numbers of in vitro-derived pro-DCs and pre-DCs. As these precursors are largely DC-restricted, they can be used to either reconstitute a mouse with DCs of desired background, to study the developmental steps in vitro or in vivo, among other purposes.

Key words: Dendritic cell, progenitors, steady state, subsets, flt3 ligand.

1. Introduction

While it is well appreciated that DCs can be separated into functionally distinct subtypes, their development from progenitors and their relationship to each other and to other leukocytes have been controversial (1, 2). DCs are of hematopoietic origin. Any further conclusions about DC development are met with great debate. This is partly due to confusion about the numerous DC subsets, their different locations, their maturation states, the distinct assumptions in human versus mouse studies, and whether the many in vitro conclusions about their development actually apply in vivo.

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Downstream of the BM hematopoietic stem cell (HSC) and multipotent progenitors (MPP), both common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) have the potential to give rise to pDCs, $CD8^+$ cDCs, and $CD8^-$ cDCs, with peak generation after 14–21 days and later (3–5). However, only progenitors that express the *fms*-like tyrosine kinase receptor 3 (flt3) are able to contribute (6, 7). Some later precursor stages immediately prior to generation of the DC subtypes (2–5 days prior) have been identified. These include the intrasplenic pre-cDCs that generate $CD8^+$ and $CD8^-$ cDCs (8–10), a $Ly49Q^-B220^+$ BM precursor of pDCs (11, 12), monocyte precursors of Langerhans cells (13, 14), and monocyte precursors of inflammatory DCs (Chapter 5 and (15–17)).

In vitro systems that generate large numbers of DCs are useful for the study of DC function and development. One common method is the culture of BM, blood precursors, or monocytes with GM-CSF with or without IL-4 (GM-DC) (18–23). These "myeloid" DCs appear to be homogeneous and have been a useful model for studying many properties of DCs. However, they have been difficult to place in the context of DC found in vivo. They are often assumed to be the equivalent of splenic $CD8^-$ cDC based on CD11b expression, although this has never been formally shown. We have shown that GM-DCs are more representative of "inflammatory" monocyte-derived DCs rather than steady-state DCs ((24) and reviewed in (1, 2).

Nevertheless, the generation in culture of GM-DCs provides a useful tool for the study of inflammatory DC development, either from monocytes or BM. In fact, Leenen and colleagues have elegantly identified the steps in development of DCs from BM progenitors in the presence of GM-CSF by tracking earlier time points in culture (25). Results from that study suggested that GM-DC development involved a sequential transition: Early Myeloid Precursors ($CD31^{hi}$ $Ly6C^-$ $CD58^{hi}$) $\Rightarrow$ Monoblasts ($CD31^+Ly6C^+$) $\Rightarrow$ Monocytes ($CD31^-Ly6C^{hi}$) $\Rightarrow$ DCs ($CD31^-Ly6C^-$). Importantly, this process clearly involved $Ly6C^{hi}$ monocytes as the immediate precursor of DC, linking the process with many studies that have demonstrated that monocytes are the immediate precursors of GM-DC.

However, for steady-state DC development Flt3 ligand appears to be the crucial cytokine rather than M-CSF or GM-CSF. This correlates with an absence of pDCs and cDCs in mice genetically deficient for Flt3 ligand and its STAT3 signaling pathway (26, 27), an increase in the numbers of splenic DCs when FL is injected (28), and the obligatory expression of flt3 on early precursors for splenic DCs (6, 7). The importance of FL in DC development also correlates with its use as a single addition cytokine in a different in vitro model for DC production

(FL-DC). In this system BM is cultured with FL for 9 days, as described in chapter 10 of this book and previously (29–32). Distinct FL-DC subtypes develop in these cultures that are equivalent to pDC, $CD8^+$ cDC, and $CD8^-$ cDC from the spleen, according to numerous functional and phenotypic parameters (29, 30, 33). Therefore, unlike GM-DC, the FL-DC culture system is a true mimetic of steady-state DC generation from BM precursors.

We presumed that at earlier time points during FL BM cultures, putative DC precursor stages en route to generation of the DC subtypes should be present. Analogous to the characterization of DC development in GM-CSF cultures (25), we identified and characterized two DC precursor populations in FL cultures. The early precursor (pro-DC) was a blasting cell that divided several times to give rise to a late-stage precursor (pre-DC). Pre-DC could then differentiate with minimal division into all of the DC subsets, but not most other lineages. Others and we have also found the in vivo equivalent of pro-DCs (34, 35) and pre-DCs (8, 9, 34). The identification of these precursor stages thus provided greater resolution of the step-wise development of DC downstream of the early BM progenitors.

Fogg et al. identified a precursor within BM that could produce cDCs (both $CD8^+$ and $CD8^-$) and macrophages, but not granulocytes, pDCs, or any other lineage (36), although recently pDC production appears to have been recognized as well (37). In contrast, pro-DCs (also known as Common DC Progenitors (CDP)) (34, 35) could generate both $CD8^+$ and $CD8^-$ cDC subtypes as well as pDCs, but not significant numbers of macrophages or any other lineage.

Whether MDPs and pro-DCs represent distinct or overlapping populations is not clear. What might account for the differences? Phenotypically, MDPs and pro-DCs are very similar (both are $Lin^-Sca\text{-}1^-IL7R^-CD117^{int}$). However, at least in our hands, most but not all pro-DCs are $CD34^-CD16/32^-$ (34), whereas MDPs are largely $CD34^+CD16/32^+$ (36). Importantly, we do not define all in vivo pro-DCs as $M\text{-}CSFR^+$ (34). It should be noted that antibodies against M-CSFR were neither used for the isolation of pro-DCs in the majority of our experiments (34), nor in some of those of Onai et al. (35), so this cannot be the reason we did not see macrophage potential in our assays, as has been suggested (38). A second important difference is that the majority of pro-DCs do not generate colonies in response to GM-CSF, whereas MDPs do. Considering that GM-CSF-derived DCs better represent inflammatory DCs (1, 2) 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, but no pDCs in vivo (36). 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.

The method described in this chapter combines the Flt3 ligand culture method described elsewhere in this book, with a CFSE-labeling step to track divided precursors and a depletion method to enrich for pro-DC and/or pre-DC (**Fig. 11.1**). These

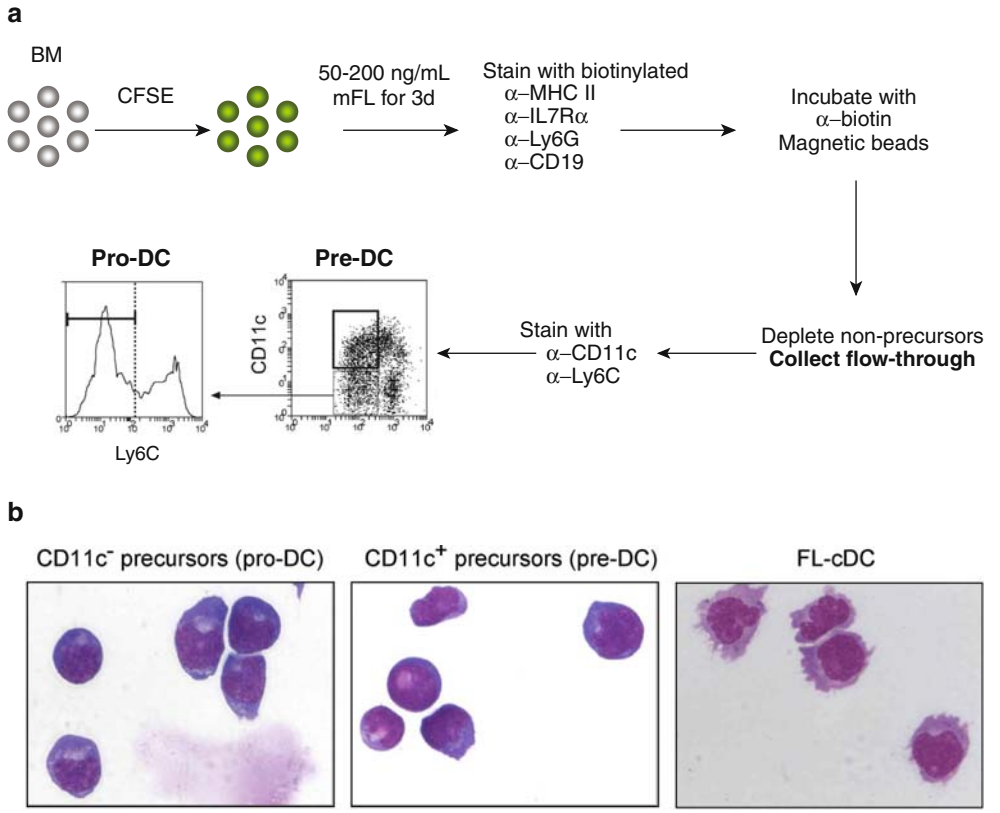

Fig. 11.1. Isolation and morphology of pro-DCs and pre-DCs. (**a**) To isolate DC precursors, BM is CFSE-labeled and cultured with FL for 3 days. Total cultured cells are collected, coated with biotinylated mAbs against non-precursor cell types, incubated with anti-biotin beads, and non-precursors depleted using a magnetic column. The flow-through-containing precursors are then stained for CD11c and Ly6C expression prior to separation by flow cytometry. Large, dividing CFSE^{low} cells are gated as CD11c$^+$ cells (pre-DCs) or CD11c$^-$Ly6C$^-$ cells (pro-DCs). (**b**) Cytospin preparations of both precursor populations as well as CD11c$^+$ CD45RA$^-$ FL-derived cDC populations stained with May-Grünwald Giemsa.

precursors can then be used for the study of DC development or to reconstitute large numbers of DCs in vitro or in vivo.

2. Materials

2.1. Bone Marrow Cell Culture

1. C57BL/6 or similar mouse strain.
2. Scissors and forceps.
3. 21-ga needle and 2-ml syringe.
4. RPMI-1640 medium (Gibco/BRL) with 5% fetal calf serum and penicillin/streptomycin (complete medium).
5. Red cell lysis buffer: 0.168 M NH_4Cl in deionized and sterile water at pH 7.2.
6. 70-μM Cell strainer, Falcon.
7. 50-ml Falcon tube.
8. Desktop centrifuge with swinging buckets and speed capability of 1,100 × *g*.
9. CFSE.
10. PBS/BSA – phosphate-buffered saline with 0.1% bovine serum albumin.
11. 37°C water bath.
12. Fetal calf serum.
13. Culture vessel – 96-well, 24-well, 6-well plate, or T25, T75, or T175 flasks.
14. Depleting monoclonal antibodies. These should all be in one form of conjugate, e.g., either all biotinylated, all PE.
    1. MHC-II (M5/114), Ly6G (1A8), CD19 (1D3), IL7-Rα (A7R34).
    2. Ly6C (5075-3.6) CD11c (N418) (if pre-DCs are not required).
15. MACS beads for the specificity of fluorochromes, e.g., anti-biotin or anti-PE. Avoid the use of FITC antibodies, as these will overlap with the CFSE fluorescence if cell sorting is to be undertaken.
16. LS column.
17. MACS magnet for LS column.
18. Antibodies in an alternative fluorochrome to the depleting antibodies against CD11c (N418) and Ly6C (if pre-DCs are required).
19. Cell sorter.

3. Methods

3.1. Isolation and CFSE Labeling

1. Isolate BM cells as per the protocol published in chapter 10.
2. To label the cells with CFSE, first wash twice in PBS/BSA.
3. Resuspend the pellet in a concentration of 1×10^7 cells/ml in PBS/BSA, and transfer to a fresh tube taking care to keep the sides of the tube dry (explained later).
4. Add 1 μl of 0.5 μM CFSE per ml of cells to the dry side of the tube which contains the cells (*see* **Note 1**).
5. Cap the tube and vortex for 10 s.
6. Place immediately in a 37°C water bath for 10 min.
7. Add complete medium to a large volume, mix, and spin.
8. Repeat this wash twice.

3.2. Culture and Harvest

9. Culture the cells at 1.5–3 $\times$ 10^6 total BM cells per ml of complete medium with flt3 ligand in any culture vessel (*see* **Note 2**).
10. Culture the CFSE-labeled BM cells in a suitable culture dish for 3.5 days. This is the time at which most cells that are not DC precursors (e.g., B cells, granulocytes, monocytes) have died from factor deprivation, but just prior to major DC precursor expansion (*see* **Fig. 1b** in Ref. (34)).
11. Harvest the cells by shaking of the culture vessel or by pipetting and then pouring cells into a new tube. Keep the culture vessel for the next step.
12. Spin down cells at 1,000 $\times$ *g* during which time add a volume of PEF to cover the bottom of the now-empty culture vessel for 5 min, e.g., 10 ml for a T175 flask.
13. Remove, but keep, the supernatant from the centrifuged cells if the precursors are to be further differentiated in vitro. This kept medium is now referred to as "conditioned medium." To eliminate cell debris from conditioned medium, allow it to pass through a 0.22-μM filter. This supernatant can also be stored at −70°C for long periods to thaw at a later stage.
14. Resuspend the cells in the PEF from the culture vessel, and use this buffer to resuspend the pellet from centrifugation.
15. Pass through a 25-ga needle and leave on ice for 5–10 min. This will ensure that the precursors are rendered as single cells, as they tend to clump together straight from culture.
16. Spin down the cells for the subsequent depletion step.

3.3. Enrichment of DC Precursors

1. The Nycodenz density centrifugation step at this stage has been excluded compared to the original protocol (34). Simplification of the protocol is the only reason for this. However, more reagents are therefore needed for the next steps.
2. Resuspend the cell pellet from **Section 3.2**, Item 16 in antibody cocktail (*see* **Note 3**). Depending on what cell type is required, the following mAbs should be incubated with the cultured cells on ice with the following:
   1. Pre- and pro-DC: biotinylated MHC-II, CD19, Ly6G, IL7R, CD11c APC, and Ly6C PE.
   2. Pro-DC only: same as 1. (above) but with biotinylated Ly6C and CD11c instead.
3. Wash in 10 volumes of PEF and spin down.
4. Resuspend in PEF containing MACS beads according to manufacturer's instructions (*see* **Note 4**). If depleting antibodies are biotinylated use anti-biotin beads, if PE-labeled use anti-PE beads. Incubate for 15 min in the fridge (not on ice).
5. Wash in 10 volumes of PEF and spin down.
6. Resuspend cells to a concentration of 100 million/ml in PEF avoiding bubble formation, and pass gently through a 25-ga needle to ensure single cells.
7. Add onto a pre-wet LS or MD column loaded on the magnet and let the cells pass through. Collect the flow-through which contains the precursors.
8. After the cells have been loaded onto the column, wash with 1 ml and then 3 ml of PEF to wash the bound cells.
9. Collect separately (a) the total flow-through and (b) elute the bound cells. Check a small fraction by flow cytometry for the efficiency of depletion, e.g., if the depleting antibodies were PE, simply run, or if they were biotinylated stain with streptavidin-PE for 5 min at a fivefold higher concentration than you normally would. This is because the anti-biotin beads have blocked many of the streptavidin sites.
10. To get a pure population of precursors, stain and then sort the cells by flow cytometry. Stain with SA-PE if biotinylated antibodies were used, along with CD11c.
    1. For pre-DC and pro-DC, sort for
       i. Pre-DC: divided ($CFSE^{lo}$), Lin^-, $CD11c^+$ cells.
       ii. Pro-DC: divided ($CFSE^{lo}$), Lin^-, $CD11c^-$, $Ly6C^-$ cells.
    2. For pro-DC only, sort for divided ($CFSE^{lo}$), Lin^- cells.

11. If you are going to culture the precursors further, plate no more than 1×10^4 pro-DC or 5×10^4 pre-DC per 100 μl of conditioned medium.
12. Peak DC production is as follows
    1. In vitro: pre-DCs after 2 days, pro-DCs after 5 days.
    2. In vivo: pre-DCs after 5 days, pro-DCs after 9 days.

4. Notes

1. Having a dry tube ensures that the subsequent vortex step allows the CFSE to label the cells homogeneously.
2. For the optimal concentration of flt3 ligand, follow the guide in the attached chapter.
3. The antibodies should be all biotin or all PE, etc. Each antibody should be titrated such that there is a clear-negative and a clear-positive population by FACS. This will allow efficient depletion but without non-specific depletion due to high background staining of the negative (precursor) population.
4. We routinely use up to 4-fold lower amounts of beads and buffer, with efficient depletion.

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Chapter 12

The Generation of Plasmacytoid and Conventional Dendritic Cells with M-CSF

Meredith O'Keeffe, Ben Fancke, and Hubertus Hochrein

Abstract

Mice lacking the ligand for Flt-3 (CD135) have a massive deficit of dendritic cells (DC) in all organs. This phenotype of FL (FL) knockout mice suggested that FL was the archetypal DC poietin in the steady state. However, FL knockout mice also have reduced numbers of common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) so it is possible that FL deficiency may limit the ability of other growth factors to drive DC development by limiting the pool of progenitor cells available. We found that DC development could be driven from BM cells of FL knockout mice using the myeloid growth factor M-CSF. The M-CSF-driven DC (MDC) developed independently of FL and resembled the DC types present in the spleen in the steady state.

Key words: Dendritic cells, bone marrow, flt3 ligand, M-CSF.

1. Introduction

Mice lacking the ligand for Flt-3 (CD135) have a massive deficit of dendritic cells (DC) in all organs (1). This phenotype of flt3-ligand (FL) knockout mice suggested that FL was the archetypal DC poietin in the steady state. Moreover, cultures of bone marrow (BM) cells with FL yielded large numbers of plasmacytoid (p) and conventional (c) DC (2,3). However, FL knockout mice also have reduced numbers of common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) (4 and our unpublished observations) so it is possible that FL deficiency may limit the ability of other growth factors to drive DC development by limiting the pool of progenitor cells available.

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Other workers have shown that DC in peripheral organs of mice express c-FMS, the receptor for the classical myeloid growth factor M-CSF (5). We found that M-CSF was able to drive the development of pDC and cDC from BM cells of FL knockout mice. The DC generated in these cultures were reduced in number relative to BM FL cultures but they more closely resembled the DC found in the spleen in the steady state (6). Most importantly these M-CSF-driven DC (MDC) developed independently of FL as shown by their development in the FL knockout mice and also by their unaffected development in M-CSF BM cultures treated with inhibitors of Flt3. The MDC were able to develop from CLP and CLP and indeed Onai et al. (7) and Naik et al. (8) have shown that a more committed DC precursor in BM expressed c-FMS.

Thus M-CSF should be considered as a new member of the DC poietins that include FL and GM-CSF. It is the only growth factor apart from FL that on its own is able to drive the generation of pDC and as such its generation and action in vivo may play key roles in the generation of pDC, both in the steady state and during viral infections.

2. Materials

Unless otherwise stated all antibodies used can be obtained from Becton Dickinson.

2.1. MDC Generation

1. RPMI (Gibco-BRL).
2. Red cell lysis buffer (Sigma–Aldrich).
3. Complete medium: RPMI-1640 medium (Gibco-BRL) supplemented with 10% FCS, 50 μM beta-mercaptoethanol, 100 IU/ml penicillin/streptomycin.
4. 70-μm Nylon cell strainer (BD Falcon).
5. FACS buffer: PBS containing 2% FCS and 2 mM EDTA.
6. Antibodies: rat anti-mouse CD11c (clone 223H7, Biozol Diagnostica Vertrieb GmbH, Eching, Germany) and CD45R (clone RA36B2).
7. Goat anti-rat magnetic beads (Qiagen, Hilden, Germany).
8. Recombinant murine M-CSF (Tebu-Bio, Frankfurt, Germany) and recombinant human M-CSF (R&D Systems, Wiesbaden, Germany).

2.2. Surface Staining of Cells from M-CSF Cultures

1. CD16/32 mAb (clone 2.4G2).
2. Antibodies for surface staining including at least CD11c and CD45R or CD45RA (not the clones used for depletion).

2.3. Inhibitor Treatment of M-CSF Cultures

1. The cFMS receptor tyrosine kinase inhibitor (Cat. no. 344036) and Flt3 inhibitor II or III (EMD Biosciences, Darmstadt, Germany).
2. Flt-3 ligand.

3. Methods

The following procedures allow the purification of M-CSF-driven DC (MDC) from BM cells and include the procedures to discriminate any involvement of CD135 in this process.

3.1. MDC Generation

1. The femurs and tibiae of mice are removed, trimmed of as much sinew as possible and placed into RPMI containing 2% FCS. The ends of the bones are nicked with sharp scissors and whilst holding a bone with forceps, a 10-ml syringe equipped with a 24-ga needle is used to flush the BM cells into a 50-ml tube. The flushed cells are suspended well by plunging through the syringe, without needle attached, several times. The cell suspension is pelletted by centrifugation at 400 × g, 7 min, 4°C.
2. The supernatant is removed and the cell pellet is gently resuspended in red cell lysis buffer and left for 1 min at RT (*see* **Note 1**); 1 ml of lysis buffer is used for the lysis of BM cells from two mice. After 1 min the tubes are filled with RPMI/2% FCS and the cells are pelletted by centrifugation at 400 × g, 7 min, 4°C. Cells are resuspended in RPMI/2% FCS, passed through a 70-μm cell strainer to remove clumps and re-centrifuged.
3. The BM cells can then either be cultured directly (jump to Step 4) or cultured after 'depletion' (*see* **Note 2**). For depletion, BM cells are incubated for 30 min on ice with FACS buffer containing rat antibodies to CD11c and CD45R. The antibodies are used at saturating concentrations as predetermined by titration on spleen cells; 10 μl of the ab cocktail is used per 1×10^6 cells. Following the incubation period the cells are washed in a large volume of FACS buffer and then pelletted by centrifugation at 400 × g, 7 min, 4°C.
4. Cells are resuspended in FACS buffer (5×10^7 cells/ml) followed by 20 min agitated incubation with goat anti-rat magnetic beads (*see* **Note 3**). At the end of the incubation period the cell/bead suspension is diluted with FACS buffer (*see* **Note 3**), then applied to a magnet and the supernatant containing the cells not expressing CD11c and CD45R is retained.

5. Remaining cells are counted and cultured in flasks or plates at 1.5×10^6 cells/ml in complete medium containing either 20 ng/ml recmuM-CSF or rechu-M-CSF. The cultures are fed with fresh M-CSF every 3 days, without medium change and allowed to proceed for 6–8 days (*see* **Note 4**).

3.2. Surface Staining of Cells from M-CSF Cultures

1. Cells are gently harvested using complete medium, so as not to dislodge adherent macrophages, then are washed in FACS buffer. The cell pellets are incubated with 1 mg/ml purified anti-CD16/32 mAb for 20 min on ice (10 μl per 1×10^6 cells, minimum 10 μl) (*see* **Note 5**).
2. An equal volume of 2X concentrated specific antibody stain is added to the cell suspension and incubated for a further 20 min (*see* **Note 6**). Cells are washed in FACS buffer and resuspended in FACS buffer containing 1 μg/ml propidium iodide.
3. For analysis of DC populations within the cultures, cells are first gated on non-dead, non-autofluorescent cells by gating on propidium iodide-negative cells. CD11c and CD45R or CD45RA can be used for distinguishing between M-pDC and M-cDC within the cultures (*see* Fig **12.1**).

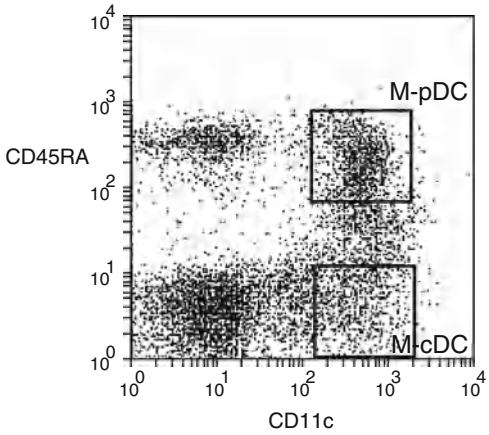

Fig. 12.1. The generation of M-pDC and M-cDC with M-CSF. Bone marrow cells cultured for 6 days with M-CSF yield non-adherent $CD11c^+CD45RA^{hi}$ M-pDC and $CD11c^+CD45RA^{neg}$ M-cDC.

3.3. M-CSF Generated DC Develop in the Absence of FL and Independently of Its Receptor

1. Total or bead-depleted BM is processed as in **Section 3.1**, Step 4, without the addition of M-CSF
2. The BM cells are separated into at least six groups and incubated with or without inhibitors (c-FMS inhibitor or Flt3 inhibitor (II or III) for 30 min at 37°C (*see* **Note 7**)).

3. M-CSF is added to the cultures as in **Section 3.1**, Step 4. To control cultures add FL (*see* **Note 8**). Allow cultures to proceed for 6 days.
4. Cells are harvested, counted and analysed for surface phenotype as in **Section 3.2**.

4. Notes

1. In our hands, longer incubation times in red cell lysis buffer lead to decreased cell yield. Often a 15-s incubation in the lysis buffer is ample to kill all RBCs.
2. The depletion procedure removes any resident pDC, cDC, $CD11c^+$ NK cells and T cells and B cells from the BM preparation. The depletion procedure removes more than 65% of the cells from BM. It should be noted that depletion with the beads only, in the absence of the rat antibodies, also depletes about 50% of the BM cells, presumably via FcR/Ig interactions. The cultures resulting from depleted BM preparations can be assumed free from contaminating resident DC and moreover this can be tested by taking a small aliquot of the cells pre- and post-depletion for stimulation with CpG2216. When supernatants of the stimulated cells are screened by ELISA for type-I interferons the depleted samples should be negative. In addition, although the depletion procedure utilises antibodies and bead reagents, culture with depleted cells requires far less expensive M-CSF.
3. The goat anti-rat magnetic beads are stored in liquid containing preservative. The beads to be used for the depletion experiment should be washed at least three times with FACS buffer, using a tube magnet to pellet the beads between each wash. The supernatant can be easily sucked away using a vacuum aspirator. After the final wash the supernatant is sucked from the beads and this is replaced by the cell suspension. Gently mix the bead/cell suspension before placing on a rocker or tube inverter for the 20-min incubation. After incubation the bead/cell suspension is diluted to about 7 ml if using 15-ml tubes and a Dynal magnet.
4. Feeding cultures 20 ng/ml M-CSF every 2 days can give slightly higher M-DC output. Care must be taken when culturing for longer than 6 days as the medium can quickly become acidic due to the high macrophage content in

the cultures. Feeding M-CSF and shorter culture times ensure a good cytokine production potential in the M-DC. Preventing this acidification without reducing M-DC output appears difficult whilst 6 days incubation is ample to see M-DC development. Also, as M-DC cultures prefer cell contact during incubation, the volume of culture per flask/well must be sufficient to allow a confluent layer of cells to settle on the flask/well floor (i.e. ≥1 ml/well of a 24-well plate).

5. The incubation with CD16/32 blocks non-specific Fc-receptor binding of the subsequently applied antibodies. This is particularly important in M-CSF cultures to ensure that other macrophage-like cells in the culture that express high levels of Fc receptor are not mistakenly sorted as dendritic cells.
6. For analysis and sorting of M-DC from the M-CSF cultures they are stained with CD45R or CD45RA and CD11c to allow the discrimination of M-pDC ($CD11c^{+}CD45R/RA^{hi}$) and M-cDC ($CD11c^{+}CD45R/RA^{neg}$).
7. BM cells should be split into groups as follows:
   a. M-CSF treatment only.
   b. M-CSF and c-FMS inhibitor.
   c. M-CSF and Flt3 inhibitor (II or III or if preferred an additional group could be added and both inhibitors tested).
   d. FL treatment only.
   e. FL and c-FMS inhibitor.
   f. FL and Flt3 inhibitor.

   In our hands the c-FMS inhibitor and Flt3 inhibitor II are optimal at a concentration of 1.25 μM. The Flt3 inhibitor III has a higher toxicity and the optimal concentration in our hands is about 0.31 μM. Although the M-CSF cultures are fed, inhibitors only need to be added during this preincubation time, not at any other time during the culture period.
8. FL is added to cultures d-f above as a positive control for the effectiveness of the Flt3 inhibitors and to show its independence of c-FMS. We have produced FL in house from the CHO-Flk2 cell line provided by Dr Nic Nicola at the Walter and Eliza Hall Institute, Melbourne, Australia. The optimal concentration of this FL in our culture systems is 35–50 ng/ml. FL purchased from commercial sources or self-produced from this cell line must be titrated for optimal concentration. The chapter by Naik et al. in this book outlines the procedure for FL DC cultures.

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Chapter 13

Isolation of Common Dendritic Cell Progenitors (CDP) from Mouse Bone Marrow

Nobuyuki Onai, Markus G. Manz, and Michael A. Schmid

Abstract

In the steady-state lymphoid organ, dendritic cells (DCs) are classified into two major subsets, plasmacytoid DC (pDC) and conventional DC (cDC). A standing question was whether a common progenitor for plasmacytoid and conventional dendritic cells exists during the sequential differentiation from hematopoietic stem cells to dendritic cells. We have recently identified such a common clonogenic plasmacytoid and dendritic cell progenitor (CDP) from mouse bone marrow using antibodies for c-kit, Flt3, and M-CSFR. CDPs generated almost exclusively pDC and cDC in vitro and upon transfer in irradiated and steady-state mice in vivo. Single-cell analysis revealed the existence of clonal progenitors giving rise to both pDC and cDC within the CDP population. Thus, these results prove the existence of a common developmental pathway for at least some pDCs and cDCs in lymphoid organs in vivo.

Key words: Flt3, M-CSFR, hematopoietic progenitor, dendritic cell (DC), plasmacytoid DC (pDC), conventional DC (cDC).

1. Introduction

Dendritic cells (DCs) possess strong antigen-presenting ability and are spread throughout the body (1). In the peripheral tissue, DCs capture the antigen, migrate to the draining lymph nodes, and present it to T cells to initiate adaptive immunity (2). DCs also act as an important regulator for tolerance in the steady state (3). In the secondary lymphoid tissue, resident DCs comprise a heterogeneous group and can be classified into at least two groups, plasmacytoid DC (pDC) and conventional DC (cDC). cDCs are further subdivided into three groups such as

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$CD11c^+CD8\alpha^-CD4^-CD11b^+$, $CD11c^+CD8\alpha^-CD4^+CD11b^+$, and $CD11c^+CD8\alpha^+CD4^-CD11b^-$ dendritic cells. cDCs efficiently activate naïve T cells by presentation of antigen on MHC class II. $CD8\alpha^+$ DCs, however, have a high potential to uptake and cross-present exogenous antigen via MHC class I. pDCs produce high amounts of type-I interferon upon stimulation by viruses and CpG and are consequently as well called type-I interferon-producing cells (IPCs). Thus, pDCs are crucial regulator of antiviral immunity (4).

All DC subsets continuously differentiate from hematopoietic stem cells via intermediate committed progenitors (5, 6). However, it was not clear so far, whether DC subsets derive from separate progenitors or one common progenitor. Based on shared cytokine dependencies, the analysis of gene-deficient mice and the biology of plasmacytoid and conventional dendritic cells, it has been suggested that these cells might proceed through DC-restricted common developmental intermediates.

Indeed, Flt3–L ligand is the only cytokine to induce differentiation of both pDC and cDC from mouse bone marrow cells (7). Flt3L-deficient mice show only about one-tenth the numbers of lymphoid tissue pDCs and cDCs compared to wild-type mice (8). Furthermore, either STAT3- or PU.1-deficient mice, which are down-stream transcription factors for Flt3-signaling, showed severe reduction of both pDC and cDC numbers (6, 9, 10). These results suggest that a common DC progenitor should exist and it might express the cytokine receptor Flt3.

Recently, we identified and characterized lin^- c-$kit^{int}Flt3^+$M-$CSFR^+$ cells in mouse bone marrow that on a clonal level in vitro, and as a population in vitro and in vivo, efficiently generate bone marrow, spleen, and lymph node pDCs and cDCs, but no other lineage read out (11, 12). Thus, we call these cells common dendritic cell progenitors (CDP) (11). These cells thus define a common Flt3 responsive pathway for steady-state DC maintenance.

2. Materials

2.1. Preparation of Bone Marrow Cell Suspension

1. C57BL/6 mice, 8–12 weeks old.
2. 70% Ethanol.
3. Phosphate-buffered saline (PBS).
4. 10-ml Syringes with 21-ga needles.
5. Mortar and pestle.
6. Nylon meshes (70 μm pore size).
7. Histopaque-1077 (Sigma–Aldrich).

2.2. Pre-enrichment of the Lineage Negative Fraction of Bone Marrow Cells

1. PE-Cy5-conjugated antibodies against lineage antigens (CD3ϵ, 145-2C11; CD4, GK1.5; CD8α, 53-6.7; B220, RA3-6B2; CD19, MB19-1; CD11b, M1/70; Gr-1, RB6-8C5; TER119, TER119; NK1.1, PK136).
2. Staining buffer: 2% fetal calf serum (FCS), 2 mM EDTA in PBS.
3. Anti-Cy5/Anti-Alexa Flour 647 microbeads (Miltenyi Biotec).
4. LS MACS columns and MidiMACS Separator or AutoMACS (Miltenyi Biotec).

2.3. Antibody Staining and Cell Sorting

1. Staining buffer: 1% FCS, 2 mM EDTA in PBS stored at 4°C.
2. Primary antibodies: FITC-conjugated anti-CD127 (A7R34), PE-conjugated anti-CD135 (A2F10.1), APC-conjugated anti-c-kit (ACK2), and biotin-conjugated anti-CD115 (AFS-98) (eBioscience).
3. Second antibody: streptavidin-APC-Cy7 (eBioscience).
4. Propidium iodide solution (1,000X) (Sigma) is dissolved at 10 mg/ml in PBS and stored at 4°C in the dark (light sensitive).
5. Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin.
6. Cell sorter: BD FACSAria (Becton Dickinson Immunocytometry Systems) or MoFlo (Beckman Coulter).

2.4. In Vitro Myeloid, Erythroid, and Lymphoid Differentiation Culture

1. Methylcellulose medium: MethoCult M3231 (StemCell Technologies).
2. Recombinant cytokines: mSCF (R&D), mIL-3 (R&D), mIL-11(R&D), human Flt3-ligand (R&D), mGM-CSF (R&D), mTpo (R&D), hEpo (Roche), mM-CSF (R&D), mIL-7 (R&D).
3. 5-ml Syringes with 18-ga needles.

2.5. In Vitro DC Differentiation Culture

1. IMDM supplemented with 10% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin.
2. Recombinant human Flt3-ligand (R&D).
3. Staining buffer: 1% FCS, 2 mM EDTA in PBS stored at 4°C.
4. Staining antibodies: FITC-conjugated anti-I-A/I-E (M5/114.15.2) (eBioscience), PE-conjugated anti-PDCA-1 (Miltenyi Biotec), PE-conjugated anti-CD45RA (14.8) (BD Bioscience), APC-conjugated anti-CD11c (N418) (eBioscience).

5. Propidium iodide solution (1,000X) (Sigma) is dissolved at 10 mg/ml in PBS and stored at 4°C in the dark (light sensitive).

3. Method

3.1. Preparation of Bone Marrow Cell Suspension

1. Wet the whole body of the mouse with 70% ethanol for sterilization.
2. Remove femurs, tibias, and the backbone from five mice and place them into ice-cold PBS. Remove the muscles from the bones using scissors and forceps and transfer them into a new Petri dish containing PBS.
3. Add 10 ml of ice-cold PBS into a mortar and crash/grind the bones using a pestle, or add 10 ml of ice-cold PBS into dish, and flushing out marrow using syringe with needle to obtain a bone marrow cell suspension from bone shaft. Pass the cell suspension through a nylon mesh to remove debris.
4. Add 10 ml of ice-cold PBS into mortar and transfer cleaned backbone. Crash and grind the backbone using pestle to obtain spinal marrow. Remove and discard the white funiculus that as well will be extracted during the crushing. Pass the cell suspension through the nylon mesh to remove debris. Mix bone marrow and spinal marrow cell suspensions and centrifuge for 5 min at room temperature.
5. During centrifugation, add 5 ml of room temperature Histopaque-1077 into a 15-ml tube.
6. Remove supernatant and resuspend cells in 5 ml of PBS at room temperature. Carefully overlay 5 ml of cell suspension onto Histopaque-1077.
7. Centrifuge for 30 min at 18–20°C, 900 × *g* with acceleration and brakes set to "zero."
8. After centrifugation, carefully aspirate the uppermost layer. Subsequently, transfer the intermediate mononuclear cell layer into a new tube. Wash the cells with an excess of ice-cold PBS (5–10 volume) and centrifuge for 5 min at 4°C.
9. Cells are resuspended in PBS and counted.

3.2. Pre-enrich the Lineage Negative Cell Fraction

1. Centrifuge cell suspension at 400 × *g* for 5 min at 4°C and aspirate supernatant.
2. Add PE-Cy5-conjugated antibody cocktail against lineage antigens (CD3, CD4, CD8, B220, CD19, CD11b, Gr-1, TER119, and NK1.1) to the cells, mix well, and incubate for 30 min at 4°C in the dark.

3. Wash the cells with ice-cold staining buffer in excess (5–10 × volume), centrifuge for 5 min at 4°C, and aspirate the supernatant.
4. Resuspend the cells in staining buffer, add appropriate volume of anti-Cy5/Anti-Alexa Flour 647 microbeads according to manufacturer's instructions, and incubate for 15 min at 4°C in the dark.
5. Wash the cells with ice-cold staining buffer in excess, centrifuge for 5 min at 4°C, and aspirate supernatant.
6. After resuspending the cells in staining buffer, proceed with magnetic separation to obtain the lineage-negative cell fraction using MidiMACS Separator or AutoMACS according to manufacturer's instructions.

3.3. Antibody Staining and Cell Sorting

1. Centrifuge lineage negative cell suspension at 400 × *g* for 5 min at 4°C and aspirate supernatant.
2. Add primary antibody mix to the cell suspension, mix well, and incubate for 30 min at 4°C in the dark.
3. Wash the cells with ice-cold staining buffer in excess, centrifuge for 5 min, and aspirate supernatant.
4. Add secondary antibody to the cells, mix well, and incubate for 30 min at 4°C in the dark.
5. Wash the cells with ice-cold staining buffer in excess, centrifuge for 5 min, and aspirate supernatant.
6. Cells are resuspended in staining buffer containing propidium iodide (final concentration 10 μg/ml) to stain and exclude dead cells.
7. Sort the lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7Rα$^-$ cell fraction by using a cell sorter, as shown in **Fig.13.1 A.** Make sure to include as well M-CSFR low positive cells.
8. Target cells are sorted into a tube containing 1 ml of 10% FCS–IMDM medium.
9. Freshly isolated lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7Rα$^-$ cells do not express DC-related cell surface marker such as CD11c, MHC class II, PDCA-1, and CD40, as shown in **Fig. 13.1 B.**

3.4. In Vitro Myeloid, Erythroid, and Lymphoid Differentiation Assay

1. Add sorted lin$^-$c-kit^{high} cells, lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7R$^-$ cells (R1), or lin$^-$c-kit^{int}Flt3$^+$IL-7R$^+$ (R2) cells to 3 ml of methylcellulose medium, MethoCult M3231, and add cytokine cocktail for myeloid erythroid colony-forming assay (10 ng/ml mSCF (R&D), 10 ng/ml mIL-3 (R&D), 10 ng/ml mIL-11(R&D), 10 ng/ml human Flt3-ligand (R&D), 10 ng/ml mGM-CSF (R&D), 10 ng/ml mTpo

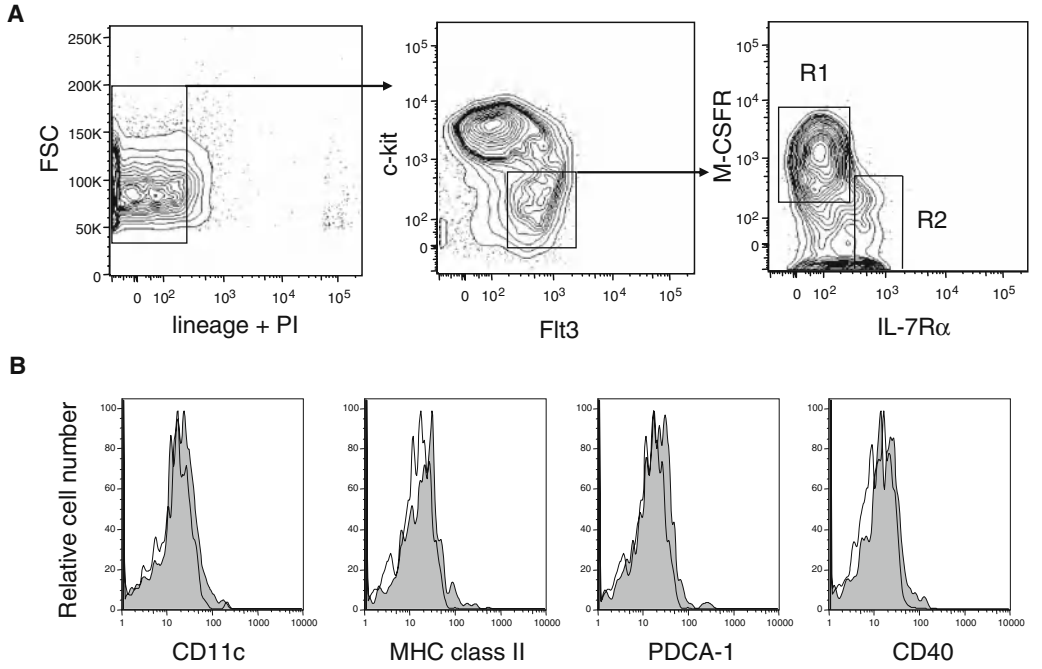

Fig. 13.1. Identification of lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$ progenitor cells in mouse bone marrow. (**A**) lin$^-$ cells were divided by c-kit and Flt3 expression (*middle panel*). lin$^-$c-kit^{int}Flt3$^+$ cells contained Flt3$^+$M-CSFR$^+$ (R1) and Flt3$^+$IL-7Rα^+ (R2) cells (*right panel*). (**B**) Additional surface marker expression on lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$ cells (closed histograms) and respective isotype controls (open histograms). Freshly isolated lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$ cells from bone marrow do not express the DC-related markers CD11c, MHC class II, PDCA-1, and CD40.

(R&D), 1 U/ml hEpo (Roche)), for macrophage colony forming assay; 10 ng/ml M-CSF, for pre-B cell colony-forming assay; 10 ng/ml SCF, 10 ng/ml mIL-7, 10 ng/ml human Flt3-ligand.

2. Mix vigorously because methylcellulose medium has a high viscosity.
3. Leave the mixture to stand for 10 min at room temperature until air bubbles have disappeared.
4. Take up the methylcellulose medium by a 5-ml syringe with a 18-ga needle, and pour them into a cell culture dish.
5. Determine and enumerate colonies under an inverted microscope consecutively from day 3 to day 8.
6. To confirm colony-types, pick colonies using a fine-drawn Pasteur pipette, spin them on slides, Giemsa stain them and evaluate by light microscopy.
7. Lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7Rα^- cells completely lacked CFU-GEMM, CFU-G, CFU-MegE, CHU-Meg, BFU-E, and CFU-B as shown in **Fig. 13.2A** and **C.** Less than 4% of

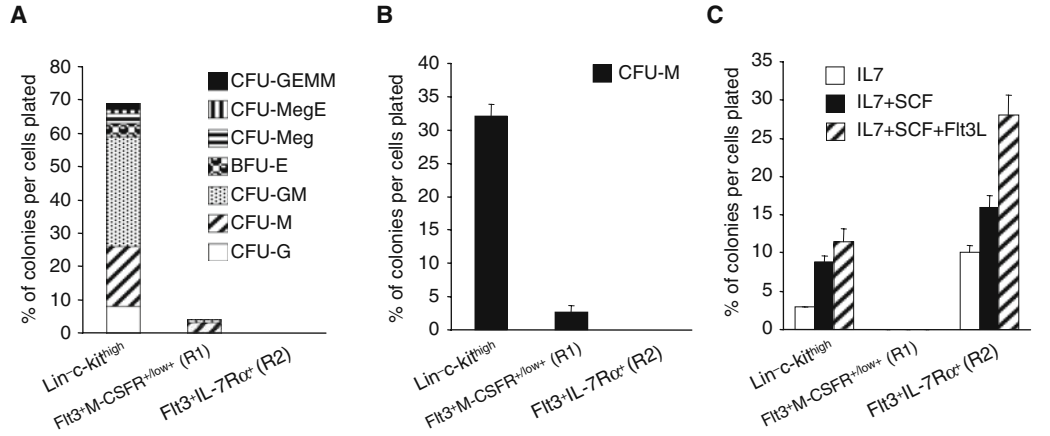

Fig. 13.2. In vitro colony-forming activity of lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$ progenitor cells. (**A**) Myeloid, (**B**) macrophage, (**C**) and pre-B cell colony-forming unit activity of lin$^-$c-kit^{high} cells, lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$ cells (R1), and lin$^-$c-kit^{int}Flt3$^+$IL-7Rα^+ (R2) cells (as indicated in **Fig.13.1A**); 200 cells were plated each. Colony-forming unit (CFU) activity. GEMM, granulocyte–erythrocyte–macrophage–megakaryocyte; MegE, megakaryocyte–erythrocyte; Meg, megakaryocyte; BFU-E, burst-forming unit, erythrocyte; GM, granulocyte-macrophage; M, macrophage; G, granulocyte; B, B cell.

lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7Rα^- cells gave rise to CFU-GM and CFU-M as shown in **Fig. 13.2 A** and **B**.

3.5. In Vitro Culture and FCM Analysis

1. Culture the sorted lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7Rα^- cells in IMDM supplemented with 10% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 ng/ml Flt3-ligand (R&D).
2. Replace half of the medium every 3 days with medium containing twofold cytokines added.
3. After 8 days of culture, harvest offspring cells, transfer into a tube, centrifuge at 400 × *g* for 5 min at 4°C, and aspirate supernatant.
4. Add appropriate amount of monoclonal antibody mixture to the cell pellet and mix well.
5. Incubate for 30 min at 4°C in the dark.
6. Add 500 μl of buffer; centrifuge at 250 × *g* for 5 min.
7. Resuspend samples in 1 ml of buffer containing propidium iodide (final concentration 10 μg/ml) to stain and exclude dead cells and keep at 4°C.
8. Cells are analyzed using a FACSCalibur and a FACSCanto (Becton Dickinson Immunocytometry Systems) according to manufacturer's instructions.
9. The lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7Rα^- cells gave rise to both CD11c$^+$B220$^+$CD45RA$^+$PDCA-1$^+$ pDC and CD11c$^+$B220$^-$CD45RA$^-$PDCA-1$^-$ conventional DCs in this culture condition, as shown in **Fig.13.3.**

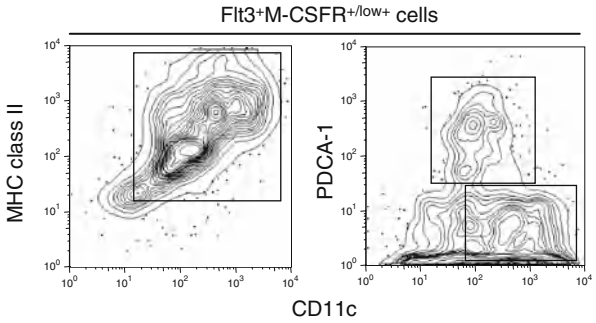

Fig. 13.3. lin^-c-$kit^{int}Flt3^+M\text{-}CSFR^+$ progenitors generate pDCs and cDCs in vitro. Lin^-c-$kit^{int}Flt3^+M\text{-}CSFR^+$ cells were cultured with recombinant human Flt3-ligand supplemented media for 8 days.

4. Notes

It is important to perform all procedures under sterilize conditions.

For the sorting, it is important to set up compensation tubes of stains in single colors for multi-color analysis.

Acknowledgments

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Chapter 14

The Isolation of Mouse Dendritic Cells from Lymphoid Tissues and the Identification of Dendritic Cell Subtypes by Multiparameter Flow Cytometry

David Vremec

Abstract

Dendritic cells (DCs) are powerful initiators of the adaptive immune system and their manipulation is seen as a viable approach in the treatment of many human ailments. Our isolation method purifies DCs from mouse lymphoid organs by efficiently removing them from the tissue using collagenase, selecting the light density fraction of cells and then negatively selecting for DCs using a combination of monoclonal antibodies directed against non-DC lineage cells together with anti-rat immunoglobulin-coated magnetic beads. Remaining contaminating cells can be depleted using a combination of specific biotinylated antibodies and anti-biotin-coated magnetic beads, in the case of natural killer (NK) cells, or by FACS gating, during sorting or analysis, in the case of autofluorescent macrophages. The heterogenous DCs can then be separated into various functionally different populations via immunofluorescent labelling and cell sorting. The study of mouse DC populations may enable us to align them to their human counterparts and subsequently to DC populations produced in vitro.

Key words: Dendritic cell, DC isolation, DC subpopulations, migratory DCs, tissue resident DCs, conventional DCs, plasmacytoid DCs, NK cell depletion.

1. Introduction

Dendritic cells (DCs) are an essential component of the immune system. Mice depleted of DCs have a defective immune response to bacteria (1), parasites (2) and viruses (3). Immature DCs are thought to be crucial in the maintenance of self-tolerance by presenting self-antigen and inducing unresponsiveness, or deletion, of developing T cells in the thymus (4) and mature T cells in the

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periphery (5) and by inducing or expanding regulatory T cells (6). This dampening of self-reactivity is balanced by the role of DCs as immunogens. When exposed to microbial antigens or other perceived "danger signals" or "alarmins", DCs can undergo a maturation process involving a cessation of antigen uptake, the upregulation of MHC class II and numerous families of membrane-associated costimulatory molecules and the production of many cytokines and chemokines (7). They are then capable of presenting antigen to naïve T cells and generating an effector T-cell response (8).

It is clear that the maturation status of DCs as well as their surface phenotype has a bearing on their function. Any study of DC phenotype or function must therefore ensure that the DCs isolated are equivalent to those found in vivo in the steady state and have not been induced to undergo this maturation process inadvertently by the methods used in their isolation. Early DC isolation procedures separated "transiently" adherent DCs from adherent macrophages and other non-adherent cells after overnight culture (9). Even short culture periods have subsequently been shown to induce DC maturation (unpublished observation). Our procedure has been designed with this limitation in mind. Our short digestion with collagenase at room temperature does not appear to activate DCs, based on the observed surface expression of MHC class II and costimulatory molecules which remain at levels seen on the much lower yield of DCs that can be isolated without collagenase using mechanical disruption at 4°C (unpublished observation).

When designing our DC isolation procedure it was important to consider the vast heterogeneity of the DC network and to include all DC subtypes in our analysis. Although all DCs share their classical role in antigen processing and presentation and activation of T cells, they differ in function due to differences in their location, their array of cell surface molecules, including pathogen receptors such as Toll-like receptors and C-type lectins, and the cytokines they secrete (10, 11). Our DC isolation procedure enables us to efficiently (and without detectable bias) extract all DC subpopulations based on differences in their expression of selected cell surface molecules. DCs are extracted from tissue by digestion with collagenase at room temperature, followed by a short treatment with EDTA to dissociate multicellular complexes between DCs and T cells. DCs are effectively enriched, while simultaneously removing dead cells and most erythrocytes, by selecting the 3–5% lightest density cells. It is possible to isolate DCs directly after tissue digestion or after density separation using either flow cytometric cell sorting or positive selection using immunomagnetic beads. However, due to the rarity of DCs (1% of total splenocytes, but even less in thymus and lymph node and only 10–15% of light density cells) it is a difficult and

expensive task, so further enrichment is advised via negative selection. Cells of non-DC lineages are coated with a cocktail of monoclonal antibodies and depleted with anti-immunoglobulin-coated immunomagnetic beads. Care must be taken when selecting monoclonal antibodies to include in the cocktail in order to avoid losing DC subtypes that bear molecules found more commonly on T cells, B cells and macrophages. Contaminating macrophages can be a problem, but can be removed during fluorescence-activated cell sorting or analysis by gating out autofluorescent cells (12). NK cells, including the recently described "interferon-producing killer dendritic cell" (IKDC), can also be a major contaminant of this purified DC fraction and make it difficult to cleanly separate DCs, as they express intermediate levels of various markers useful for DC identification (13, 14). Unfortunately we have not been able to locate an anti-NK cell-specific monoclonal antibody suitable for inclusion in our monoclonal antibody depletion cocktail. NK cells may be removed with a further round of negative selection, using biotinylated monoclonal antibodies against CD49b and/or CD161c and anti-biotin-coated magnetic beads or removed later by fluorescence-activated cell sorting using the same monoclonal antibodies (*see* **Fig. 14.1**).

Specialized DC subtypes can be grouped into migratory DCs, lymphoid tissue resident DCs and inflammatory DCs. The inflammatory DC are not present in the steady state but develop during infection or inflammation, and will not be included here (15). A selection of surface markers can be used to identify and separate these different DC subtypes after multicolour immunofluorescent labelling and flow cytometry.

Plasmacytoid pre-DCs (pDCs), also referred to as interferon-producing cells, are migratory DCs formed in the bone marrow and found in all lymphoid organs after migration via the blood stream. Upon activation by virus, pDCs acquire characteristic DC morphology, upregulate MHC class II and rapidly produce high levels of type I interferons (16). All pDCs have the phenotype $CD11c^{int}$ $120G8^{+}$ (17) and are enriched if the appropriate monoclonal antibody depletion cocktail is used in the procedure. High levels of CD45R and/or CD45RA can substitute for 120G8, but they have certain limitations. Neither is specific for pDCs and their expression levels decrease upon pDC activation. In lymphoid tissue pDCs occur in four phenotypes: $CD4^{-}CD8^{-}$, $CD4^{-}CD8^{+}$, $CD4^{+}CD8^{-}$ and $CD4^{+}CD8^{+}$. It is not clear what role CD8 has but CD4 is a maturation marker as $CD4^{-}$ pDCs become $CD4^{+}$ (16). Ly6C and Ly49Q are also useful as differentiation markers dividing pDCs into an immature $Ly6C^{-}Ly49Q^{-}$ ($CD4^{-}CD8^{-}$) population and a more mature $Ly6C^{+}Ly49Q^{+}$ population (14, 18, 19).

Conventional dendritic cells (cDCs) exhibit typical DC morphology and function in the steady state, with CD11c the marker

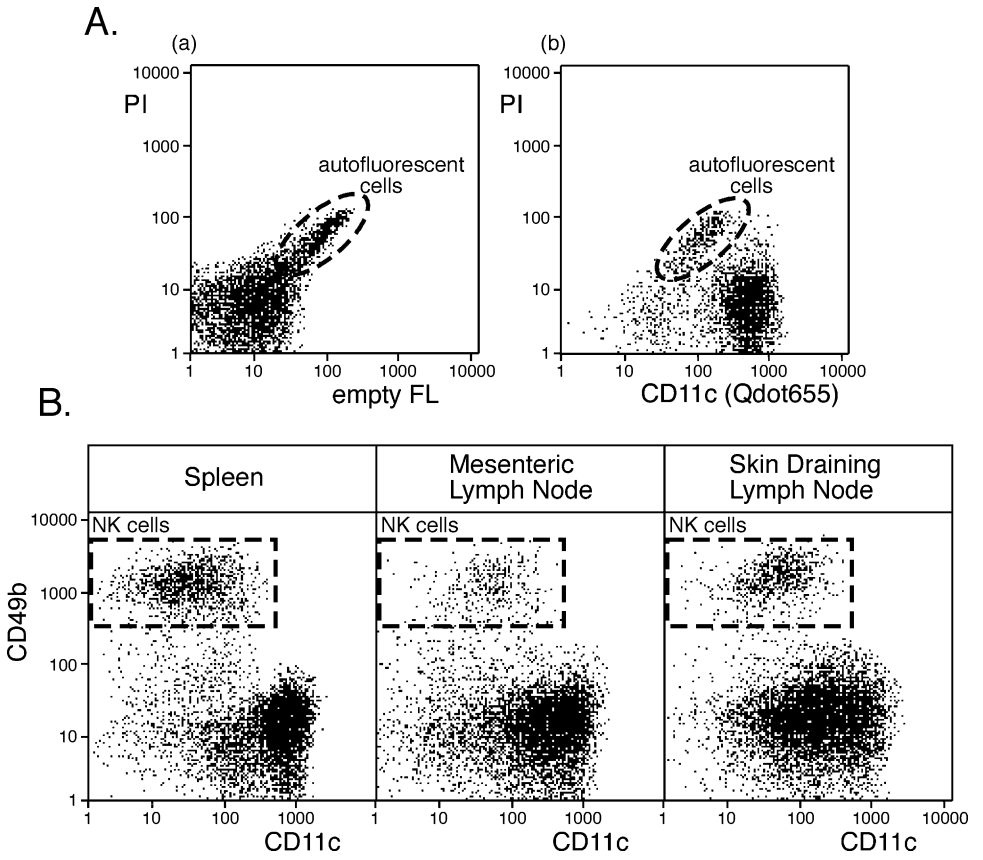

Fig. 14.1. Contaminants in mouse dendritic cell preparations. (**A**) Autofluorescent macrophages are found as contaminants in DCs isolated from all mouse lymphoid organs. They may be gated out during flow cytometric sorting or analysis using the PI channel in combination with either (**a**) an unused fluorescence channel or with (**b**) a fluorescence channel in which the expected signal will be much higher than the autofluorescence, allowing only cells displaying high levels of fluorescence to be retained and cells displaying low levels of autofluorescence in both channels to be gated out. (**B**) NK cells are a significant contaminant in spleen and lymph node DC preparations, contributing up to 15% of the recovered cells. They are gated out during flow cytometric sorting or analysis using the pan NK cell marker CD49b or removed prior to this stage using a combination of NK cell-specific monoclonal antibodies conjugated to biotin and anti-biotin-coated immunomagnetic beads.

of choice to identify and separate them from contaminating cells. Other cells such as NK cells also express CD11c but at intermediate levels and must be removed by a second round of depletion or by fluorescence-activated cell sorting using monoclonal antibodies against NK-specific molecules. CD11c can be used in conjunction with 120G8 to separate cDCs from pDCs as cDCs have higher levels of CD11c and do not express 120G8 (17). Combining CD11c with MHC class II and only selecting $CD11c^{hi}$ MHC class II^{+} cells will ensure a more stringent separation (9). In the lymphoid organs, cDCs can be comprised of

both tissue resident DCs that have developed in that organ from a blood-borne precursor and migratory DCs that have arrived from the periphery (20).

Traditionally, cDCs in the spleen have been divided using CD4 and CD8α into three distinct subtypes: $CD4^{-}CD8\alpha^{-}$, $CD4^{+}CD8\alpha^{-}$ and $CD4^{-}CD8\alpha^{+}$. The $CD8\alpha^{+}$ cDCs are found in the T-cell areas of the spleen and are the major producers of the pro-inflammatory cytokine IL-12p70, causing polarization of T cells to a Th1 response (21). They are also the most efficient at cross-presenting antigens (22). The $CD4^{+}$ population is found in non-T-cell areas of the spleen and is the population that produces the highest levels of inflammatory chemokines (23) and together with the $CD4^{-}CD8\alpha^{-}$ subtype are the most potent presenters of MHC-II–antigen complexes to $CD4^{+}$ T cells (22). Until recently the $CD4^{-}CD8\alpha^{-}$ subtype was also thought to be the major producer of interferon gamma, but this has now been shown to be due to a minor contaminating population of $CD8\alpha^{-}$ precursors of the $CD8\alpha^{+}$ cDCs that are yet to upregulate CD8α (14). Unfortunately, CD4 and CD8α are not adequate for aligning the cDC subtypes with DCs produced from bone marrow precursors cultured with Flt3L or with those isolated from human lymphoid tissue. Those produced in culture do not express CD4 or CD8 and human DCs express CD4 but not CD8. A satisfactory alignment of cDCs from different sources is made possible by introducing new markers, which separate them into $CD8\alpha^{+}CD24^{hi}CD205^{+}CD11b^{lo}CD172a^{lo}$ and $CD8\alpha^{-}CD24^{lo}CD205^{-}CD11b^{+}CD172a^{hi}$ subtypes (24) (*see* **Fig. 14.2**). Further division of these subtypes is possible; the $CD8^{+}CD172a^{lo}$ DCs can be $CD103^{+}$ or $CD103^{-}$ (unpublished observation) and the $CD8^{-}$ $CD172a^{hi}$ DCs can be Dectin-1 $(Clec7a)^{+}$ or Dectin-1^{-} (25); however, the significance of these differences within each subtype is yet to be determined (*see* **Fig. 14.3**). The precursor of the $CD8\alpha^{+}$ cDCs can also be readily identified as its phenotype is $CD8\alpha^{-}CD4^{-}CD24^{hi}CD11b^{lo}CD172a^{lo}$ (14).

It is also difficult to separate the two cDC populations found in thymus solely on the basis of CD8α, as both stain positively for CD8αβ and CD4 due to pickup of these molecules from T cells (12). However, if we apply a similar strategy to that used in spleen, it is possible to identify a $CD8\alpha^{+}CD24^{hi}CD11b^{lo}$ $CD172a^{lo}$ subtype and a $CD8\alpha^{-}CD24^{lo}CD11b^{+}CD172a^{hi}$ subtype (24) (*see* **Fig. 14.4**). The $CD172a^{hi}$ population can be divided further using CD103, but again the significance of this is unknown.

The cDC subpopulations found in the lymph node are far more complex to resolve and six have been identified. They include the three populations found in spleen as well as

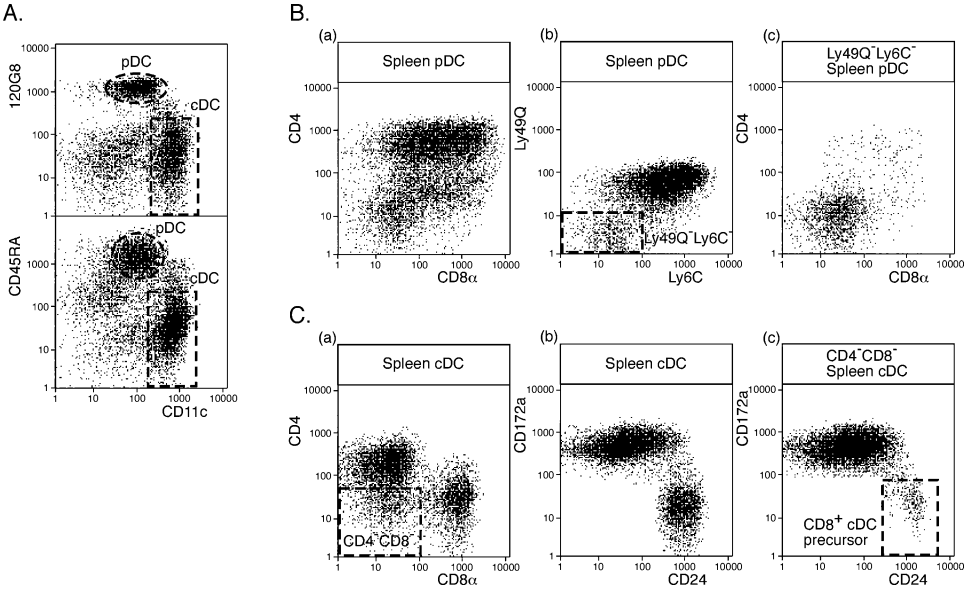

Fig. 14.2. Separation of mouse spleen dendritic cells into discrete subtypes. (**A**) Conventional DCs and plasmacytoid pre-DCs are distinguished using a combination of CD11c and 120G8 or CD11c and CD45RA. cDCs are $CD11c^{+}120G8^{-}/CD45RA^{-}$ and pDCs are $CD11c^{int}120G8^{+}/CD45RA^{+}$. (**B**) pDCs are further segregated using (**a**) CD4 and CD8 or (**b**) Ly49Q and Ly6C. CD4, Ly49Q and Ly6C are upregulated during pDC development. (**c**) $Ly49Q^{-}Ly6C^{-}$ pDC precursors are largely $CD4^{-}CD8^{-}$. **(C)** (**a**) Traditionally spleen cDC subtypes are divided using a combination of CD4 and CD8 into three populations: $CD4^{+}CD8^{-}$, $CD4^{-}CD8^{-}$ and $CD4^{-}CD8^{+}$. (**b**) Alignment of these subtypes with DC produced in culture (and not expressing CD4 and CD8) is achieved using other markers including CD172a and CD24 to distinguish $CD172a^{hi}CD24^{lo}CD11b^{hi}CD205^{-}$ and $CD172a^{lo}CD24^{hi}CD11b^{lo}CD205^{+}$ populations that correspond to the $CD8^{-}$ and $CD8^{+}$ populations, respectively. (**c**) A precursor of the $CD8^{+}$ cDC, which does not express CD8α, but is capable of IL-12p70 production and cross-presentation of antigens is found in the $CD4^{-}CD8^{-}$ fraction of spleen. It is distinguished by its $CD172a^{lo}CD24^{hi}$ phenotype.

three migratory DC populations originating from the skin, mucosa and interstitial tissue (26, 27). Mesenteric lymph nodes drain the gut and contain interstitial DCs, which are $CD4^{-}CD8^{-/lo}CD205^{int}CD103^{+}CD11b^{-/lo}CD24^{hi}CD172a^{-}$langerin^{+}. Skin-draining lymph nodes contain specialized interstitial DCs, called dermal DCs, that have migrated from the dermis along with a $CD4^{-}CD8^{lo}CD205^{hi}CD103^{-}CD11b^{+}CD24^{int}CD172a^{+}$ langerin^{+} population that corresponds to the mature form of Langerhans cells that have migrated from the epidermis (28, 29). A third migratory, airway-derived, cDC population has been described in mediastinal lymph nodes and has a $CD8^{-}CD205^{+}CD11b^{-}CD24^{hi}F4/80^{+}$ surface phenotype. This subtype is also present in renal and hepatic lymph nodes but is absent from both mesenteric- and skin-draining lymph nodes (27). The skin-derived DCs within the lymph node are the only cDCs with an activated phenotype in the steady state, expressing higher levels of MHC class II, FcγR and costimulatory molecules

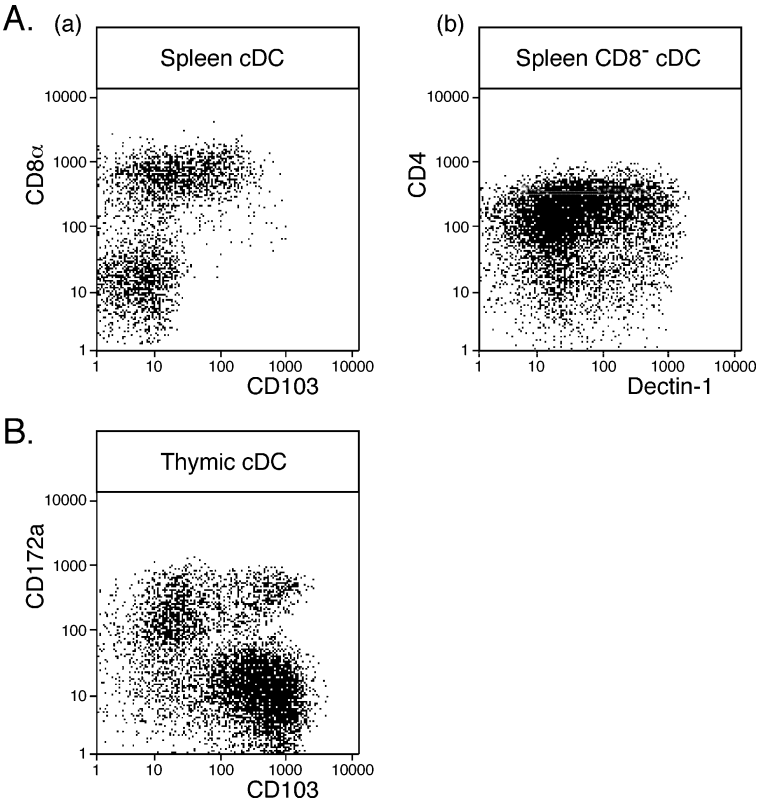

Fig. 14.3. Further subdivision of mouse cDC subtypes. (**A**) (**a**) Some $CD8^+$ spleen cDCs express CD103, while all spleen $CD8^-$ cDCs are $CD103^-$. (**b**) Some spleen $CD8^-$ cDCs express Dectin-1. (**B**) Thymic $CD172a^+$ cDC, which corresponds to the $CD8^-$ population, can be separated into $CD103^-$ and $CD103^+$ populations. The significance of the expression of these molecules on only a proportion of these subtypes is still to be determined.

such as CD40, CD80 and CD86, but slightly lower levels of CD11c, than other cDCs (28) (*see* **Figs. 14.5–14.7**).

Complex phenotyping of the DC subtypes isolated from mouse lymphoid tissue is essential if we are to align these subtypes to their counterparts isolated from human tissue or to those produced in culture. Differential expression of molecules on one DC subtype but not others may also reflect functional specialization and these molecules may be used to selectively deliver antigen to one subtype only, thereby manipulating the immune responses obtained (25, 30–33).

The procedure described here to isolate and enrich mouse DCs can be readily modified for isolation of human DCs if appropriate adjustments are made to provide human osmolarity media and to cater for the different buoyant density of human DCs (34). The final division into DC subtypes, however, awaits development of appropriate markers for the human DC system.

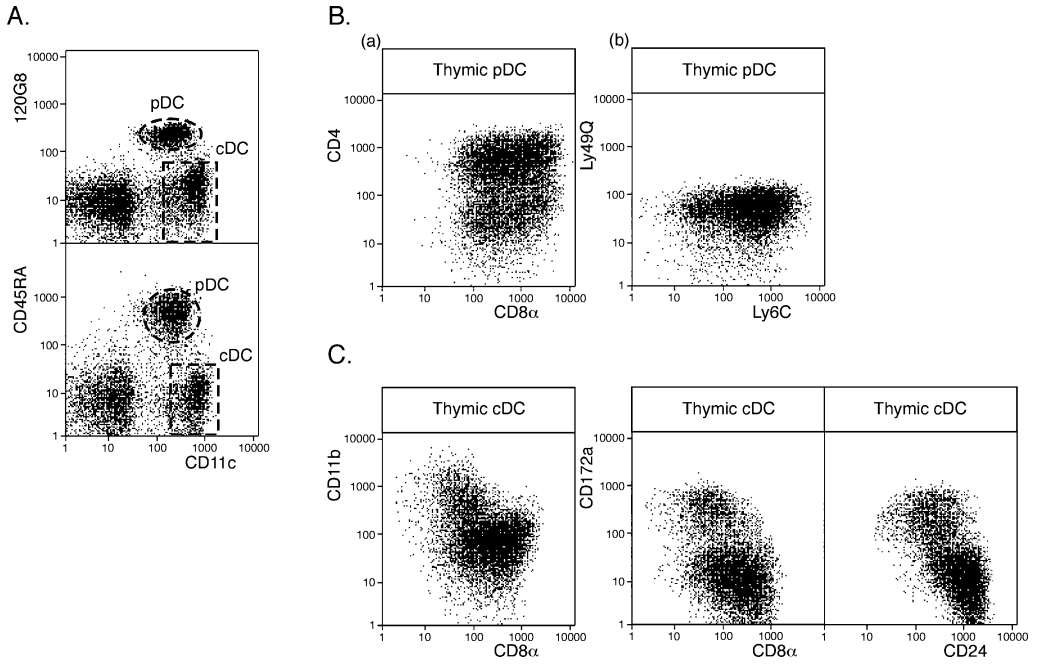

Fig. 14.4. Separation of mouse thymic dendritic cells into discrete subtypes. (**A**) CD11c^{int}120G8^{+}/CD45RA^{+} plasmacytoid pre-DCs are separated from CD11c^{+}120G8^{-}/CD45RA^{-} conventional DCs. (**B**) pDCs found in thymus are separated into populations similar to those found in spleen using either **(a)** CD4 and CD8 or **(b)** Ly49Q and Ly6C. The higher level of expression of CD8 on thymic pDC is presumably due to pick up from T cells. This artefact has previously been demonstrated to occur on thymic cDCs. (**B**) Two distinct cDC subtypes can be identified in the thymus: a CD11b^{hi}CD172a^{hi}CD24^{lo} population and a CD11b^{lo}CD172^{lo}CD24^{hi} population that correspond to the splenic CD8^{-} and CD8^{+} populations, respectively.

2. Materials

2.1. Organ Removal

1. FCS: foetal calf serum. Aliquot and store at −20°C.
2. RPMI–FCS: Modify RPMI-1640 to mouse osmolarity (308 mOsm/kg) and include additional pH 7.2 HEPES buffering to reduce dependence on CO_2 concentration. After adjusting to ~pH 7 with CO_2, filter sterilize and store at 4°C. Add FCS to a final concentration of 2% prior to use.

2.2. Digestion and Release of DCs

1. Enzyme digestion mix: Dissolve collagenase (Type III, Worthington Biochemicals, Freehold, NJ, USA) at 7 mg/ml and DNase I (Boehringer Mannheim, Mannheim, Germany) at 140 μg/ml in RPMI–FCS. Ensure the collagenase used is free of trypsin and other trypsin-like proteases (*see* **Note 1**). Aliquot into 1-ml amounts and store as a 7X stock solution frozen at −20°C. Dilute each 1-ml aliquot with 6 ml RPMI–FCS immediately prior to use. This solution must be filtered if sterility is required.

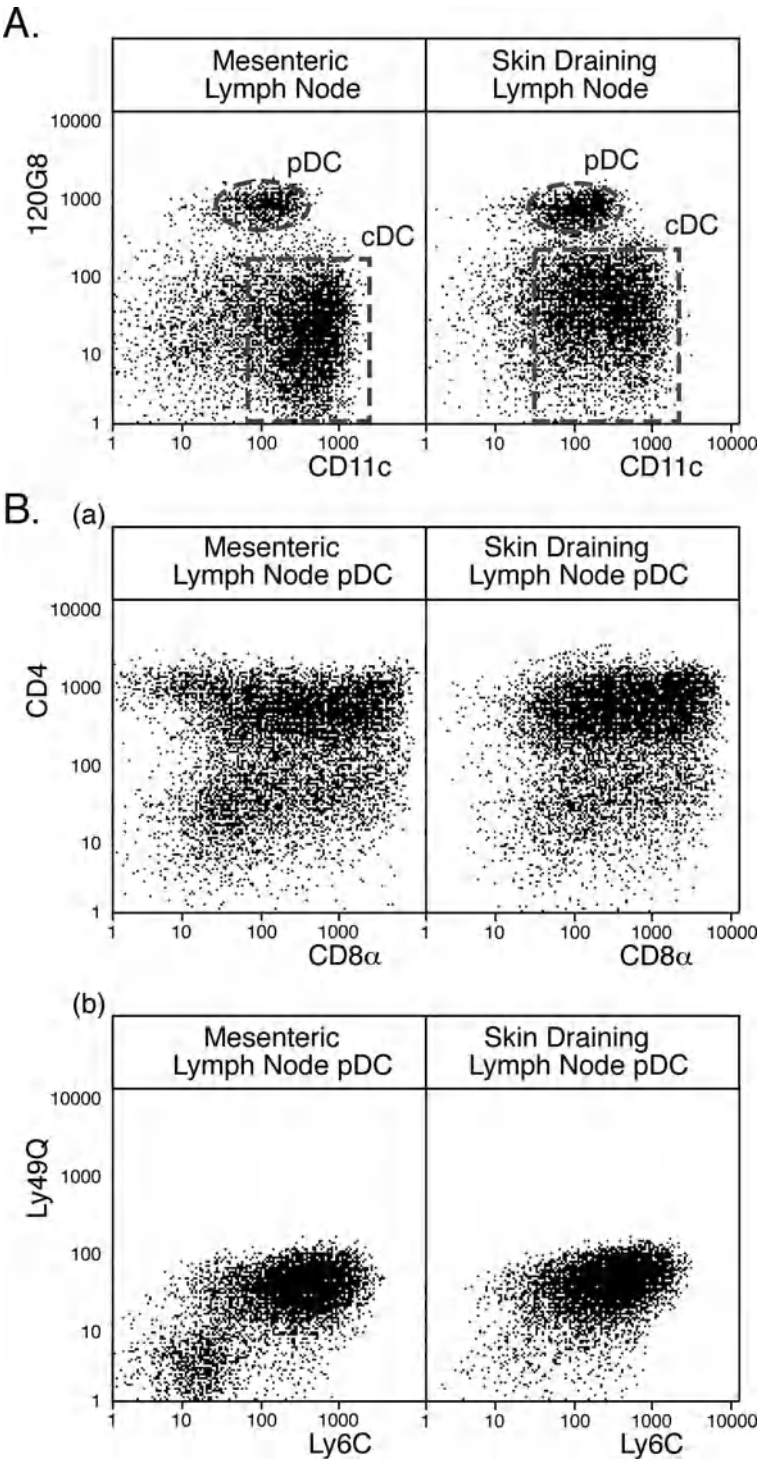

Fig. 14.5. Discrete dendritic cell subtypes of mouse mesenteric and skin-draining lymph nodes. (**A**) A combination of CD11c and 120G8 separates $CD11c^{+}120G8^{-}$ conventional DC and $CD11c^{int}120G8^{+}$ plasmacytoid pre-DC. Similar results are obtained substituting CD45RA in place of 120G8. (**B**) Lymph node pDCs are subdivided using (**a**) CD4 and CD8 or (**b**) Ly49Q and Ly6C resulting in populations similar to those found in spleen and thymus.

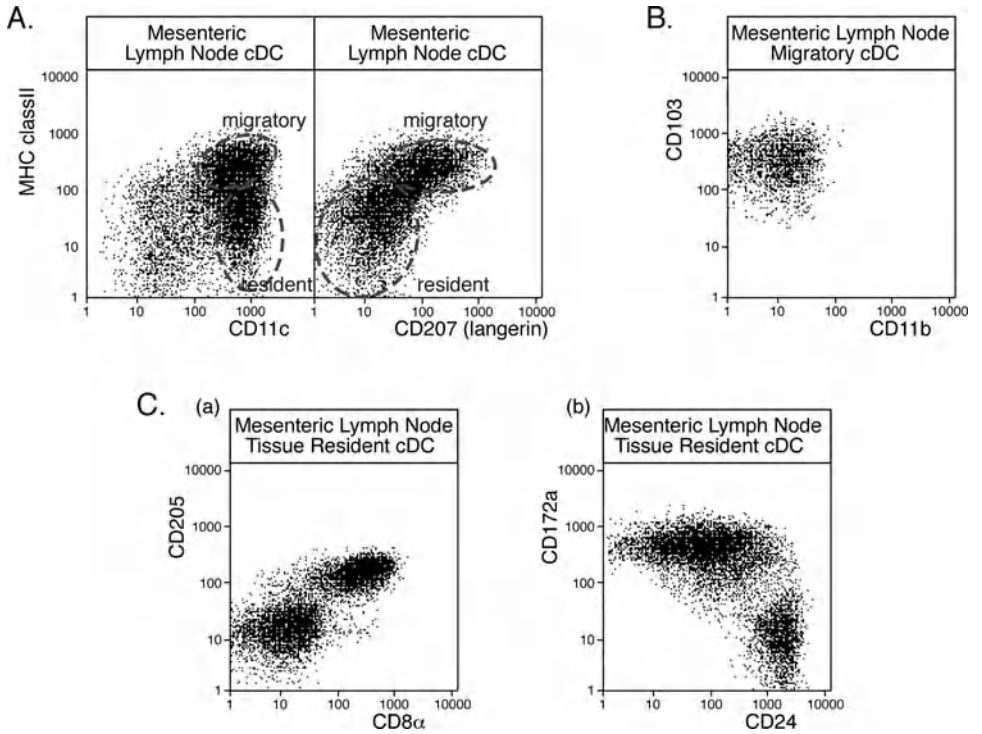

Fig. 14.6. Conventional dendritic cell subtypes of mouse mesenteric lymph node. (**A**) cDCs from mesenteric lymph node can be separated into MHC class II^{++}langerin^{+} migratory, interstitial DC and MHC class II^{+}langerin^{-} tissue resident DC. (**B**) The migratory, interstitial DCs are CD11b$^{-/lo}$CD103^{+}. (**C**) (**a**) The tissue resident DCs are divided into CD8^{+}CD205^{+} and CD8^{-}CD205^{-} populations. (**b**) The CD8^{+} and CD8^{-} populations are aligned to CD172a^{lo}CD24^{hi} and CD172a^{hi}CD24^{lo} populations, respectively.

2. EDTA solution: 0.1 M ethylenediamine tetra-acetic acid disodium salt adjusted to pH 7.2. Filter to sterilize and store at 4°C.
3. EDTA–FCS: Add 1 ml of 0.1 M EDTA per 10 ml FCS prior to use.

2.3. Selection of Light Density Cells

1. BSS–EDTA: Modified salt solution containing 150 mM NaCl and 3.75 mM KCl (no Ca^{2+} or Mg^{2+}) and 5 mM EDTA. Adjust to pH 7.2 and mouse osmolarity (308 mOsm/kg). Filter to sterilize and store at 4°C.
2. BSS–EDTA–FCS: BSS–EDTA containing 2% EDTA–FCS.
3. Nycodenz–EDTA: Nycodenz AG powder (Nycomed Pharma AS, Oslo, Norway) is prepared as a 0.372 M stock solution in water, then diluted and adjusted to the appropriate density (at 4°C) and osmolarity (308 mOsm/kg) using BSS–EDTA (for spleen, 1.077 g/cm^3, for thymus, 1.076 g/cm^3, for lymph nodes, 1.082 g/cm^3) (*see* **Note 2**). Filter to sterilize and store in sealed 10-ml aliquots at

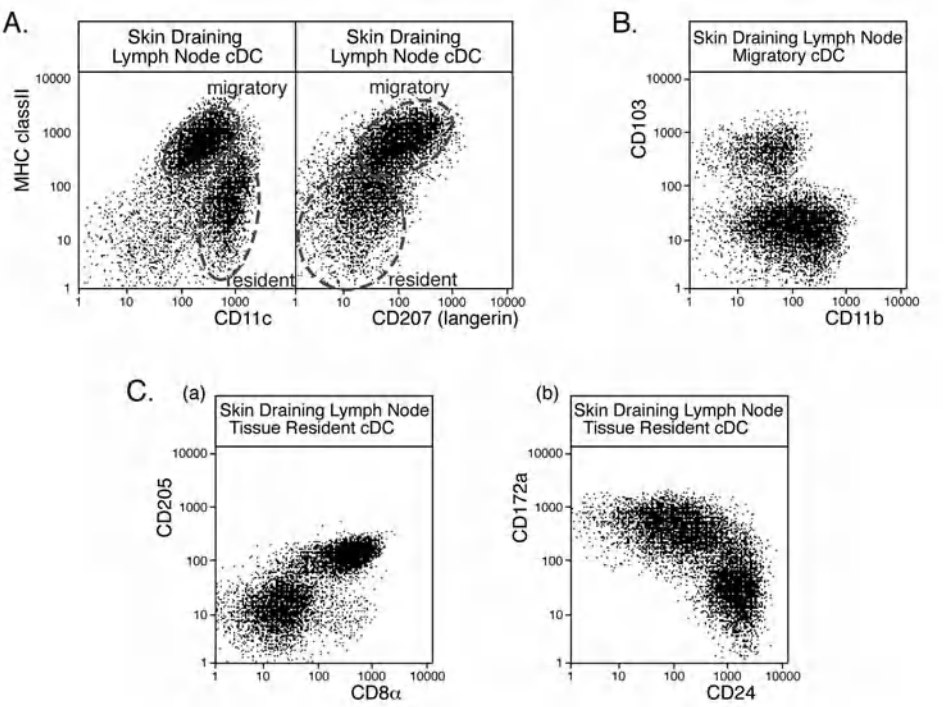

Fig. 14.7. Conventional dendritic cell subtypes of mouse skin-draining lymph nodes. (**A**) cDCs from skin-draining lymph nodes are separated into MHC class II^{+++} langerin^{+} migratory DCs and MHC class II^{+}langerin^{-} tissue resident DCs. The skin-derived migratory DCs within the lymph node are the only DCs with an activated phenotype in the steady state, expressing the highest levels of MHC class II and costimulatory molecules. (**B**) Skin-derived migratory cDCs are separated into CD11b$^{-/lo}$CD103^{+} dermal-derived DCs and CD11b^{+}CD103^{-} epidermal-derived DCs, which are the mature form of the Langerhans cell. (**C**) Tissue resident cDCs are divided into CD8^{-}CD205^{-}CD172a^{hi}CD24^{lo} and CD8^{+}CD205^{+}CD172a^{lo}CD24^{hi} populations similar to those found in the spleen, thymus and mesenteric lymph node.

−20°C. Thaw at room temperature, mix thoroughly and cool to 4°C prior to use (*see* **Note 3**).

4. Polypropylene tubes: 14 ml polypropylene round-bottom tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA).

2.4. Depletion of Non-DC Lineages

1. Monoclonal antibody depletion cocktail: Add together pre-titrated amounts of rat monoclonal antibodies (*see* **Note 4**) specific for non-DC lineage cells and dilute to the appropriate volume with BSS–EDTA–FCS. Saturating levels of all monoclonal antibodies are used except anti-CD11b and anti-F4/80 (*see* **Note 5**). The monoclonal antibodies added can be varied depending on the DC populations required (*see* **Table 14.1**). Filter to sterilize, aliquot and store at −20°C.

Table 14.1
The monoclonal antibodies contained in the various monoclonal antibody depletion cocktails. The contents of these cocktails can be varied depending on which DC populations are to be isolated. As all the monoclonal antibodies are rat derived, a simple depletion using anti-rat IgG-coated immunomagnetic beads is possible

Clone	KT3-1.1	GK1.5	M1/70[a]	ID3	RA36B2	T24/31.7	1A8	F4/80[a]	TER119
Specificity	CD3ε	CD4	CD11b	CD19	CD45R	CD90	Ly6G	F4/80Ag	Erythroid lineage
Spleen and LN cDC only	√	–	–	–	√	√	√	–	√
Spleen and LN cDC + pDC	√	–	–	√	–	√	√	–	√
Thymus cDC only	√	√	√	–	√	√	√	√	√
Thymus cDC + pDC	√	–	√	√	–	√	√	√	√

[a] *see* **Note 5**

2. Immunomagnetic beads: Either BioMag goat anti-rat IgG-coated beads (Qiagen, Clifton Hill, Australia) or Dynabead sheep anti-rat IgG-coated beads (Dynal, Oslo, Norway).
3. Spiral rotator: Spiramix 10 (Denley, Billingshurst, England).

2.5. Depletion of NK Cells

1. Biotinylated antibody: Conjugate DX5 and PK136 to biotin-XX succinimidyl ester (Molecular Probes, Inc., Eugene, OR, USA) following the manufacturer's directions. Add FCS to 1% and NaN_3 to a final concentration of 10 mM (*see* **Note 6**). Aliquot and store stocks at −70°C but a working stock may be kept at 4°C. Titrate to determine optimal concentrations (*see* **Table 14.2**).
2. Anti-biotin beads: Anti-biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany).
3. BSS–EDTA–0.5% FCS: BSS–EDTA containing 0.5% EDTA–FCS.
4. MACS column: MACS LS column (Miltenyi Biotec, Bergisch Gladbach, Germany).
5. Magnet and stand: miniMACS magnet and MACS multistand (Miltenyi Biotec, Bergisch Gladbach, Germany).

Table 14.2
The monoclonal antibodies used to deplete NK cells. Their isotype (DX5) or species of origin (PK136) precludes their inclusion in the depletion cocktails outlined in Table 14.1. Instead they are conjugated to biotin, used to coat cells and positive cells removed during a second round of depletion using anti-biotin-coated immunomagnetic beads

Clone	DX5	PK136
Specificity	CD49b (pan NK)	CD161c (NK1.1)
Isotype	Rat IgM	Mouse IgG_{2a}
Conjugate	Biotin	Biotin

2.6. Immunofluorescent Staining and Flow Cytometric Analysis

1. The majority of monoclonal antibodies were purified from hybridoma culture supernatant using Protein G Sepharose (Amersham Biosciences, Castle Hill, Australia) and subsequently conjugated to fluorochromes in-house. Conjugated monoclonal antibodies purchased were 2E6-FITC (Medical and Biological Laboratories, Nagoya, Japan), HMα2-PE, RM4-5-PerCp.Cy5.5 and 53-6.7-PerCp.Cy5.5 (BD Pharmingen, San Jose, CA, USA), PO3.1-PE, N418-PE.Cy7 and eBioL31-biotin (eBioscience, San Diego, CA, USA) and 2A11-biotin (Serotec, Kidlington, UK) (*see* **Table 14.3**). All were titrated to determine saturating levels.
2. Fluorochrome-conjugated mAb: Conjugate P84 and NLDC145 to FITC (Molecular Probes, Inc., Eugene, OR, USA), YTS169.4 and M2/90 to phycoerythrin ((PE) Prozyme, San Leandro, CA, USA), 14.8 and M5/114 to allophycocyanin ((APC) Prozyme, San Leandro, CA, USA), M1/69 and M1/70 to AlexaFluor633 and 120G8, YTS169.4 and M1/69 to AlexaFluor680 (Molecular Probes, Inc., Eugene, OR, USA), N418 and 5075–3.6 to Quantum Dot 655 (Invitrogen) and M1/70 to biotin-XX succinimidyl ester (Molecular Probes, Inc., Eugene, OR) following the manufacturer's instructions. Add FCS to 1% and NaN_3 to a final concentration of 10 mM (*see* **Note 6**). Titrate to determine saturating levels. Aliquot and store stocks of FITC, AlexaFluor633, AlexaFluor680 and biotin conjugates at −70°C. Stocks of PE, APC, Quantum Dot 655 and PerCp.Cy5.5 conjugates (*see* **Note 7**) and working stocks of FITC, AlexaFluor633, AlexaFluor680 and biotin conjugates are stored at 4°C, protected from light. Dilute to final concentration immediately prior to use.

Table 14.3
Distinct DC subtypes in spleen, thymus and lymph node can be defined by seven-colour flow cytometric analysis using monoclonal antibodies

Specificity	**I-A^{bdq} I-E^{dk}**	**CD4**	**CD8α**	**CD8α**	**CD11b**	**CD11c**
Clone	M5/114	RM4-5	YTS169.4	53-6.7	M1/70	N418
Conjugates	APC	PerCp.Cy5.5	PE, Alexa680	PerCp.Cy5.5	Biotin Alexa633	PE.Cy7 QDot655
Specificity	**CD24**	**CD45RA**	**CD49b**	**CD86**	**CD103**	**CD172a**
Clone	M1/69	14.8	HMα2	PO3.1	M2/90	P84
Conjugates	Alexa633 Alexa680	APC	PE	PE	PE	FITC
Specificity	**CD205**	**CD207 (Langerin)**	**pDC**	**Ly6C**	**Ly49Q**	**Dectin-1**
Clone	NLDC145	eBioL31	120G8	5075-3.6	2E6	2A11
Conjugates	FITC	Biotin	Alexa680	QDot655	FITC	Biotin

3. PE-Streptavidin, PE.Cy7-Streptavidin and PerCp.Cy5.5-Streptavidin (BD Pharmingen, San Jose, CA, USA) are titrated to determine saturating levels and stored protected from light at 4°C. Dilute immediately before use.
4. PI: Prepare a 100 μg/ml propidium iodide (Calbiochem, La Jolla, CA, USA) stock solution in normal saline. Aliquot and store at 4°C protected from light (*see* **Note 8**).
5. BSS–EDTA–FCS–PI: Dilute the PI stock in BSS–EDTA–FCS to a final working concentration of 500 ng/ml before addition to cells.

3. Methods

3.1. Organ Removal

1. Remove spleens, thymus or lymph nodes (*see* **Note 9**) from eight mice into cold RPMI–FCS (*see* **Note 10**). Aim to remove the organs with as little fat or connective tissue as possible.

3.2. Digestion and Release of DCs

1. Prepare the enzyme digestion mix immediately prior to use and allow it to warm to room temperature.
2. Remove any remaining fat or connective tissue (*see* **Note 11**) from the organs and transfer them to a small plastic Petri dish containing 7 ml of the enzyme digestion mix. Cut the organs

into small fragments using sharp scissors (*see* **Note 12**) and transfer them to a 10-ml polypropylene tube using a wide-bore Pasteur pipette. Begin digesting the fragments, mixing frequently using the same wide-bore Pasteur pipette. Continue the digestion for 20–25 min at room temperature (~22°C) (*see* **Note 13**).

3. Add 600 μl of EDTA solution to the digestion mix and continue the incubation for a further 5 min (*see* **Note 14**).
4. Run the digestion mix through a sieve to remove any residual tissue fragments. Add RPMI–FCS to a final volume of 9 ml, underlay with 1 ml of cold FCS–EDTA and recover the cells by centrifugation (*see* **Note 15**).

3.3. Selection of Light Density Cells

1. Thaw two 10-ml aliquots of Nycodenz–EDTA of appropriate density at room temperature. Once thawed ensure they are mixed well and maintain them at 4°C until required (*see* **Note 16**).
2. Produce a cell suspension by resuspending the cell pellet in 10 ml of Nycodenz–EDTA (*see* **Note 17**).
3. Transfer 5 ml of the remaining Nycodenz–EDTA into the bottom of each of two polypropylene tubes.
4. Gently layer 5 ml of the cell suspension over the Nycodenz–EDTA in each of the two tubes (*see* **Note 18**). Layer 1–2 ml of EDTA–FCS over the cell suspension.
5. Gently mix the interface by inserting the tip of a Pasteur pipette, swirling and removing it (*see* **Note 19**).
6. Perform the density cut in a swing-out head, refrigerated (4°C) centrifuge for 10 min at 1,700 × *g* with the brake set on low.
7. Collect the light density fraction in the upper zones down to the 4 ml mark using a Pasteur pipette (*see* **Note 20**). Discard the bottom 4 ml and the cell pellet.
8. Transfer the light density fraction to a 50-ml tube, dilute up to 50 ml with BSS–EDTA, mix thoroughly and centrifuge to recover the cells (*see* **Note 21**).
9. Resuspend the cells in 5 ml BSS–EDTA–FCS and count (*see* **Note 22**).

3.4. Immunomagnetic Bead Depletion of Non-DC Lineage Cells

1. Calculate the volume of monoclonal antibody depletion cocktail required if 10 μl is needed per 10^6 cells.
2. Add the required volume of the appropriate monoclonal antibody depletion cocktail to the cell pellet, resuspend and incubate at 4°C for 30 min.

3. Calculate the required volume of immunomagnetic beads required (*see* **Note 23**) and transfer them to a 5-ml polypropylene tube. Wash the beads with BSS–EDTA–FCS (*see* **Note 24**) by diluting, placing the tube into the magnet, allowing the beads to move to the side of the tube and removing the supernatant. Repeat the wash three to four times. After the final wash, pellet the beads at the bottom of the tube in a small amount of BSS–EDTA–FCS and place them at 4°C until they are required.
4. Dilute the cells up to 9 ml with BSS–EDTA–FCS and underlay with 1 ml of FCS–EDTA. Centrifuge the cells and remove the supernatant from the top, leaving the FCS layer over the cells (*see* **Note 25**). Sit the tube at 4°C for a short time to allow any remaining supernatant to run down the wall of the tube. Then remove the supernatant and the FCS (*see* **Note 26**). Resuspend the cells in 400–500 μl of BSS–EDTA–FCS.
5. Remove BSS–EDTA–FCS remaining on the magnetic bead pellet and add the cells. Produce a slurry of beads and cells by vortexing the tube very briefly (*see* **Note 27**). Seal the tube and mix the slurry continuously for 20 min at 4°C at an angle of 30° on a spiral mixer (*see* **Note 28**).
6. Dilute the bead–cell slurry with 3 ml of BSS–EDTA–FCS, mix very gently and attach the tube to the magnet for 2 min.
7. Recover the supernatant containing unbound DCs with a Pasteur pipette and transfer to a second 5-ml polypropylene tube. Discard the tube containing magnetic beads bound to non-DCs (*see* **Note 29**). Place the tube containing the supernatant into the magnet for a further 2 min to remove any remaining beads. Transfer the supernatant to a 10-ml tube.
8. Layer 1 ml of FCS–EDTA under the cell suspension and centrifuge to recover the DC fraction. Resuspend the cells in 2 ml of BSS–EDTA–FCS and count.
9. Maintain the cells at 4°C until they are required.

3.5. Depletion of NK Cells

1. If depletion of NK cells is deemed necessary, centrifuge cells to a pellet.
2. Add a cocktail of subsaturating levels (*see* **Note 30**) of DX5-biotin and PK136-biotin at 10 μl/10^6 cells, resuspend and incubate at 4°C for 30 min.
3. Resuspend up to a larger volume with BSS–EDTA–FCS and underlay with FCS–EDTA. Centrifuge to wash the cells (*see* **Note 15**).
5. Remove the supernatant leaving the FCS–EDTA layer above the cells and centrifuge again for 30 s to allow

any remaining media to wash down the wall of the tube. Remove the remaining media and the FCS–EDTA layer.

6. Add anti-biotin microbeads at 1 μl/4 × 10^6 cells (*see* **Note 31**) in a final volume of 2.5 μl/10^6 cells BSS–EDTA–0.5%FCS, resuspend and incubate for 15 min at 4°C.
7. Resuspend to a larger volume with BSS–EDTA–FCS, underlay with FCS–EDTA and centrifuge to wash away unbound microbeads.
8. Prepare a MACS LS column (*see* **Note 32**) by placing into a cold miniMACS magnet (*see* **Note 33**) suspended on a MACS multistand and washing with 1 ml BSS–EDTA–0.5%FCS.
9. Resuspend the cells in 500 μl BSS–EDTA–0.5%FCS and apply them to the column using a syringe and 27 gauge needle to ensure a single-cell suspension. Rinse the column with 3 × 1 ml of BSS–EDTA–0.5% FCS and collect the flow-through containing the unbound cells (*see* **Note 34**).
10. Centrifuge to recover the cells.
11. Resuspend the cells in BSS–EDTA–FCS and count.

3.6. Immunofluorescent Staining and Flow Cytometric Analysis

1. Prepare a cocktail of pre-titrated fluorochrome-conjugated monoclonal antibodies (*see* **Table 14.3**) at the appropriate concentration immediately prior to use.
2. Centrifuge the cells and remove the supernatant.
3. Add 10 μl of the fluorochrome-conjugated antibody cocktail per 10^6 cells, resuspend by flicking and incubate at 4°C for 30 min (*see* **Note 35**).
4. Resuspend up to a larger volume with BSS–EDTA–FCS and underlay with FCS–EDTA. Centrifuge to wash the cells (*see* **Note 15**).
5. Remove the supernatant leaving the FCS–EDTA layer above the cells and centrifuge again for 30 s to allow any remaining media to wash down the wall of the tube. Remove the remaining media and the FCS–EDTA layer.
6. If a biotinylated antibody was included in the first stage, add a second stage of pre-titrated fluorochrome-conjugated Streptavidin at 10 μl per 10^6 cells. Incubate and wash as for the first stage.
7. Resuspend cells in BSS–EDTA–FCS–PI and maintain cells at 4°C until ready for flow cytometry (*see* **Note 36**).
8. Sort the DCs using a FACSAria (BD Biosciences, San Jose, CA, USA) or analyze using an LSRII (BD

Biosciences, San Jose, CA, USA) (*see* **Note 37**). The flow cytometers should be set up with their standard lasers: a blue 488-nm emitting laser for detection of FITC, PE, PI, PerCp.Cy5.5 and PE.Cy7, a UV laser, emitting a wavelength of 355 nm (LSRII), or a violet laser emitting a wavelength of 405 nm (FACSAria), to detect Quantum Dot 655 and a red laser emitting a wavelength of 633 nm to detect AlexaFluor633/APC (*see* **Note 38**) and AlexaFluor680, as well as the appropriate filters and dichroic mirrors (BD Biosciences, San Jose, CA, USA) (*see* **Table 14.4**). Select DCs on the basis of high forward and side-light scatter, excluding dead cells with high PI fluorescence. Remove contaminating macrophages by gating out autofluorescent cells using the PI channel in combination with another fluorescence

Table 14.4
The optical requirements for detection of fluorochromes used in the flow cytometric separation of mouse dendritic cell subtypes

Laser	Fluorochrome	ABS. max.	EM. max.	Bandpass filter	Longpass dichroic mirror
Laser 1	FITC	490 nm	525 nm	530/30	505LP
488 nm	PE	565 nm	575 nm	575/26 (LSRII) 575/25 (ARIA)	550LP
	PI	536 nm	620 nm	610/20	600LP
	PerCpCy5.5	490 nm	694 nm	695/40 (LSRII) 710/50 (ARIA)	635LP (LSRII) 685LP (ARIA)
	PE.Cy7	496 nm	767 nm	780/60	735LP (LSRII) 755LP (ARIA)
Laser 2	Quantum Dot655	405 nm	655 nm	660/40 or	635LP (LSRII)
355 nm (LSRII)				665/40 (LSRII)	
405 nm (ARIA)				670/30 (ARIA)	630LP (ARIA)
Laser 3	AlexaFluor633[a]	621 nm	639 nm	660/20	-
633 nm	APC[a]	650 nm	660 nm	660/20 (LSRII) 670/30 (ARIA)	-
	AlexaFluor680	679 nm	702 nm	710/50	685LP/680LP

[a]APC and AlexaFluor633 require the same detector and cannot be used together.

channel as shown in **Fig.14.1** (*see* **Note 39**). Identify $CD11c^{int}$ $120G8^{+}$ pDCs and $CD11c^{hi}120G8^{-}$ cDCs. CD45RA and CD45R (B220) are alternative markers to 120G8. Use combinations of conjugated antibodies to identify other dendritic cell subtypes (*see* **Note 40**).

9. Appropriate software is used to detail the DC subpopulations as shown in **Figs. 14.2–14.7**.

4. Notes

1. Contamination of the collagenase with trypsin or trypsin-like proteases varies between batches, so each should be tested prior to use. These proteases can degrade cell surface molecules and alter the surface phenotype of the DCs. We test for their presence by incubating thymocytes with each new batch of collagenase for 30 min at 37°C and then screening for the loss of the trypsin-sensitive cell surface markers CD4 and CD8 by flow cytometry.
2. Density is measured using a pycnometer. The pycnometer is a glass flask with a close-fitting ground glass stopper with a capillary tube in it, so that air bubbles may escape from the apparatus. This enables the density of a fluid to be measured accurately, by reference to water, using an analytical balance. The flask is weighed empty, full of water and full of Nycodenz, and the specific gravity of the Nycodenz is calculated. A correction will need to be made as the density of water will not be 1 g/cm^3 at 4°C.
3. Temperature, pH and osmolarity all affect the buoyant density of cells. We calculate the density of Nycodenz at pH 7.2, 308 mOsm/kg and 4°C. Temperature is of particular importance during the density cut, so care must be taken to ensure that the Nycodenz and the centrifuge to be used are at 4°C. Using Nycodenz of higher than recommended density will reduce purity while one of lower than recommended density will decrease the yield.
4. Monoclonal antibodies that we have utilized for depletion of non-DC lineage cells or the identification of DCs and DC subpopulations are generally available commercially.
5. The rat anti-mouse monoclonal antibodies added to the cocktail are individually titrated via flow cytometry using a fluorochrome-conjugated anti-rat Ig secondary reagent in order to determine their working dilution. Dilutions resulting in cell surface saturation of the antigen are

considered adequate for efficient depletion. Monoclonal antibodies against CD11b and F4/80 are necessary for the depletion of thymic macrophages, which express high levels of these molecules. Thymic dendritic cells express much lower levels and are spared from depletion if subsaturating levels (five to tenfold less in our hands) of the monoclonal antibodies are used. Appropriate levels should be determined for each batch of antibody.

6. All proper precaution should be taken when using sodium azide as a preservative. It is extremely toxic if ingested. Appropriate gloves and face mask should be worn during preparation of the stock solution.
7. Some fluorochromes, including phycobiliproteins, are extremely sensitive to freezing and thawing. Do not freeze phycoerythrin (PE), allophycocyanin, PE.Cy7, PerCp.Cy5.5 or Quantum Dots.
8. Propidium iodide is irritating to the eyes, the respiratory system and skin and its toxicological properties have not been thoroughly investigated. Precautions such as safety glasses, gloves and face mask should be worn during preparation of the stock solution.
9. We routinely use mesenteric, inguinal, brachial and axillary lymph nodes, pooling the inguinal, brachial and axillary lymph nodes as skin-draining lymph nodes. We have also used this method successfully with auricular and pancreatic lymph nodes.
10. A proportional increase or decrease of all listed amounts and volumes should be made to cater for any change in the starting number of organs, or in the case of treated mice, with increased or decreased organ size, the total number of cells in those organs.
11. It is important to clean the organs as much as possible before commencement of the digestion. Residual fat will reduce cell viability and cause clumping. Connective tissue will also accumulate and not be digested. We use two 20 gauge needles to perform the cleaning but forceps, scissors or any other suitable instrument may be used.
12. The organs should be cut into very small fragments to increase the surface area available to the enzymes. This ensures adequate digestion and maximizes cell yield. Any other suitable sharp implement, such as a single-sided razor blade, may be used instead of scissors.
13. A digestion time of 20–25 min should prove sufficient to digest all but the outer capsule of lymph nodes and the pulpy tissue from spleen, provided the tissue was cut up

adequately prior to the digestion and adequate mixing occurred during the digestion. Inadequate digestion will result in a lower recovery and the preferential loss of certain DC populations that are more firmly entrenched in the tissue.

14. EDTA will chelate Ca^{2+} and Mg^{2+} ions and dissociate lymphocytes complexed to DCs. EDTA must be added to all media from this point onwards to stop the reformation of these multicellular complexes. Failure to do so will cause loss of DCs during purification and possible contamination of the recovered DCs with lymphocytes.
15. All centrifugation steps are performed at $1{,}000 \times g$ for 7 min at 4°C unless otherwise stated. It is possible to eliminate the need for repetitive 'washing' of the cells by layering FCS under the sample, thereby incorporating a zonal centrifugation step to increase the efficiency of separation of the cells from smaller particles and soluble material in the supernatant.
16. Mix Nycodenz thoroughly prior to aliquoting and prior to use to ensure a solution of uniform density.
17. Efficiency of separation will be lost and yields reduced if the density separation is overloaded. Do not load more than four organs ($\sim 10^9$ cells) per 10 ml of Nycodenz.
18. A discrepancy between the density of the Nycodenz at the top of an aliquot and the bottom, or between different aliquots, will affect the ability to layer Nycodenz containing cells over the Nycodenz at the bottom of the tube when setting up the density cut. Ensure Nycodenz has been adequately mixed and is of uniform density before use.
19. A sharp interface will reduce the efficiency of the density separation. Disrupt the interface slightly using a pipette to create a density gradient rather than a sharp cut-off.
20. After centrifugation, the light density fraction of cells will have formed a band at the interface zone between the FCS and Nycodenz while dense cells will have formed a pellet. Cells of intermediate density will be found in the gradient between these zones, so collect all cells down to the 4 ml mark while concentrating on the light density band at the interface.
21. Adequate dilution and mixing of the light density fraction of cells with EDTA–BSS is essential to recover them as a pellet during centrifugation.
22. All cells, including any remaining erythrocytes, should be counted to calculate the appropriate volume of mAb depletion cocktail and immunomagnetic beads required in sub-

sequent steps. As a rough guide the light density fraction should represent 3–7% of the starting number of cells.

23. For the isolation of spleen DCs we recommend BioMag beads at a 10:1 bead to cell ratio. This provides optimal economy and reasonably good efficiency. For optimal efficiency we recommend Dynabeads at a 5:1 bead to cell ratio, but this will inflate the cost of the procedure. Alternatively, a two-stage depletion can be performed to optimize efficiency but reduce cost by using an initial 5:1 BioMag to cell ratio followed by Dynabeads at a 3:1 bead to cell ratio on the reduced number of cells. Due to the smaller number of DCs recovered from thymus and lymph node we recommend using Dynabeads at a 5:1 bead to cell ratio to maximize recovery.
24. Immunomagnetic beads are stored in preservative, which must be removed by washing prior to use.
25. Centrifuging through a layer of FCS separates cells from unbound (excess) mAb.
26. Any excess mAb will compete for binding to the beads thus decreasing the efficiency of the depletion. It is therefore important to carefully remove all the supernatant after washing.
27. The efficiency of the depletion is greatly increased by maximizing contact between beads and cells in the concentrated slurry.
28. The bead–cell slurry is kept concentrated at the bottom of the tube by rotating the tube at a 30° angle. We create the angle by fitting a wide ring around the top of the tube to elevate it from the horizontal. The wide ring also serves to slow the rate of rotation.
29. If numbers are critical, more DCs can be recovered by washing the beads and attaching the tube to the magnet a second time, however, this will result in reduced purity.
30. Too high a concentration of antibody added at this stage tends to increase the non-specific loss of DCs and lower the yield. We routinely use antibodies at a quarter of their saturating levels, but would recommend that each user carefully titrate their antibody for optimal performance.
31. This is a lower bead to cell ratio than recommended by the manufacturer. We have compensated for this by reducing the volume in order to increase the final concentration and maximize bead to cell contact. This has resulted in an economical and more efficient process.
32. Do not overload the column. This will result in a lower recovery and reduced purity. If using cells isolated from

more than eight mice check the manufacturer's instructions to determine whether more than one column is required.

33. Cell viability is increased at 4°C so cooling the magnet before use ensures that the column and cell suspension passing through it are kept cold during the separation. Placing the magnet at −20°C for 15 min before use is recommended.
34. If necessary purity may be increased by running the flow through over a second MACS LS column. An alternative is to detect any remaining biotin-coated cells using a fluorochrome-conjugated Streptavidin and gating them out during fluorescence-activated cell sorting or analysis.
35. All immunostaining steps should be performed at 4°C to promote cell viability and to prevent capping of monoclonal antibody from the cell surface.
36. Propidium iodide is used for dead cell exclusion during flow cytometric analysis.
37. Any flow cytometer with appropriate lasers and optical setup can be used.
38. AlexaFluor633 and APC are measured by the same detector, so only one of these fluorochromes can be used in each sample.
39. Remove autoflourescent cells (mainly macrophages) during fluorescence-activated cell sorting or analysis by gating out cells that have low levels of fluorescence in two or more fluorescent channels. Ideally use a combination of the PI and an unused channel. During multicolour sorting or analysis, it may be necessary to combine autofluorescence in the PI channel with low fluorescence in a channel that is being used. Choose a parameter where all DCs will fluoresce brightly (i.e. CD11c) and gate out cells of low fluorescence.
40. If functional studies are to be undertaken we would advise against staining the cells for anti–MHC class II as we have observed that this may interfere with the DCs ability to interact appropriately with T cells. The cells should be washed to remove propidium iodide post-sorting.

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Chapter 15

Isolation of Cutaneous Dendritic Cells

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Abstract

Cutaneous dendritic cells represent the first immunological interface with the environment and play a key role in the defense against pathogens that breach the skin. This protocol describes how to isolate cutaneous dendritic cells from mouse ears for flow cytometry analysis and functional assay studies.

Key words: Epidermis, dermis, dendritic cells, Langerhans cells, langerin.

1. Introduction

As the body's largest and most exposed interface with the environment, the skin provides the first line of defense against a broad array of microbial pathogens. The skin is equipped with a highly sophisticated system of immune surveillance that relies on a rich network of professional antigen-presenting dendritic cells (DCs) that populate the epidermis and the dermis (1). DCs in the epidermis are also known as Langerhans cells (LCs), whereas dermal DCs belong to a broader subset of interstitial DCs (1). Recent data established that the dermal DC network is more complex than originally anticipated and contain at least two phenotypically distinct DC populations that include the langerin$^+$ CD11b^{lo}CD103$^+$DCs and the langerin$^-$CD11b$^+$ CD103$^-$ DC subsets (2–4). Here, we describe how to isolate and phenotypically characterize each cutaneous DC subset.

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2. Materials

1. Very fine forceps.
2. 3-ml syringe and a 19 gauge needle.
3. 12-well plates.
4. Hank's buffered salt solution (HBSS) with calcium and magnesium (CellGro).
5. Dispase from *Bacillus polymyxa* (GIBCO). The lyophilized powder is kept at 4°C. A 50X stock solution prepared in HBSS can be kept at −20°C. The working solution is made extemporaneously.
6. Collagenase from *Clostridium histolyticum*, type IV (Sigma–Aldrich). Need to be stored at −20°C. The working solution is made extemporaneously.
7. Fetal bovine serum (FBS) (HyClone, Ogden, UT, USA).
8. Dulbecco's phosphate-buffered saline (DPBS) (CellGro).
9. Bovine serum albumin (BSA) (Sigma).
10. EDTA (Invitrogen).

3. Methods

1. Ears are cut at the base. The dorsal and ventral layers are separated with a fine forceps and incubated epidermal face down in 3 ml of HBSS–dispase (2 U/ml) in a 12-well plate for 90 min at 37°C (*see* **Note 1**) to allow for the separation of the epidermal and dermal sheets.
2. The epidermal sheets are delicately separated from the dermal sheets using fine forceps and disposed separately in 3 ml of HBSS–10% FSB–collagenase (working activity of 154 U/mg). The sheets are then cut into small pieces prior to incubation for 2 h at 37°C (*see* **Note 2**).
3. A homogeneous cell suspension is recovered by flushing three to five times the 3 ml solution with a 3-ml syringe and a 19-ga needle (*see* **Note 2**). The cell suspension is washed in a solution of PBS supplemented with BSA (0.5%) and EDTA (2 mM).
4. For flow cytometry analysis, skin cells are resuspended in PBS supplemented with BSA (0.5%) and EDTA (2 mM) and incubated with anti-CD16/32 monoclonal antibody (mAb) (clone 24G2) for 10 min and then stained

with the appropriate Abs. CD45 (clone 30F11), IA/IE (clone M5/114.15.2), CD103 (clone 2E7), CD11b (clone M1/70), CD11c (clone N418) and the corresponding isotype controls and the secondary reagents (allophycocyanin, peridinin chlorophyll protein, and phycoerythrin–indotricarbocyanine-conjugated streptavidin) can be purchased from BD Biosciences (San Jose, USA) or from eBioscience (San Diego, USA). Anti-langerin Ab (clone E-17) recognizing an intracellular epitope can be purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Intracellular staining for langerin is performed with the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocol.

Analysis of the three cutaneous DC populations is done by gating on $DAPI^{-}CD45^{+}$ $CD11c^{+}MHC\text{-}II^{+}$ cells. The epidermis includes only the LC population characterized as $langerin^{hi}CD11b^{high}CD103^{-}$ (1). The dermis contains three DC populations that include migratory LCs, in their way to the draining lymph node (with a similar phenotype to the epidermal LCs), and two dermal DC subsets including the $langerin^{-}CD11b^{+}CD103^{-}$ DC subset and the dermal $langerin^{+}CD11b^{-}$ $CD103^{+}$ DC subset (2–4).

4. Notes

1. *Avoiding contamination:* Careful dissociation of the epidermal and dermal sheets after the dispase treatment helps decrease the contamination of the dermal cell compartment with epidermal cells. Another alternative is to incubate the dorsal and ventral ear layers in dispase overnight at 4°C.
2. *Improving cell viability:* Decreasing the duration of the collagenase treatment should improve the cell viability. Careful syringe-based cell dissociation should also improve cell viability.

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Chapter 16

Isolation of Skin Dendritic Cells from Mouse and Man

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Abstract

Dendritic cells (DC) are crucial for the induction of immune responses and populate various tissues to fulfil their special role. The skin harbours different DC subsets, the Langerhans cells (LC) in the epidermis and the dermal DC in the dermis. The investigation of skin DC is cumbersome since these cells are rare in the skin. As a consequence, it is laborious to receive enough cells from the tissue for experiments. Several approaches have been developed to isolate skin DC based on either enzymatic digestion of the tissue or skin explant culture. Immature LC can be obtained by trypsinization of epidermis, cultured in vitro and be highly enriched with gradient centrifugation and magnetic bead sorting. Mature skin DC can be easily received from skin explant culture. For this purpose skin pieces are cultured for several days and migratory DC emigrate from epidermis and dermis. Both techniques are described for human and mouse skin in the following chapter of the book.

Key words: Skin, dendritic cells, Langerhans cells, isolation, skin explant culture.

1. Introduction

Skin dendritic cells (DC) are mandatory for the induction of an immune response against pathogens invading the body through the skin. As sentinels of the immune system they are capable of incorporating and presenting antigen to naïve T cells in the skin-draining lymph nodes (1). Several subsets of skin DC exist and their respective function and role in the skin immune system are still not entirely clear. The two main subsets are the Langerhans cells (LC), situated in the suprabasal layers of the epidermis and the dermal DC residing in the connective tissue of the dermis. Besides these cells, there are

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numerous macrophages located in the dermis which are positive for mMGL (murine macrophage galactose–type C-type lectin/CD301), F4/80 and CD11b (2).

In the murine system, Langerin is a useful marker for LC in the epidermis and skin-draining lymph nodes (3, 4), though it also reacts with a subset of dermal DC that are distinct from migratory LC on their way to the lymph nodes (5–7). There are two markers that allow discrimination of Langerin$^+$ LC and Langerin$^+$ dermal DC in the skin and lymph node, the integrin CD103 and the membrane glycoprotein Ep-CAM/CD326 (7, 8). Hence, we can distinguish three types of migratory skin DC (**Fig. 16.1**): LC as Langerin$^+$CD103$^-$Ep-CAM$^+$, dermal

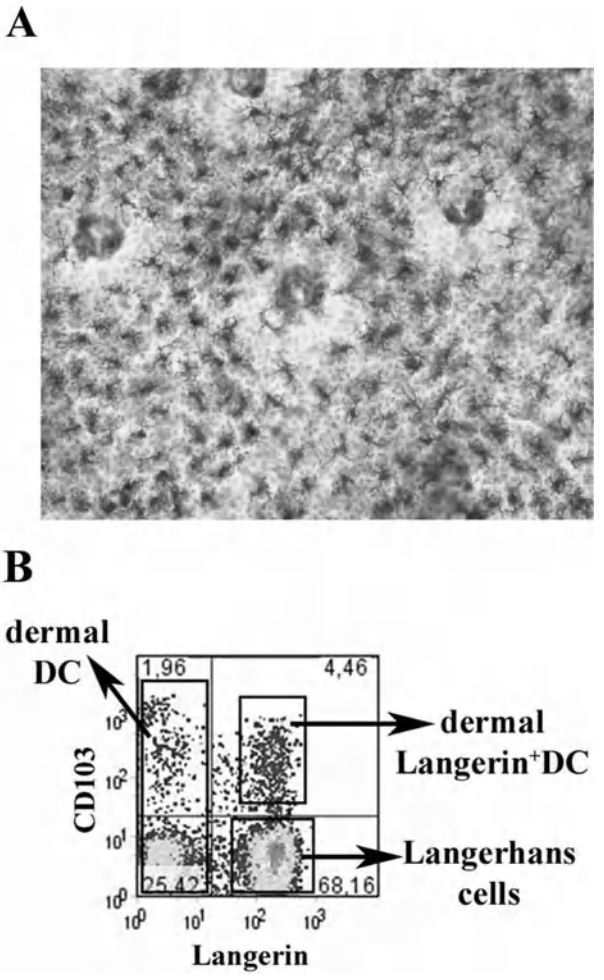

Fig. 16.1. LC form a dense network in the epidermis as visualized here with MHC class II staining on an epidermal sheet (**A**). Skin DC migrating out of whole skin explants can be subdivided into three subsets based on their Langerin and CD103 expression as shown in (**B**).

Langerin$^+$ DC as Langerin$^+$ CD103$^+$ Ep-CAM$^-$ and the dermal DC as Langerin$^-$ partly CD103$^+$ Ep-CAM$^-$. As a common feature they all express CD11c and high levels of MHC class II as well as costimulatory molecules like CD40 and CD86 after migration to the lymph nodes (9). Differences in the function of these three subsets are less clear and further research is required to unravel their function in vivo.

In the human system, Langerin and CD1a allow identification of LC in the epidermis, whereas the dermal DC are typical myeloid DC which can be identified by DC-SIGN/CD209, CD11c and BDCA-1/CD1c. Few plasmacytoid DC identified by IL-3Rα/CD123 and BDCA-2/CD303 can be observed (10, 11). In the dermis there are many macrophages which are positive for the markers M130/CD163 and factor XIIIa in human skin (11).

In recent years skin DC gained more and more interest since their in vivo function is still unclear. The development of transgenic mice in which Langerin$^+$ DC can be selectively depleted allowed for the first time to study specifically the role of LC as well as dermal Langerin$^+$ DC (12–14). However, the first results are not entirely conclusive and there is an ongoing debate whether LC are required for induction of immunity or simply exert tolerogenic functions (15). For this reason, further research is required to illuminate the physiological role of the different skin DC subsets. As a prerequisite we need to isolate skin DC from skin and draining lymph nodes to study their phenotype and function in vitro. In the recent years we have developed and refined established methods (16–18) to isolate, culture and enrich murine and human LC. Skin DC can be received from the skin by enzymatic digestion of the tissue or by skin explant culture. These protocols will be described in this chapter.

2. Materials

2.1. Mechanical Tools for Skin Preparation

1. Two strong forceps with rounded tips to split ear halves and prepare body skin.
2. Two thin curved forceps to peel off epidermis.
3. Scissors to cut skin.
4. Scalpel with size 10 blades to cut skin into smaller pieces and scrape off subcutaneous fat.
5. Bacteriological 100-mm Petri dishes (Falcon 1029) for skin preparations, tissue culture Petri dishes (100 mm) for skin DC culture (Falcon 3003).

6. Sterile gauze to dry ear skin.
7. 100-μm Cell strainers (Falcon 2360).
8. Silver-type hand dermatome (Martin 10-882-19; right handed).
9. Biopsy punches (Kai Medical, Kai Europe).

2.2. Solutions/Saline Solutions

1. 70% ethanol
2. Fetal bovine serum (FBS).
3. Phosphate-buffered saline (PBS) and Hank's balanced salt solution (HBSS) both without calcium and magnesium salts.
4. RPMI.
5. 1 M HEPES.
6. Bovine serum albumin (BSA).
7. 0.5 M EDTA solution.
8. 200 mM L-glutamine solution.
9. 50 M 2-mercaptoethanol solution.
10. 50 mg/ml Gentamicin solution.

2.3. Enzymes

1. For murine LC preparation use lyophilized trypsin from Merck (cat. no. 1.08350.0001) which is dissolved at room temperature in pre-warmed PBS to prepare a 0.8% solution. When completely dissolved sterile-filter solution and freeze aliquots at −20°C. For human LC preparation use 0.8% trypsin for *full-thickness* skin or 0.05% trypsin/EDTA (cat. no. 25300; Gibco–Invitrogen) for *split-thickness* skin.
2. For enzymatical separation of epidermis and dermis use lyophilized dispase II from Roche (cat. no. 04942078001) which is dissolved in HEPES-buffered saline (50 mM HEPES/KOH pH 7.4, 150 mM NaCl at 10 mg/ml according to manufactures' protocol. Dispase solution should be sterile-filtered and can be stored at −20°C. For use dispase II is diluted to 1.2 U/ml with RPMI.
3. For "Wuerzburger buffer" use lyophilized desoxyribonuclease (DNase) I from Roche (cat. no.11284932001) which is dissolved in PBS to make up a stock solution of 4 mg/ml. Solution should be sterile-filtered and can be stored at −20°C.

2.4. Buffers

1. "Wuerzburger buffer": PBS supplemented with 1% BSA or 1% FBS, 5 mM EDTA and 20 μg/ml DNase I.
2. MACS buffer: PBS supplemented with 0.5% BSA and 2 mM EDTA according to manufactures' protocol.

2.5. Culture Medium

RPMI-1640 or IMDM (which seems to be superior for human and murine skin culture) supplemented with 5–10% FBS (sources and batches are not critical), 2 mM L-glutamine, 50 nM 2-mercaptoethanol and 50 μg/ml gentamicin (penicillin/streptomycin may be added as precaution to minimize contamination).

2.6. Growth Factors

1. Murine GM-CSF: supernatant of the transfected plasmacytoma cell line X38-Ag8, kind gift of A. Lanzavecchia, Bellinzona, CH.
2. Human GM-CSF: Leukine (sargramostim), Bayer Health Care Pharmaceuticals, Berkley, CA, USA).

2.7. Gradients

1. 14% Nycodenz (Histodenz, Sigma D2158):
   Solution A: 500 ml of 308 mOsm (EDTA-SS) "Shortman buffer" (19) 0.154 M NaCl (4.5 g), 4 mM KCl (0.1491 g), 14.8 mM HEPES (2.96 ml of 2.5 M stock; pH 7.2), 5 mM EDTA (5 ml of 0.5 M), make up to 500 ml volumetrically with dH_2O.
   Solution B: 230.78 ml of 30.55% Nycodenz ($d = 1.16$) 70.5 g Nycodenz powder, make up to 230.78 volumetrically.
2. Lymphoprep 1077: density 1.077+/–0.001 g/ml (20°C), from Fresenius Kabi Norge AS.

2.8. Magnetically Activated Cell Sorting (MACS) Materials (Miltenyi Biotec, Bergisch-Gladbach, Germany)

1. Large cell columns (#130-042-202) or MS columns (#130-042-201).
2. Pre-separation filters (#130-041-407).
3. Anti-mouse MHC class II MACS beads (#130-052-401).
4. Anti-human CD1a MACS beads (#130-051-001).

3. Methods

3.1. Isolation of Langerhans Cells from Mouse Skin

3.1.1. Skin Preparation and Trypsinization

LC can be prepared from mouse ear and/or trunk skin. The mouse ears are cut off at the base with scissors and rinsed briefly in 70% ethanol. Before trypsinization ear skin has to be air-dried on sterile gauze for 20 min. For preparation of trunk skin remove hair by plucking against the growth direction of hair. Mouse body is placed into a Petri dish and thoroughly rinsed with 70% ethanol. Make a transverse cut with scissors near the base of the tail of the back and cut upwards on both sides to the axilla. Peel off the skin with forceps and cut off the skin at the neck. Turn mouse around and remove the abdominal skin the same way. Trunk skin is placed

dermal side up in a Petri dish and with a scalpel the subcutaneous fat is scraped off. For trypsinization skin is placed on 10 ml of a 0.8% trypsin solution in a Petri dish. The body skin is cut into 2 × 2 cm pieces and incubated dermal side down on the trypsin solution. Mouse ears are split into dorsal and ventral halves (containing the cartilage) with two strong forceps and placed dermal side down on the trypsin solution (*see* **Note 1**). The body skin and ventral halves of ear skin are incubated for 45 min, the thinner dorsal halves of ear skin for 25 min at 37°C.

3.1.2. Isolation of LC

After 25 min take dorsal ear halves off the trypsin and transfer them onto 10 ml pure FBS in a Petri dish (*see* **Note 2**). After another 20 min remove all other skin pieces from the trypsin solution and place them onto FBS. Peel off the epidermis with the thin curved forceps while the skin is floating on FBS, leave epidermis in FBS but discard the dermal pieces. Transfer epidermal pieces to a 50-ml tube containing 20 ml complete medium (*see* **Note 3**) and shake the pieces for 30 min in the water bath at 37°C to release LC. Afterwards remaining epidermal pieces and cell suspension are filtered through a 100-μm cell strainer into a 50-ml tube followed by centrifugation at 450 × *g* for 5 min at 4°C. While epidermis is peeled off the dermis, many epidermal cells are released and fall into the FBS solution. Thus, it is worthwhile to also filter the FBS solution through a 100-μm cell strainer into a 50-ml tube, rinse Petri dish carefully with complete medium and spin the cells with 450 × *g* for 5 min at 4°C. An alternate method to that described above is to place a sterile tea strainer into medium in a Petri dish and move the pieces of epidermis across the surface of this metal sieve to dislodge the basal epidermal cells as described earlier (17). Resuspend cells in complete medium and wash once more in complete medium.

3.1.3. Cell Yields and Culture of Epidermal Cells

The epidermal cells are counted in the hemocytometer. Cell viability should be 80–90% and LC are indistinguishable from the keratinocytes. You should obtain roughly 2–4 × 10^6 cells per mouse ear and 15–30 × 10^6 cells from the dorsal and abdominal skin of one mouse. The LC contribute roughly 1–3% of all the epidermal cells; the other cells are keratinocytes and variable numbers of dendritic epidermal T cells (DETC) depending on the mouse strain (16). Epidermal cells can be cultured at 7 × 10^6 cells per culture Petri dish in complete medium supplemented with 200 U/ml GM-CSF for 3 days (*see* **Note 4**). After 3 days of culture around 3 × 10^4–1.5 × 10^5 mature LC per Petri dish can be obtained.

3.1.4. Enrichment of LC

Separation of immature LC from keratinocytes is very difficult, yet there are protocols available to enrich freshly isolated LC (17). In contrast, mature LC can be easily separated from epidermal

cells after 1–3 days of culture. Some keratinocytes will develop a monolayer at the bottom of the Petri dish, however, most of them will die during the 3 days of culture since they require a special medium and growth factors for extended survival and/or differentiation. Mature LC can be separated from dead keratinocytes and enriched by gradient centrifugation. We routinely use Nycodenz gradient to enrich DC from mixed cell suspensions. Epidermal cells derived from 1 to 5 culture Petri dishes can be loaded onto one gradient. Cells are resuspended in 5 ml Nycodenz solution, overlaid with 2 ml of "Shortman buffer" and centrifuged at 680 × *g* for 20 min at 4°C (*see* **Note 5**). Mature LC can be found in the interphase around the 5 ml mark. This layer should be removed using a glass Pasteur pipette and washed two times in 10 ml of "Wuerzburger buffer". Purity of LC should be roughly around 40–80% of viable cells.

Further enrichment of LC can be achieved by magnetic cell sorting. For this purpose epidermal cells derived from the interphase of Nycodenz gradients are labelled with anti-MHC class II MACS beads after blocking Fc receptors (by addition of a mAb (clone 2.4G2) for 5 min in the fridge). For less than 10^7 cells use at least 10 µl MHC class II-MACS beads and add 90 µl "Wuerzburger buffer". For more cells use the recommended 10 µl MHC class II-MACS beads per 10^7 cells according to the manufacturers' protocol. Cells are incubated for 15 min in the fridge, mixed every 5 min to prevent clumping and washed in "Wuerzburger buffer". For magnetic cell separation we use mini or large cell columns with pre-separation filters. The usage of large cell columns and pre-separation filters prevents blockages of columns. Furthermore, the matrix of large cell columns is more open and results in higher yields of LC. The labelled cells are resuspended in 2 ml "Wuerzburger buffer" and loaded onto the column in four subsequent portions, each at 500 µl. The columns are washed two times with "Wuerzburger buffer" before LC are eluted from the column outside the magnetic field with 3–4 loads of 2 ml "Wuerzburger buffer". Cell yields can be increased by running the negative fraction through the column once again. The purity can be improved by eluting the cells from the column with 2 ml "Wuerzburger buffer" and running them through the same column. During the whole purification process up to half of the LC can be lost meaning that you can obtain roughly 1.5×10^4 -7×10^4 mature LC per Petri dish (purity was routinely above 90%).

3.2. Generation of Migratory Murine Skin DC

Skin DC migrate spontaneously from skin explants cultured on complete medium (20) and cells derived from these cultures represent mature DC. At onset of culture, skin compartments can be separated enzymatically or can be cultured as whole skin explants as described in the following part.

3.2.1. Whole Skin Explant Culture

Mouse ears are cut off at the base and rinsed briefly in 70% ethanol before they are air-dried on sterile gauze for 20 min. With two strong forceps the ventral (containing the cartilage) and dorsal ear halves can be split. The cartilage-free dorsal halves are floated dermal side down on 2 ml complete medium in 24-well plates (one ear/well) and cultured for 3 days at 37°C (*see* **Note 6**). After removal of the skin explants the migratory DC can be harvested from the culture medium. The cells are centrifuged at 450 × *g* for 5 min at 4°C and resuspended in a small volume for counting in the hemocytometer. Roughly a third to a half of the cells are mature DC that can be recognized easily by their numerous dendrites (**Fig. 16.2**), both in phase contrast and under the hemocytometer. The remaining cells are smaller T cells. On average 5×10^3–2×10^4 DC (two third of them are Langerin$^+$CD103$^-$ LC, rest dermal DC, (*see* **Fig. 16.1**) can be obtained from one mouse ear (*see* **Note 7**). Mouse trunk skin is not suitable for whole skin explant culture since DC get stuck in the dermis and cell yields are poor. Furthermore, addition of chemokines into the culture medium is not required to attract skin DC. Skin explant culture reflects an inflammatory setting and the release of proinflammatory cytokines is sufficient to induce emigration of skin DC.

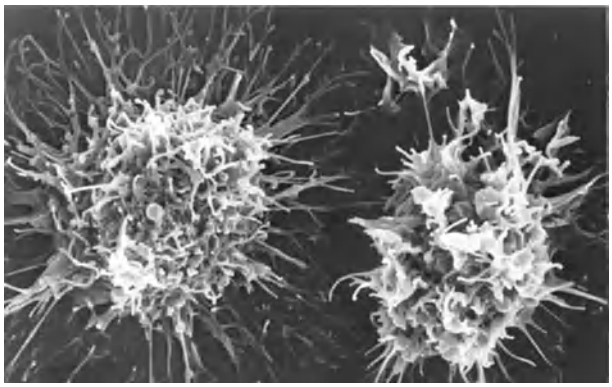

Fig. 16.2. Migratory skin DC can easily be recognized by their many dendrites as shown here in a scanning electron microscopy picture of DC from whole skin explants. (Courtesy of K. Pfaller, Department of Histology and Embryology, Innsbruck Medical University.)

3.2.2. Epidermal Explant Culture

For culture of epidermal explants both ear and trunk skin can be used. Trunk and ear skin is prepared as described above for the LC isolation procedure. Small pieces of body skin (squares of 2 × 2 cm) and ventral as well as dorsal ear halves are incubated dermal side down on 10 ml of 1.2 U/ml dispase II in Petri dishes for 30 min for dorsal ear halves and 60 min for ventral ear halves and trunk skin at 37°C (*see* **Note 8**). Skin is transferred to 10 ml pure FBS in a Petri dish and epidermis is peeled off. The

epidermal pieces are cultured on 2 ml complete medium in 24-well plates for 3 days at 37°C. After removal of epidermal pieces emigrated cells can be harvested from culture medium. Cells are centrifuged at 450 × *g* for 5 min at 4°C and resuspended in a small volume for counting in the hemocytometer. Half of the cells will be mature LC, the rest dendritic epidermal T cells and keratinocytes. In general, culture of epidermal explants from trunk skin results in a lower enrichment of LC than from ear skin. On average 1.5×10^4–3×10^4 LC can be expected from one ear (both ear halves) and roughly 1×10^5–2×10^5 LC from the entire back and abdominal skin of one mouse.

3.3. Isolation of Langerhans Cells from Human Skin

3.3.1. Skin Preparation and Trypsinization

Human skin from breast or abdominal reduction surgery can be used. *Full-thickness* skin should be cleaned of subcutaneous fat and part of the dermis should be removed by slicing pieces with a dermatome. For this purpose, the skin is fixed onto a rubber cylinder (the curved surface facilitates the cutting process) against a piece of immobilized, alcohol-disinfected, dry (bench-coat) paper. Dampen the skin slightly and dip the dermatome into a sterile beaker of PBS to keep the skin moist during cutting. With the right hand, move the knife gently from side to side (~2 mm each direction), turning the cylinder very slowly in the direction of cutting, keeping pace with the speed of the cut. This lifts up the split skin onto the back of the knife. The razor blade may need turning around as it becomes blunt with large skin specimens (**Fig. 16.3**). *Split-thickness* skin is cut into 0.5 cm × 0.5 cm pieces with a scalpel and incubated dermal side down overnight on 0.05% trypsin/EDTA solution in Petri dishes in the fridge at 4°C. *Full-thickness* skin needs a higher trypsin concentration of 0.8% for overnight digestion in the fridge (*see* **Note 1**).

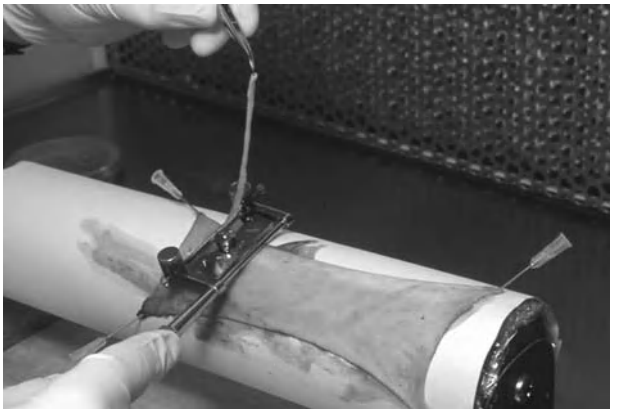

Fig. 16.3. Full thickness skin should be cut with a dermatome to obtain thinner skin pieces for optimal enzymatic digestion as shown in the picture above.

Next morning, transfer plates to the incubator for 1 h at 37°C (*see* **Note 2**).

3.3.2. Isolation of LC

Epidermis can be peeled off the skin pieces with two thin curved forceps. The epidermal pieces are collected in Petri dishes with RPMI for *split-thickness* skin (trypsin activity should not be stopped) or complete medium for *full-thickness* skin (to stop the higher concentrated trypsin). The epidermal pieces are squeezed with the plunger of a syringe in the Petri dish so that cells are released from the epidermis and RPMI/complete medium becomes cloudy. The resulting cell suspension should be filtered through 100-μm cell strainers into 50-ml tubes. The epidermal pieces that remain in the cell strainers are transferred into new plate with RPMI/complete medium and squeezed once more with the plunger of a syringe to obtain as many cells as possible. The epidermal cells are centrifuged at 300 × *g* for 10 min at 4°C. After centrifugation resuspend the cells in complete medium to stop digestion by trypsin (*see* **Note 3**).

3.3.3. Enrichment of LC

Density gradients with 10 ml Lymphoprep gradient into 50-ml tubes are overlaid with 30 ml epidermal cell suspension (1 gradient/2–3 Petri dishes used for the trypsinization). After spinning for 30 min with 450 × *g* at 20°C, collect cells from interphase and transfer them to 50-ml tubes with 20 ml PBS/EDTA. Two gradients can be combined into one tube and are centrifuged at 300 × g for 10 min at 4°C. For magnetic cell separation resuspend 10^7 cells in 80 μl MACS buffer and add 20 μl of CD1a-MACS beads in a 15-ml tube, incubate cells for 15 min in the fridge and shake tube every 5 min. Wash cells with MACS buffer and spin at 450 × *g* for 10 min at 4°C. The labelled cells are loaded onto large cell columns and after running through, the columns are washed three times with 500 μl MACS buffer. Remove the column from magnet, place column on a 15-ml tube and flush out cells with 1 ml MACS buffer using a plunger. From one Petri dish (ca. 60–70% covered with pieces of skin; roughly equivalent to 45–55 cm^2 of skin) you should obtain 7.5×10^4–4.5×10^5 cells after MACS sorting. The LC suspension should contain 62–93% CD1a positive cells.

3.3.4. Culture of LC

We routinely culture 5×10^5 cells in 1 ml complete medium supplemented with 1,000 U/ml GM-CSF per well in 48-well plates for 2 days to obtain mature LC (*see* **Note 9**). Culture can be performed as well in serum-free medium but cell loss will be higher than with medium containing serum.

3.4. Generation of Migratory Human Skin DC

For this purpose it is advisable to use *split-thickness* skin (best 0.3 mm thick) prepared with a dermatome since more DC emigrate from skin explants and separation of epidermis and dermis

with dispase II is easier (*see* **Note 10**). It should also be noted that DC/LC obtained by this method are always mature and that the addition of chemokines is not required to induce migration of skin DC.

3.4.1. Whole Skin Explant Culture

Standardized pieces of healthy skin are prepared with an 8-mm biopsy punch from *split-thickness* skin. Skin explants are floated on 1.5 ml complete medium in 24-well plates (one skin explant per well) for 3–5 days at 37°C. Alternatively, 0.5 × 0.5 mm pieces of skin can be incubated on 15 ml complete medium in a culture Petri dish and should be covered maximal 50–60% with skin pieces (*see* **Note 11**). Cells that have emigrated into the medium during culture can be harvested and counted in the hemocytometer. The cell suspensions consist of 50–60% DC and the rest are T cells. Regarding cell yields, 3.5×10^3–1.2×10^4 DC can be obtained from one punch biopsy (i.e., approx. 0.5 cm^2). A minority of the skin DC are LC (10–30%), as defined by immunolabelling for Langerin/CD207 and the rest are dermal DC.

3.4.2. Epidermal or Dermal Skin Culture

Before the onset of culture, the epidermis and dermis can be split with dispase II. Skin pieces of 0.5 × 0.5 mm are incubated on 1.2 U/ml dispase II for 20–40 min at 37°C. The epidermis is peeled off the dermis and with 8-mm biopsy punches epidermal and dermal skin explants can be prepared before they are placed onto 1 ml complete medium in a 24-well plate (one skin explant per well). The skin explant culture can be performed in a culture Petri dish as well (cut 0.5 × 0.5 mm pieces); however, enrichment of LC/DC is routinely lower. Migratory LC/DC can be harvested after 3–5 days of culture at 37°C. From one epidermal skin explant you can expect 2×10^3–1.3×10^4 LC which are enriched up to 80–90%. In the dermal explant cultures 8×10^3–2×10^4 DC can be obtained which are 30–70% enriched (other cells are T cells). Like in murine explant cultures, DC can readily be recognized by their pronounced dendritic, "hairy" shape in phase contrast and under the hemocytometer.

4. Notes

1. Skin pieces should float on the trypsin solution to achieve digestion through the dermal side. This prevents over digestion of epidermis so that epidermis can be peeled off in one piece.
2. At the end of the incubation on trypsin you should test with one or two skin pieces if the epidermis can be peeled off easily in one piece. If this is not the case transfer Petri

dish back into the incubator for a few more minutes and try again. Depending on the trypsin the digestion can take longer than stated in this protocol.

3. It is important to use complete medium for the incubation step in the water bath to stop the trypsin activity with the FBS in the medium. There might be still enough trypsin in the skin pieces that keeps digesting the tissue and the cells.
4. The addition of GM-CSF is not essential in bulk cultures containing keratinocytes since they produce enough GM-CSF for the LC to survive and mature. However, to ensure that there will be enough GM-CSF around, we routinely supplement the medium with GM-CSF.
5. The enrichment of LC with gradient depends greatly on how carefully the "Shortman buffer" is overlaid onto the cells suspended in the Nycodenz solution.
6. The ear skin explants spread out when placed on the medium when they have been dried carefully after disinfection with ethanol. More migratory skin DC can be obtained when culture is performed in 24-well plates than in 100-mm Petri dishes for unknown reason.
7. The number of migratory skin DC obtained from one ear depends on the mouse strain used since more DC migrate out of BALB/c ear skin than from C57BL/6.
8. As described earlier for LC isolation with trypsin, the skin pieces should float on the dispase solution. Again test with one or two skin pieces after incubation period if epidermis can be peeled off easily or if the digestion takes longer.
9. Depending on the grade of enrichment after MACS sorting LC will need GM-CSF for culture. When the LC enrichment is low after sorting meaning more keratinocytes left in the cell suspension, it is not absolutely essential to add GM-CSF to the culture.
10. The DC yields derived from human skin explants vary a lot depending on skin thickness and the handling of skin during surgery and preparation.
11. When Petri dish contains more skin pieces and is completely covered, cells might starve and undergo apoptosis depending on duration of culture.

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Chapter 17

Identification and Isolation of Rodent Respiratory Tract Dendritic Cells

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Abstract

This chapter describes the preparation of respiratory tract tissue from both mice and rats for the isolation of respiratory tract dendritic cells (RTDC). The methods describe in detail the preparation of cells from the respiratory tract tissue of the main conducting airways (representing mucosal populations) and peripheral lung (representing predominantly interstitial populations) in both rodent species. Our research in this area has found that these anatomical sites differ in their composition of antigen-presenting cell (APC) types including RTDC, and that phenotypic and functional differences exist in RTDC isolated from these sites. We predominantly use a flow cytometry-based approach to identify and sort RTDC as this is the most accurate way of isolating RTDC subsets in an environment where many typical dendritic cell surface markers are shared by other APC populations.

Key words: Dendritic cell, respiratory tract, lung, airways, mice, rats, isolation, cell sorting.

1. Introduction

Respiratory tract dendritic cells (RTDC) are a trace cell population found within the different anatomical compartments that constitute the respiratory tract, that display potent antigen acquisition and presentation capabilities and are unique amongst antigen-presenting cell types of these tissues in their ability to traffic antigen to draining lymph nodes and activate naive T cells (1). RTDC are present as an interstitial population within lung parenchymal tissue and also within the nasal turbinates and are particularly prominent at the mucosal surfaces of the conducting airways, where they are thought to play a critical role in

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the regulation of immune responses to environmental antigens and infectious agents (2–5). In airways, RTDC are found in the airway epithelium and underlying mucosa where they form an integrated network of cells (6). Studies in rats (which appear to resemble humans most closely in terms of DC distribution) have indicated that this network of epithelial RTDC develops slowly after birth from MHC class II negative precursors arriving from the bone marrow: cell numbers and MHC class II expression slowly increases with time, presumably in response to environmental antigens, reaching adult-equivalent levels at 4–6 weeks of age (4). Whether this epithelial network exists in humans, however, and the rate at which it develops from birth still remain unclear. A unique feature of the airway intraepithelial population of RTDC is their rapid rate of turnover: under steady-state conditions, these cells display turnover rates in the order of 2–3 days which is markedly more rapid than the lung parenchymal population (7–10 days) or epidermal populations (>21 days) (7, 8). This turnover rate can be further increased by exposure to inflammatory stimuli including bacteria, virus and also inert protein (9). This unique responsiveness of epithelial RTDC to inflammation highlights the importance of these cells in antigen surveillance at this site.

RTDC have been shown to have a complex life history, with evidence suggesting that these cells are poor stimulators of T-cell responses but highly specialized for antigen uptake and processing when resident in airway tissue (10, 11). In response to maturation stimuli, however, these cells will then enter the afferent lymph and migrate to draining secondary lymphoid organs where they act as potent stimulators of naive T cells (12). This pattern of DC maturation also appears to apply to lung and airway mucosal DC. Freshly isolated RTDC express moderately high levels of MHC class II but are relatively poor stimulators of naive T-cell responses, although these cells show high levels of endocytic and antigen uptake activity in vitro and acquire soluble antigen in vivo (13). In response to maturation factors such as LPS or GM-CSF, however, marked increases in cell surface expression of MHC class II and co-stimulatory molecules, with concurrent increases in naive $CD4^+$ T-cell stimulating activity, are observed and this is associated with a decrease in endocytic activity (14). This maturation process in association with directed migration to draining lymph nodes appears to be typical of DC of myeloid origin and is thought to limit the potential of these cells to induce local tissue inflammation (15). RTDC have been shown to express a range of surface markers typical of most tissue DC populations. In rodents, these cells express moderately high levels of the typical DC markers MHC class II and CD11c, although care must be taken as other cell types within the respiratory tract such as alveolar and interstitial macrophages also express these markers (8).

Rat and mouse RTDC also express a range of other markers such as CD4 (rat only), CD11b, CD103 and CD205 which may be useful in the identification of functional subsets of these cells (8) (*see* **Table 17.1**). The co-stimulatory molecules CD40, CD80 and CD86 tend to be expressed at low–moderate levels on freshly isolated RTDC and are upregulated to varying degrees following maturation depending on the species (CD40 more readily in mouse than rat; CD86 more readily in rat than mouse) and the nature of the maturation signal (13, 16). More recently, we have found the use of both in vitro and in vivo antigen uptake and processing assays useful in the identification of highly endocytic populations of RTDC in the airways and lungs and also antigen-bearing DC in draining lymph nodes (12).

Table 17.1
Surface phenotype of mouse respiratory tract APC populations (adapted from (8))

	Surface markers[1]							
APC type	**I-A^d**	**CD11c**	**CD11b**	**CD2**	**CD103**	**CD115**	**F4/80**	**Gr1**
CD11b$^-$ DC	high[2]	high	–	–	high	–	–	low
CD11b$^+$ DC	high	high	high	int	high	–	mod	int
B cell	high	–	–	high	–	–	–	–
Macrophage	high	high	high	high	high	–	high	mod

[1] All populations are uniformly high for CD16/32, CD54 and CD205
[2] <10%; low = 11–20%; int = 21–40%; mod = 41–60%; high > 61%

Another important consideration in the isolation of RTDC is the anatomical location of the cells. In addition to the difference in turnover rates described above, RTDC isolated from the mucosal surfaces of the conducting airways of both rats and mice differ in their phenotype and functional activity and their behaviours during the induction of respiratory inflammatory diseases such as allergic asthma. For example, during the induction of experimental allergic airways disease in mice we have found that a hallmark of the initiation phase is the upregulation of CD40 preferentially on airway mucosal DC as opposed to lung tissue DC following allergen inhalation, suggesting that the mucosal population of cells are more responsive to maturation stimuli provided by inhaled innocuous stimuli. In addition, studies in rats have shown that the airway mucosal population of RTDC are the first to interact early during disease induction with inflammatory CD4$^+$ T cells and may be closely regulated by regulatory T cells within the respiratory mucosa (16, 17). In contrast, a range of other mechanisms may act within the deeper interstitial lung tissue to regulate RTDC activity, including interactions with alveolar

macrophages and the secretion of nitric oxide which is inhibitory to RTDC and T-cell activity (18, 19).

The following chapter describes the basic protocols that we use primarily for the isolation and purification of RTDC from the airways and lungs of rats and mice, principally by flow cytometry and cell sorting. Although other non-sorting methods can be used, these typically result in less pure populations of cells that may contain other potential antigen-presenting cell types such as B cells and macrophages that are present within rodent respiratory tissue and which can express equivalent levels of CD11c (alveolar macrophages and to a lesser extent airway mucosal macrophages) and MHC class II (B cells in rats and mice) in some cases (8). In rats, sorting approaches are based on the unique expression of MHC class II by RTDC following rigorous removal of other non-DC, MHC class II-expressing cells such as B cells and macrophages that are found in lung cell preparations. In mice, our primary approach is to sort based on the relative expression levels in RTDC when compared to other populations such as B cells and macrophages that are present in lung tissue. These cells show all the properties of immature RTDC in terms of antigen acquisition and T-cell stimulation and are responsive to maturation factors such as GM-CSF and are thus suitable for a variety of functional studies.

2. Materials

2.1. Animals

Animals to be used as respiratory tissue donors should be between 8 and 12 weeks of age to ensure maximum cell yields. For rat studies, we routinely use male or female PVG or BN strain rats bred under clean conditions in-house, with the BN strain giving the greatest recoveries. Care should be taken, however, when working with different rat strains as RTDC populations may vary slightly in phenotype. For mouse studies, we routinely use female BALB/c mice sourced SPF from a commercial supplier and housed under clean conditions. All animals are given free access to food and water and housed under standard conditions on low-dust bedding.

2.2. General Reagents

1. Sodium pentobarbital solution (Nembutal) –325 mg/ml.
2. Bovine serum albumin (BSA) (Sigma Chemicals, MO, USA). Store at −20°C as a 5% w/v solution in PBS.
3. Fetal calf serum (FCS) (TRACE Biosciences, Melbourne, Australia). Heat-inactivate at 56°C for 30 min and store at −20°C.

4. Type IV collagenase (Worthington, Lakewood, NJ, USA). Store desiccated at −20°C (*see* **Note 1**).
5. Deoxyribonuclease I from bovine pancreas (DNAse I; Sigma Chemicals). Store dessicated at −20°C.
6. Mouse recombinant granulocyte macrophage-colony stimulating factor (rGM-CSF) (Biosource International, CA, USA). Make a 1 μg/ml stock solution in PBS–BSA and store at −20°C
7. Cotton wool filters – 5-ml syringe barrel plugged with a small volume (approximately 1 ml) of loosely packed cotton wool and autoclaved.

2.3. General Buffers and Media

1. Glucose–potassium–sodium (GKN) buffer made as follows (*see* **Note 2**): 11 mM D-glucose, 5.5 mM KCl, 137 mM NaCl, 25 mM $Na_2HPO_4 \cdot 12H_2O$, 5.5 mM $NaH_2PO_4 \cdot 2H_2O$. Filter-sterilize and store at 4°C.
2. GKN containing 0.2% w/v BSA (GKN–BSA).
3. GKN containing 5% FCS (G-5).
4. GKN containing 10% FCS (G-10).
5. GKN containing 50% FCS (G-50).
6. Phosphate-buffered saline (PBS).
7. PBS containing 0.5% w/v BSA (PBS–BSA).
8. Digestion buffer: G-10 containing 1.5 mg/ml type IV collagenase and 0.1 mg/ml DNAse I. Make fresh on day of use.
9. RPMI containing L-glutamine (Gibco BRL, NY, USA) supplemented with 5% FCS (R-5).

2.4. Monoclonal Antibodies and Fluorescence Staining Reagents

1. *Anti-rat monoclonal antibodies*
   The monoclonal antibodies routinely used for the isolation of rat RTDC by flow cytometric sorting are available commercially from BD Biosciences, Sydney, Australia, eBioscience, San Diego, USA or Serotec, Kidlington, UK. The basic set of antibodies for phenotyping and sorting of RTDC is used as direct conjugates (FITC, PE, biotin or APC). For both mouse and rat, the specific combinations of antibody–fluorochrome conjugates used will depend on antibody availability and cytometer configuration.
   a. Anti-rat MHC class II (clone OX6)-FITC, -PE, -biotin, -APC. (Care should be taken with OX6-APC as at least some rat strains (BN) will not stain with this conjugate.)

b. Anti-rat immunoglobulin light chain (clone OX12)-FITC, -PE.

c. Anti-rat CD4 (clone W3/25 or OX35)-PE, -APC.

d. Anti-rat CD103 (clone OX62)-biotin.

e. Anti-human C3b inactivator as isotype control (clone OX21).

f. Biotinylated anti-rat CD172, CD80 and CD86 (Serotec, UK).

2. *Anti-mouse monoclonal antibodies*
The following antibodies represent a basic set of markers routinely used for the isolation of mouse RTDC by flow cytometric sorting. A variety of other markers can be used to label different subsets of antigen-presenting cells based on surface phenotype using the markers described in **Table 17.1**; however, most require a flow cytometry-based approach to differentiate high- and low-expressing populations of cells. Antibodies are typically sourced commercially from BD Biosciences, Sydney, Australia, eBioscience, San Diego, USA or Biolegend, San Diego, USA as direct conjugates to FITC, PE, biotin or APC (*see* **Note 3**).
   a. Anti-mouse I-A/I-E (2G9 or M5/114.15.2, BD Biosciences).

   b. Anti-mouse CD11c (HL3, BD Biosciences or N418, BioLegend).

   c. Anti-mouse CD11b (M1/70, BD Biosciences).

   d. CD205 (DEC-205, Cedarlane Laboratories).

   e. Co-stimulatory molecule expression can be assessed using biotinylated mAbs against CD40, CD80 or CD86 (clones 3/23, 16-10A1 and GL1, respectively, BD Biosciences).

3. Other fluorescence staining reagents
   a. Purified mouse IgG1 conjugated to FITC (IgG1-FITC) (Dako, Denmark). Used at a final concentration of 5 μg/ml.

   b. Streptavidin conjugated to FITC (SA-FITC), PE (SA-PE), PerCP (SA-PerCP) or APC (SA-APC) – BD Biosciences, Australia.

   c. Polyclonal goat anti-mouse IgG conjugated to phycoerythrin (GAM-PE) (Dako, Denmark).

   d. Normal mouse serum (NMS) – heat-inactivated and stored at −20°C.

   e. Anti-PE Dynabeads® and depletion magnet (Invitrogen Australia, Victoria, Australia).

3. Methods

3.1. Isolation of Single Cells from the Respiratory Tract

This section describes the removal of whole respiratory tract tissue for enzymatic digestion. This will include conducting airway, hilar and parenchymal regions, often referred to as 'whole lung' and will allow isolation of a population of total respiratory tract DC (RTDC). We also have protocols for further dissection and isolation of airway mucosal DC and peripheral lung DC (*see* **Note 4**) as we have found the distribution of DC populations to differ between these anatomical sites (8). We usually work with groups of five to six animals in order to provide enough tissue for cell isolation. All steps have been optimized to avoid delay in removing and labelling tissue following euthanasia. Keep tissue and cells cold in order to limit autolysis.

3.1.1. Removal of Lungs

1. Euthanize up to three animals at a time by i.p. sodium pentobarbitone overdose.
2. Open the abdominal cavity and exsanguinate the animal by severing the abdominal aorta and inferior vena cava. Blot with tissue paper.
3. Cut around the diaphragm and remove the ribcage. Take care not to nick the lungs with scissors or forceps as this will cause bleeding into the tissue that cannot be removed by perfusion. Once the ribcage has been removed, cut through the clavicles and muscles of the neck to expose the trachea.
4. Perfuse gently through the heart with cold PBS until the lungs have turned white and are free of blood; 5–10 ml (rats) or 1–2 ml (mice) PBS is slowly administered via the right atrium/ventricle over approximately 1 min. The majority of the blood is washed out within a few seconds with this method. Avoid perfusion at too high pressure and administration of PBS via the left side of the heart as this will create pulmonary oedema. One indication of pulmonary oedema is the appearance of PBS running out of the nose of the animal by the end of perfusion, and oedematous lungs acquire a spongy watery appearance.
5. Clamp the great vessels and excise the heart after removing the mediastinal connective tissue.
6. Remove thymus and associated lymph nodes and connective tissue. Grasp the trachea with forceps and separate it from the oesophagus. Cut the trachea as close to the larynx as possible and lift the lungs and trachea out of the thoracic cavity, freeing it from connective tissue and the mediastinal lymph node while dissecting towards the tracheal bifurcation

and hili. Care should be taken to remove as much of the connective tissue as possible from the outside of the trachea. Flush the trachea with PBS.

7. Separate the main conducting airways from the lung tissue by cutting the main bronchi at the hili.

3.1.2. Enzymatic Digestion of Lung Tissue

1. Slice lung tissue into 0.125-mm slices (we use a McIlwain tissue chopper from Mickle Laboratory Engineering, Surrey, UK). Take care to blot the lung tissue before placing on the tissue chopper to remove excess buffer. For the airways, cut it in half in the longitudinal direction using a scalpel, then half again before very finely chopping the strips in the transverse direction.
2. Place the chopped peripheral lungs from up to five mice or two rats into 30 ml of digestion buffer in a 50-ml sterile conical flask. Separate tissue pieces by pipetting with a plastic transfer pipette.
3. Incubate for 60 min at 37°C in a shaking water bath, then add another 3 mg DNAse in no more than 1 ml digestion buffer and incubate for another 30 min.
4. Disrupt tissue with a plastic transfer pipette or wide-bore Pasteur pipette until most of the larger pieces of tissue have dispersed by pipetting up and down without frothing the cell suspension.
5. Pass the digest mixture through a cotton wool filter to remove tissue debris. Rinse the flask and filter with ice-cold G-5 to maximize cell recoveries.
6. Pellet cells and resuspend in NH_4Cl if red cell lysis is required.
7. Pellet cells and resuspend in cold G-5.
8. Expect >80% viable cells in final lung cell preparation.

3.2. Isolation of RTDC by Cell Sorting (see Note 5)

3.2.1. Labelling of Rat Leucocyte Cell Surface Antigens for Cell Sorting

1. Label all lung digest cells with OX12 for B-cell depletion. As a general rule use 10 μg of antibody for every 1×10^8 cells.
2. Incubate for 15 min at 4°C and wash once in GKN–BSA.
3. Resuspend in 1 ml of anti-mouse Ig Dynabeads® according to manufacturer's instructions.
4. Place cells in magnet to deplete B cells and harvest non-adherent cells according to manufacturer's instructions.
5. Wash once in GKN–BSA at 4°C.
6. Resuspend in warm GKN–BSA up to 1×10^7 cells/ml and deplete macrophages by adherence to plastic (100 mm

tissue culture grade dishes containing 10 ml cell solution) for 60 min at 37°C in GKN–BSA.

7. Collect non-adherent cells and wash once in GKN–BSA.
8. Resuspend in OX6-FITC and CD4-APC at 10 μg/ml in GKN–BSA containing 10% NMS, using 1 ml for every 1×10^8 cells. Add 5 μg/ml of mouse IgG1-FITC and other appropriate Ig control mAbs in GKN–BSA to control cells.
9. Incubate for 15 min at 4°C and wash once in GKN–BSA at 4°C.
10. Pellet cells and resuspend to 1×10^7 cells/ml in G-5. Keep on ice.

3.2.2. Labelling of Mouse Leucocyte Cell Surface Antigens

1. For every 1×10^8 viable cells, resuspend in optimized dilutions (typically in the range of 1:200–1:1,000 depending on the clone and supplier) of anti-CD11c, anti-IA/E and anti-CD11b mAbs conjugated to biotin or preferred fluorochrome combinations (*see* **Section 2.3.3**) using a 1 ml volume of diluted antibody in GKN–BSA.
2. Incubate for 30 min at 4°C and wash once in GKN–BSA.
3. If a biotinylated mAb is used in the primary labelling, incubate for a further 30 min at 4°C in an optimized dilution of streptavidin–fluorochrome conjugate. Wash once in GKN–BSA – for cell sorting, we recommend adding propidium iodide (1 μg/ml) to this wash to label dead cells.
4. Pellet cells and resuspend to 1×10^7 cells/ml in G-5 for sorting. Keep on ice.

3.2.3. Cell Sorting

RTDC, B cell and macrophage populations can be identified in lung and airway tissue of mice and rats on the basis of forward- and side-scatter measurements and class II MHC, CD11c, CD11b and other marker expression depending on species. The protocol described here is a standard setup for most standard and high-speed cell sorters, although we have found that the high fluidics pressures of high-speed cell sorters can affect RTDC function and viability and therefore we recommend sorting at cell rates of no greater than 10,000 cells/s. Best cell recoveries and purities will be obtained if cells are sorted at a low cell concentration ($<1 \times 10^7$/ml) in order to reduce coincidence aborts. If preferable, an enrichment sort can be run initially at a high data rate with coincidence abort OFF, followed by a second high-purity sort at low a data rate with coincidence abort ON, however, this will result in reduced cell recoveries. Always keep cells cold during the sort and collect into tubes containing 2 ml of cold G-50.

1. Adjust forward and side-scatter detectors in order to differentiate small lymphocytes from larger cells.

2. Using single-stained control tubes, set fluorescence detectors and compensation.
3. Set FSC and SSC size gates to distinguish lymphocytes from larger cells (**Figs. 17.1** and **17.2**)
4. For mice, RTDC are identified on the basis of high levels of MHC class II and CD11c expression and can be subdivided into at least two subsets on the basis of a difference in CD11b expression (**Fig. 17.1**). The distinction between RTDC and macrophages in airway tissue can be difficult to discern on the basis of CD11c expression and a macrophage marker such as CD2 or F4/80 may be needed.
5. For rats, RTDC are identified on the basis of high levels of class II MHC expression and subsets can be identified based on differential CD4 expression (*see* **Fig. 17.2** and **Notes 6**

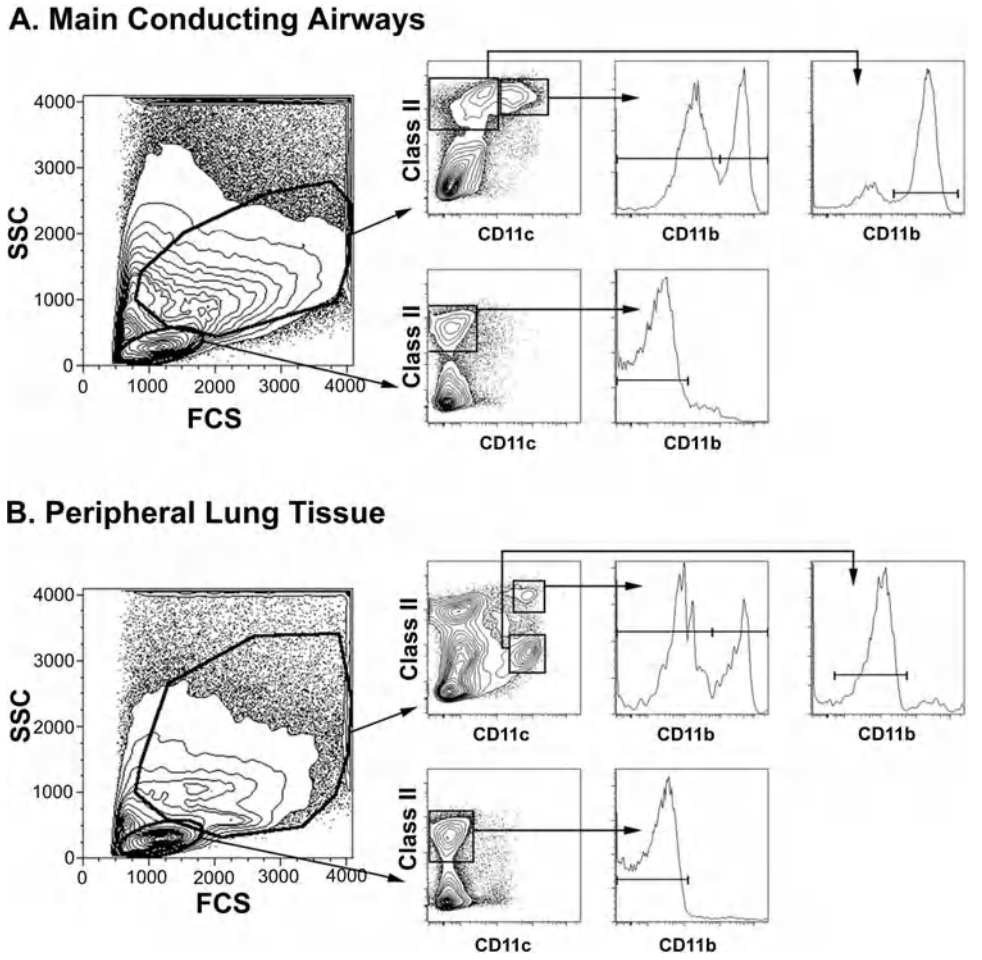

Fig. 17.1. FACS plot of pre-sorted and post-sorted mouse main conducting airways (trachea) and peripheral lung tissue cells showing typical sort/analysis regions.

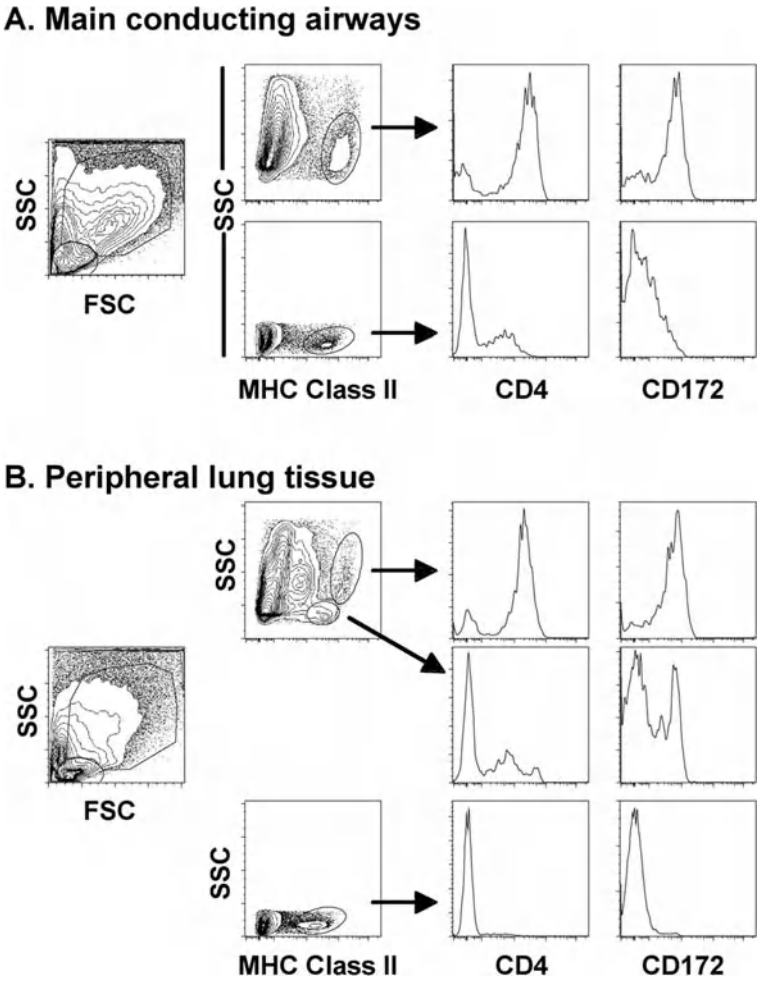

Fig. 17.2. FACS plot of pre-sorted and post-sorted rat main conducting airways (trachea) and peripheral lung tissue cells showing typical sort/analysis regions.

and 7). Further differentiation of phenotype/subsets can be achieved with other antibodies as discussed above.

6. Sort RTDC into tubes containing 2–3 ml of G-50 on ice, keeping cells at 4°C during the sort.
7. Pellet cells by centrifugation and wash once in R-5.
8. Check cell numbers and viability by Trypan Blue exclusion and resuspend to desired concentration in R-5.

3.3. In Vitro Culture of Purified RTDC

1. Wash cells twice in R-5 and resuspend to 1×10^6 cells/ml in R-5 warmed to 37°C.
2. Add 10 ng/ml of rGM-CSF and transfer cells to a 10-ml V-bottomed tube (*see* **Note 8**).

3. Incubate cells for 24–48 h at 37°C in 5% CO_2, 90% humidity with the tube cap loosened.
4. Check cell viability and re-label an aliquot of cells to confirm upregulation of surface MHC class II expression by flow cytometry. A tenfold increase in the intensity of expression of MHC class II is typically observed at this stage (*see* **Note 9**).
5. For presentation assays DC can be titrated into T-responder populations. Typically, we use 1×10^5 T-responder cells and start DC titrations at a top ratio of 5%. Culture in R-5 for a total of 96 h. ^{3}H-tymidine can be added to determine CPM or alternatively this system works well with CFSE-labelled T-responder cells.

4. Notes

1. Correct working concentrations of collagenase enzyme must be determined for each batch of enzyme. Suppliers and batches of enzyme vary considerably in terms of enzyme concentration required for optimal lung or trachea cell recovery and viability and this should be determined by titration prior to use for each new batch of enzyme. Care should be taken to choose those suppliers and batches with minimal non-specific protease activity to avoid digestion of cell surface markers.
2. GKN is an all-purpose cell handling buffer that is particularly suited to the handling of dendriform cell types (M. Puklavec, Oxford University, personal communication).
3. Mouse lung DC have been shown to express the DEC-205 antigen, recognized by the NLDC-145 mAb, as well as other markers (*see* **Table 17.1** and (8)). In addition, detailed use of the N418 hamster mAb to sort mouse DC has been published (20) and an mAb termed 33D1 that recognizes functional subsets of DC in mouse spleen and lymph node (21, 22), which may also be useful for the direct isolation of mouse lung DC although this has not been tested in our hands.
4. If the main conducting airways (trachea) are to be separated from lung parenchyma to isolate airway mucosal RTDC, cut the main bronchi at the hili. To isolate RTDC from lung parenchymal tissue only (thus avoiding the bulk of the mucosal RTDC populations), in order to avoid contamination by lymph node cells in the hilar regions, dissect a rim of peripheral lung tissue (maximal peripheral third of the lung).

5. Magnetic bead approaches may be used for RTDC isolation; however, a factor to consider when using magnetic bead sorting for rat RTDC is the inability to separate $OX6^{high}$ vs $OX6^{low}$ populations. Thus, purification of RTDC by magnetic bead sorting will result in a more heterogeneous population of cells consisting of high and low MHC class II-expressing cells, although 24 h culture in GM-CSF will enrich for MHC class II RTDC and these cells are fully functional in antigen presentation assays. Likewise for mice RTDC, magnetic bead sorting will not allow separation of populations expressing low-to-intermediate levels of MHC class II and/or CD11c and thus contamination with other cell types such as alveolar macrophages ($CD11c^{high}$ MHC class $II^{intermediate}$).
6. Flow cytometric analysis of macrophage and B-cell-depleted lung digest cells reveals a bimodal expression for MHC class II in rats, with the $OX6^{high}$ and $OX6^{low}$ populations representing approximately 1–3% and 6–8% of total lung digests, respectively. The majority of $OX6^{high}$ cells show a dendriform morphology, express CD86 (but not CD80), are actively endocytic and upregulate surface MHC class II expression and both in vivo and in vitro antigen-presenting capacity in response to GM-CSF. In contrast, less than 50% of $OX6^{low}$ lung digest cells are endocytic and the majority of these do not express CD86. Although a small proportion does upregulate MHC class II in response to GM-CSF, we generally exclude these cells from our sorting regions and collect only the $OX6^{high}$ population (*see* **Fig. 17.2**), although this will exclude a possibly more immature population of cells that may reside within the OX6 subset. Additional markers such as CD4 and CD172 also define subsets of rat RTDC with potential differences in antigen-handling capacity (unpublished data – *see* **Fig. 17.2**).
7. When sorting RTDC it is important to consider the fact that DC can form clusters with T cells. Sorting RTDC subpopulations, in both rat and mouse, can lead to the isolation of both DC and T-cell populations, from what was originally thought to be a DC region. It is extremely difficult to identify by phenotype if DC and T cells are clustered together and only after disruption by the sorting procedure does this become obvious. Doublets can be gated out, but this will thus lead to the elimination of those DC which may be of some importance.
8. We typically culture purified RTDC in 10-ml tubes to avoid cell losses during post-culture washes and use a maximum of 1×10^6 cells in 1 ml/tube. GM-CSF is required for survival of RTDC in culture, however, additional factors such

as TNFα, bacterial LPS and CD40-ligand may also influence DC maturation and may be added together with GM-CSF (23).

9. Post-culture analysis of the degree of upregulation of MHC class II induced by GM-CSF should be performed prior to the use of RTDC in presentation assays. Cell viabilities after culture in GM-CSF will be greater for $OX6^{high}$ cells sorted by flow cytometry compared with the mixed $OX6^{high+low}$ cells sorted by magnetic beads (approximately 70 and 30% viable, respectively) as a high proportion of $OX6^{low}$ cells do not respond to GM-CSF.

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Chapter 18

Isolation of Mouse Thymic Dendritic Cell Precursors

Li Wu and Angela D'Amico

Abstract

Dendritic cells (DC) are efficient antigen-presenting cells. Their ability to present antigens via MHC class I and MHC class II molecules to T cells allows them not only to initiate an immune response to exogenous pathogens but also to induce immune tolerance to self-antigens. Thymic DC play important roles in the establishment of central immune tolerance by presenting self-antigens to developing thymocytes and subsequently deleting the self-reactive thymocytes and inducing naturally occurring regulatory T cells. DC in the thymus are comprised of plasmacytoid DC (pDC) and conventional DC (cDC) populations. The cDC can be divided into two populations based on the expression of CD8α and Sirpα: $CD8\alpha^{+}Sirp\alpha^{lo}$ (~70%) and $CD8\alpha^{lo}$ $Sirp\alpha^{+}$ (~30%). The $CD8\alpha^{+}Sirp\alpha^{lo}$ cDC are generated in the thymus by the earliest intrathymic oligo-potent progenitors that are also precursors for T-lineage cells and natural killer cells (NK cells). Whereas the $CD8\alpha^{lo}Sirp\alpha^{+}$cDC and pDC are migratory DC and originate mainly from peripheral blood. The ability to isolate and purify the earliest intrathymic precursors allows us to generate thymic cDC in culture or in vivo upon intrathymic or intravenous injections. These experimental systems are crucial for studying the development and functions of thymic DC.

Key words: Dendritic cell, thymic dendritic cell, thymic precursors, precursor isolation, precursor transfer, flow cytometry.

1. Introduction

The majority of thymic cDC are generated from the earliest intrathymic precursor population first isolated as the "$CD4^{lo w}$" precursors due to the low levels of expression of CD4 on the cell surface (1–4). A similar precursor population was described by later studies as the "early T cell precursors (ETP)" or the $CD4^{-}8^{-}$ double-negative (DN) thymocyte subset 1a and 1b (DN1a + 1b) (5–7). This early precursor population has the potential to generate T cells, B cells, NK cells, DC, and myeloid cells upon adoptive transfer or in culture with various

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cytokines (1, 3, 5, 8) and represents only ~0.05% of total cells in mouse thymus. These precursors are negative for haemopoietic lineage markers ($CD11b^-Gr\text{-}1^-B220^-Ter\text{-}119^-$) and are $CD3^-CD4^{lo}CD8^-CD25^-Thy\text{-}1^{lo}$ $IL\text{-}7R\alpha^{lo}$, but express high levels of c-kit, CD44, and Sca-1 (**Fig. 18.1**). Thymic DC can also be generated with lower efficiency from a downstream thymic precursor population among the $CD4^-8^-$ DN thymocytes, namely, the $CD3^-4^-8^-CD25^+c\text{-}kit^+$ (DN2) precursors that are more mature than the $CD4^{lo}$/ETP/DN1a + 1b precursors and have the potential to generate T cells, as well as NK cells and DC, but have lost the potential to generate B cells and myeloid cells (9). The DN2 precursors further differentiate into DN3 ($CD25^+c\text{-}kit^-$) then into DN4 ($CD25^-c\text{-}kit^-$) thymocytes (**Fig. 18.2**). The full commitment to T-lineage differentiation occurs at DN3 stage and by this stage the precursors have lost the potential to generate DC.

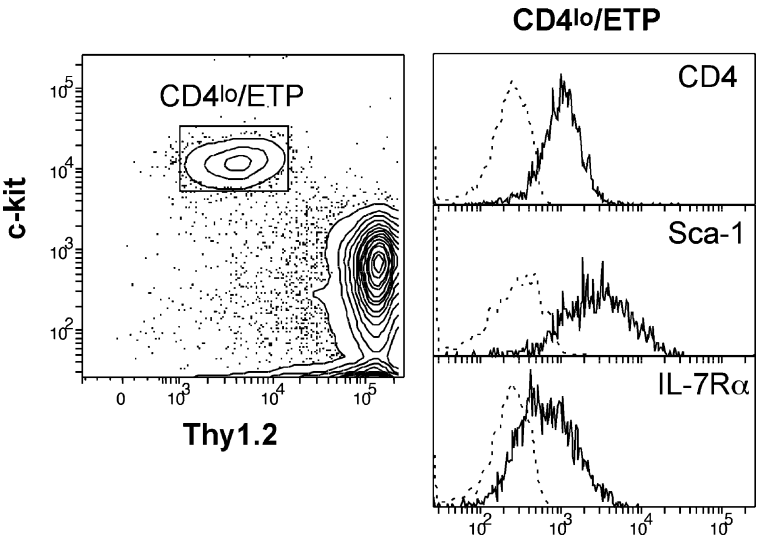

Fig. 18.1. The typical profiles of the depleted thymocytes preparation by flow cytometric analysis. The cells were stained with anti-Thy-1.2-FITC and anti-c-kit-APC. The $CD4^{lo}$/ETP population is gated as $Thy\text{-}1^{lo}c\text{-}kit^+$ cells as presented by the box. These $CD4^{lo}$/ETP also express low levels of CD4 and IL-7Rα and high levels of Sca-1. The dashed lines in the histograms represent background staining and the solid lines represent the expression levels of each indicated surface molecules on gated $CD4^{lo}$/ETP precursors.

Thymic DC contain pDC ($CD11c^+CD45RA^+MHC\text{-}class\text{-}II^{lo}$) and cDC ($CD11c^+CD45RA^-MHC\text{-}class\text{-}II^{hi}$) populations. The cDC can be subdivided into two subsets based on the expression of CD8 and Sirpα, namely, the $CD8^+Sirp\alpha^{lo}$ (~70%) and the $CD8^{lo}Sirp\alpha^+$ (~30%) cDC subsets (10). Our recent studies demonstrated that the major $CD8^+Sirp\alpha^{lo}$ cDC subset was generated within mouse thymus from the early intrathymic precursors described above, whereas the minor $CD8^{lo}Sirp\alpha^+$ cDC

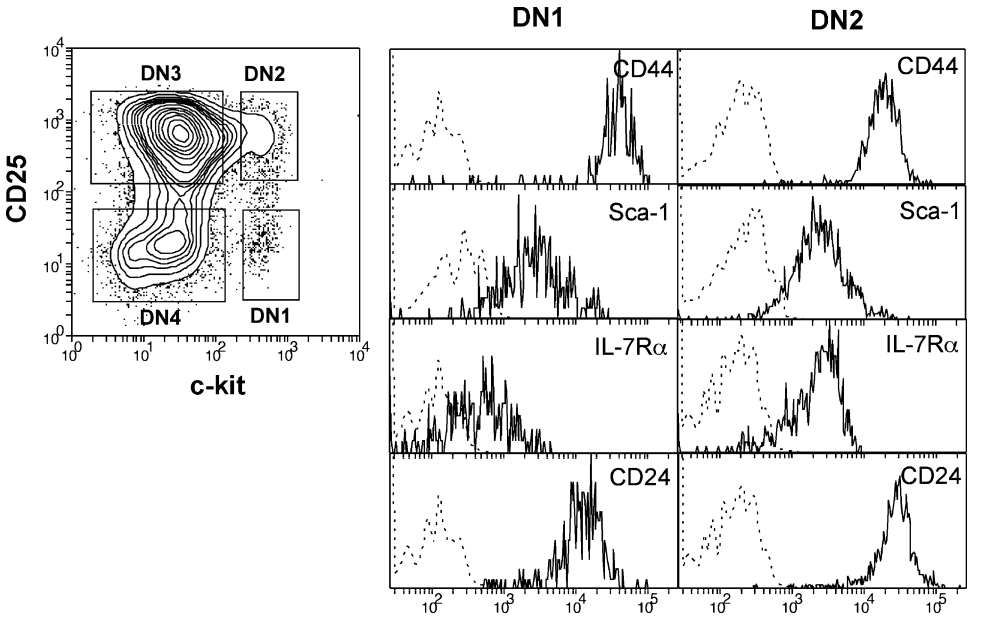

Fig. 18.2. The typical profiles by flow cytometric analysis of the depleted thymocyte preparation for $CD4^{-}8^{-}$ double-negative (DN) precursor populations. The cell preparation was stained with anti-CD25-FITC and anti-c-kit-APC. Four precursor populations are shown, namely, $CD25^{-}$c-kit^{+} (DN1, contains some of the ETP), $CD25^{+}$c-kit^{+} (DN2), $CD25^{+}$c-kit^{-} (DN3), and $CD25^{-}$c-kit^{-} (DN4). The *boxes* represent sorting gates for each population. The dashed lines in the histograms represent background staining and the solid lines represent the expression levels of each indicated surface molecules on gated DN1 and DN2 precursors. The DN1 precursors express low levels of IL-7Rα, intermediate levels of CD24, and high levels of CD44 and Sca-1. The downstream and more mature DN2 precursors upregulate the expression of IL-7Rα and CD24 and reduced the expression of CD44 moderately.

subset represents migratory DC that originate from peripheral blood (Wu et al., unpublished).

The strategy for isolating these extremely rare precursor populations with maximum purity but at a reduced cost involves an efficient enrichment of the populations prior to cell sorting by flow cytometry. This is accomplished using a combination of density separation, adhesion depletion, and immunomagnetic bead depletion which successfully remove mature and immature thymocytes and non-T-lineage cells. It is important to deplete non-T-lineage cells, including erythrocytes, macrophages, granulocytes, B cells, and DC, which may otherwise contaminate the precursor preparation.

The development of DC from the purified thymic precursors can be studied using both in vitro and in vivo systems. For in vitro studies, two culture systems can be used to generate DC from the thymic precursors. One culture system uses a combination of cytokines to culture the precursors without any feeder cells. The second culture system uses Flt3 ligand (Flt3L) alone and in the presence of the syngeneic bone marrow (BM) cells as feeders. This Flt3L-supplemented BM culture system supports

the generation of different DC populations equivalent to the DC populations found in mouse spleen (11). For in vivo studies, purified thymic precursor cells from C57BL/6 CD45.2 mice can be injected directly into a thymic lobe of a sublethally irradiated congenic CD45.1 recipient or transferred intravenously into a lethally irradiated CD45.1 recipient. The intrathymic precursor transfer system can be used to examine the differentiation of the precursors in the thymic environment. Whereas the intravenous transfer system can be used to determine the capacity of the precursors to home different tissues and their developmental potential in the tissues they entered. These systems described above provide important tools for studying the regulation of thymic DC development and function.

In the following sections the procedures for precursor isolation and the systems for in vitro and in vivo DC generation from these thymic precursors are described.

2. Materials

2.1. Mice

C57BL/6 J mice at 5–6 weeks of age are used for precursor isolation. C57BL/6 CD45.1 congenic mice at 8–10 weeks of age are used as recipients for precursor transfer analysis.

2.2. Isolation of Thymic Precursors

2.2.1. Media

1. FCS: fetal calf serum is filtered through a 0.22-μm membrane and heat-inactivated at 56°C for 40 min. Aliquot and store at –20°C.
2. Balanced salt solution (BSS): a HEPES-buffered balanced salt solution with the mouse osmolarity (308 mOsm/kg) at pH 7.2 and supplemented with 3–5% fetal calf serum (FCS) is used for single-cell suspension and immunofluorescent staining.
3. RPMI-1640–HEPES–FCS: RPMI–FCS: Modify RPMI-1640 to mouse osmolarity (308 mOsm/kg) and include additional pH 7.2 HEPES buffering to reduce dependence on CO_2 concentration. After adjusting to ~pH 7 with CO_2, filter-sterilize and store at 4°C. Add FCS to a final concentration of 5–10% prior to use.
4. Density separation medium: Nycodenz AG powder (Nycomed Pharma AS, Oslo, Norway) is prepared as a 0.372 M stock solution in water (density about 1.16 g/cm^3), then diluted and adjusted to a density of 1.086 g/cm^3 (at 4°C) and osmolarity of 308 mOsm/kg using BSS. Filter to sterilize and store in sealed 10-ml

aliquots at −20°C. Thaw at room temperature, mix thoroughly, and cool to 4°C prior to use (*see* **Note 1**).

5. EDTA solution: 0.1 M ethylenediamine tetra-acetic acid disodium salt adjusted to pH 7.2. Filter to sterilize and store at 4°C.
6. BSS–EDTA: Modified BSS containing 150 mM NaCl and 3.75 mM KCl (no Ca^{2+} or Mg^{2+}) and 5 mM EDTA. Adjust to pH 7.2 and mouse osmolarity (308 mOsm/kg). Filter-sterilize and store at 4°C.

2.2.2. Monoclonal Antibodies (mAb) for Depletion

The mAb cocktail for depletion is prepared and stored in small aliquots at –20°C. The concentration of each mAb is determined by pretitration using immunofluorescent staining with an anti-Ig second stage. Each mAb is used at saturating concentration in the final cocktail. The mAb cocktail is used at 10 μl/1 × 10^6 cells. The mAb cocktail contains anti-CD3 (KT3-1.1), anti-CD8 (53-6.7), anti-CD2 (RM2-1), anti-CD25 (PC61), anti-B220 (RA3-6B2), anti-Mac-1 (M1/70), anti-Gr-1 (RB6-8C5), anti-erythrocyte antigen (Ter-119), and anti-MHC-class-II (M5/114). Note that the anti-CD4 antibody is not used in the depletion mAb cocktail for preparation of the $CD4^{lo}$/ETP precursors because of the low levels of CD4 on these precursors. In the case of preparing DN thymic precursors, anti-CD4 (GK1.5) is added and anti-CD25 (PC61) is omitted from the mAb cocktail.

2.2.3. Immunomagnetic Beads for Depletion

1. BioMag beads: Goat anti-rat IgG-coated magnetic beads (Qiagen, Clifton Hill, Australia) are used at a bead to cell ratio of 6:1. The beads are washed three times with BSS–3% FCS prior to use to remove preservative in the stock.
2. Dynabeads: Sheep anti-rat IgG-coated M450 dynabeads (Dynal, Oslo, Norway) are used for the second round magnetic beads depletion at a bead to cell ratio of 3:1. The beads are washed three times with BSS–3% FCS prior to use to remove preservative in the stock.
3. Spiral rotator: Spiramix 10 (Denley, Billingshurst, England) is used for mixing cells and beads during incubation.
4. 5-ml Round-bottomed polypropylene Falcon tubes (Falcon, Becton Dickinson).

2.2.4. Antibodies for Immunofluorescent Staining

The fluorescent-conjugated antibodies for immunofluorescent staining are either purchased from BD PharMingen (San Diego, CA, USA) or eBioscience (San Diego, CA, USA), or made in our laboratory. All are titrated to determine saturating levels. For staining the earliest intrathymic precursors ($CD4^{lo}$/ETP), FITC-conjugated anti-Thy-1.2 (30H-12) and APC- or PE-conjugated anti-c-kit (ACK-2) are used. For staining of DN

precursors, FITC-conjugated anti-CD25 (PC61) and APC- or PE-conjugated anti-c-kit (ACK-2) are used. The combination of fluorescent-conjugated antibodies can be changed depending on the models of flow cytometers used.

2.3. Culture of Thymic Precursors for Generating DC

1. *Medium:* Mouse osmolarity (308 mOsm/kg) RPMI-1640 is buffered with HEPES at pH 7.2 and supplemented with 10% FCS, 10^{-4} M 2-mercaptoethanol (2-ME), sodium pyruvate, penicillin G 500 U/ml, and streptomycin 25 mg/ml.
2. *Cytokines:* Recombinant cytokines (Immunex Corp., Seattle, WA, USA) are used at the following concentrations: IL-1β (human) 200 U or 0.2 ng/ml, IL-3 (murine) 200 U or 400 ng/ml, IL-7 (human) 200 U or 10 ng/ml, stem cell factor (SCF, murine) 10 ng/ml, TNFα (murine) 1 ng/ml.

   Flt3 ligand (Flt3L): Flt3L is produced from a murine Flt3L/FLAG producing CHO cell line generated in the Walter and Eliza Hall Institute (Melbourne, Australia) and purified using an anti-FLAG M2 affinity column (Sigma, Castle Hill, NSW, Australia).

   All cytokines are diluted in RPMI-1640 culture medium, filtered, and stored at –20°C for no more than 1 month prior to further dilution in culture.
3. The 96-well flat-bottomed microtitre trays (Falcon, Becton Dickinson) and 20-well V-bottomed Terasaki trays (Nunclon, Denmark) are used.
4. *Red cell lysis buffer*: 0.168 M NH_4Cl is used at 4°C to lyse erythrocytes from mouse BM.
5. 45-μm Nylon cell strainers (BD Falcon, Bedford, MA, USA) to remove cell clumps from cultured cells.
6. *Flow cytometry*: For detecting DC populations generated from the precursors in culture, APC-conjugated anti-CD45RA (14.8), Alexa-594 (or Alexa-680)-conjugated anti-CD11c (N418), and FITC-conjugated anti-Sirpα (P84) are used. In the case where erythrocytes-depleted BM cells are used as feeder cells, PE-conjugated anti-CD45.2 (S.450-15.2) and PE-Cy7-conjugated anti-CD45.1 (A20-1.1) are used to stain the feeder BM and the precursor-derived cells. Again, the combination of fluorescent-conjugated antibodies can be changed depending on the models of flow cytometers used.

2.4. Intrathymic and Intravenous Precursor Transfer

2.4.1. Anaesthetizing Mice

A mixture of Ketavet 100 (Ketamine hydrochloride 0.05 mg/g body weight; Delta Veterinary Laboratories Pty. Ltd, NSW, Australia) and Rompun (a muscle relaxant, xylazine hydrochloride 0.01 mg/g body weight; Bayer AG, Germany) is injected i.p. to anaesthetize mice prior to the intrathymic injection procedure.

2.4.2. Cell Transfer

1. 50-μl Hamilton syringe (Hamilton, Reno, NV, USA) and 30 gauge needles (Precision Glide Needle 30 G1, Becton Dickinson) for intrathymic transfer.
2. 1-ml Syringe (TERUMO, Elkton, MD, USA) and 25-ga (5/8) needles (BD Precision Glide Needles) for intravenous transfer.
3. Wound clips (AUTOin the sentence CLIP 9 mm, MikRon Precision, Inc., Clay Adams, BD, NJ, USA.)

2.4.3. Analysis of the Progeny of Transferred Precursor Populations

The multi-colour flow cytometric analyses are used to detect the precursor-derived DC populations. PE-conjugated anti-CD45.2 (S.450-15.2) and PE-Cy7-conjugated anti-CD45.1 (A 20-1.1) are used to distinguish donor and host-derived cells. APC-conjugated anti-CD45RA (14.8), Alexa-594-conjugated anti-CD11c (N418), and FITC-conjugated anti-CD8 (YTS-169.4) or anti-Sirpα (P84) are used to identify different DC populations.

3. Methods

3.1. Thymus Removal and Cell Dissociation

1. Mice are sacrificed by CO_2 asphyxiation (*see* **Note 2**).
2. Remove thymus from 8 to 16 mice under sterile conditions and place in cold BSS–3% FCS.
3. Prepare a thymocyte suspension by gently forcing thymus lobes through a sieve in BSS–3% FCS.
4. Transfer the cell suspension into 10-ml conical tubes (4 thymi/tube), underlay with 1 ml FCS, and centrifuge at 1,000 × *g* for 7 min at 4°C in a benchtop centrifuge.

3.2. Purification of Thymic Precursors

3.2.1. Density Separation by Centrifugation

The early thymic precursors have a lower density (<1.086 g/cm^3) than mature thymocytes. Using this property, the thymic precursors can be separated from the mature single-positive ($CD4^+$ and $CD8^+$) and double-positive ($CD4^+8^+$) thymocytes, as well as other high density cells.

1. Aliquot 5 ml of well mixed Nycodenz medium (d= 1.086 g/cm^3) at 4°C into each 14-ml round-bottomed polypropylene Falcon tube.
2. Resuspend the thymocyte pellet of each conical tube in an additional 5 ml Nycodenz medium and overlay this cell/Nycodenz suspension onto the 5 ml Nycodenz medium in the Falcon tube (*see* **Note 3**).
3. Overlay with 2 ml FCS and slightly mix the interface bands with a Pasteur pipette (*see* **Note 4**).

4. Centrifuge the tubes in a swing-out rotor at 4°C for 10 min at 1,800 × *g*, with brake off.
5. Collect the cells at the interface and the FCS layer from each tube using a Pasteur pipette (*see* **Note 5**).
6. Pool the cells collected from two centrifugation tubes into one 50-ml tube and dilute the collected fraction immediately with BSS without FCS to a volume of 50 ml and mix well (*see* **Note 6**).
7. Take a small sample at this stage to count cell yield.
8. Centrifuge the cells to a pellet.

3.2.2. Adhesion Depletion of Macrophages

This step is to remove macrophages by adhesion to a plastic surface.

1. Resuspend the cell pellet and pool all cells in 20 ml RPMI-1640–10% FCS and transfer cells (10 ml/dish) to two 8-cm diameter plastic Petri dishes. Ensure the suspension is distributed evenly over the entire area of the dish.
2. Incubate the dishes in a 37°C CO_2-in-air incubator for 60 min.
3. Collect the non-adherent cells by collecting the supernatant first, then gently washing the surface of each dish twice with 10 ml prewarmed (37°C) RPMI-1640–10% FCS and collecting all the cells that do not adhere to the dish (*see* **Note 7**).
4. Take a small sample for a cell count and then pellet the cells by centrifugation.

3.2.3. Immunomagnetic Bead Depletion

This procedure removes most of the cells bearing markers of mature thymocytes and non-T-lineage cells. The removal of antibody-coated cells is facilitated by using two rounds of depletion with anti-rat IgG-coated beads.

1. Resuspend cells in the depletion mAb cocktail (either for $CD4^{lo}$/ETP or for DN) at 10 µl/10^6 cells (*see* **Section 2**) and incubate on ice for 30–40 min.
2. Dilute cells in 28 ml BSS–3% FCS, underlay 2 ml FCS, and then centrifuge through the FCS layer (*see* **Note 8**).
3. Remove the supernatant from the pelleted cells.
4. The amount of anti-rat IgG-coated BioMag beads required is calculated at a ratio of 6:1 (beads to cells) (*see* **Note 9**).
5. The BioMag beads are transferred and divided into two to four 5 ml round-bottomed polypropylene Falcon tubes and prewashed three times with BSS–3% FCS to remove preservative, then recover the beads with a Dynal magnet.

6. Resuspend the antibody-coated cells in 600 μl BSS–3% FCS (4 thymi/600 μl/tube), then transfer the suspension to the Falcon tubes containing the washed beads. Mix the slurry of cells and beads, avoiding bubbles.
7. Seal each tube with a cap and place a "collar" around the cap. Then place the tube on a spiral rotator and rotate continuously for 20–30 min at 4°C, at a 30° angle (*see* **Note 10**).
8. The cells uncoated by the depleting mAbs are recovered by diluting the bead to cell mix in 2 ml BSS–3% FCS per tube, then removing beads and attached cells with Dynal magnet. The bead-free cell suspension from each tube is collected using a Pasteur pipette and pooled (*see* **Note 11**).
9. Take a small sample to count and recover the cells by centrifugation.
10. The anti-rat IgG-coated Dynabeads are used at 3:1 (bead to cell) for the second round depletion to achieve the maximum enrichment. The appropriate amount of beads is transferred to one 5-ml Falcon tube and washed three times with BSS–3% FCS prior to use. Resuspend the cell pellet in 500 μl BSS–3% FCS and add to beads.
11. Mix the beads and cell slurry for 20–30 min on a spiral rotator as in **Step 7**.
12. Recover the bead-free cell suspension with a Pasteur pipette on a Dynal magnet.
13. Centrifuge to pellet the cells. At this stage, the precursor populations are enriched about 500-fold.

3.2.4. Immunofluorescent Staining and Sorting by Flow Cytometry

The depleted cell preparation can be stained in two fluorescent colours with FITC-conjugated anti-Thy-1.2 (30H-12) and APC- or PE-conjugated anti-c-kit (ACK-2) for the $CD4^{lo}$/ETP or with FITC-conjugated anti-CD25 (PC61) and APC- or PE-conjugated anti-c-kit (ACK-2) for DN precursors.

1. Resuspend the depleted cell preparation in BSS–3% FCS containing the appropriate staining antibodies (10 μl/10^6 cells) and incubate on ice for 20–30 min.
2. Remove excess staining antibodies by washing cells in BSS–3% FCS through an FCS layer (*see* **Note 8**) and add propidium iodide (PI) at 0.5–1.0 μg/ml to this final wash to stain dead cells.
3. Analyse stained cells using a FACSDiva or a FACSAria (BD Biosciences, San Jose, CA, USA) and take a file of 50,000 cells for setting up sorting gates.
4. When sorting for $CD4^{lo}$/ETP, live gates for Thy-1^{lo}c-kit^{+} cells are set (**Fig. 18.1**) and dead cells are excluded by gating

for PI^- cells (*see* **Note 12**). Usually the Thy-1^{lo}c-kit^+ population represents 5–10% of the stained cells.

5. When sorting for DN2 precursors, live gates for $CD25^+$c-kit^+ cells are set (**Fig. 18.2**) and dead cells are excluded by gating for PI^- cells (*see* **Note 13**). Usually the $CD25^+$c-kit^+population represents ~3% of the stained cells.
6. The purity of sorted cells is determined by reanalysis of a small sample of collected cells. It is usually > 98%.

3.3. Culture of Thymic Precursors

Two culture systems can be used to generate DC from the thymic precursors as described below.

3.3.1. Culture of Thymic Precursors in a Combination of Cytokines

1. The sorted precursor cells are counted and diluted in RPMI-1640–10% FCS culture medium containing a combination of cytokines to a concentration of 2.5×10^4 cells/ml. The combination of IL-1β, IL-3, IL-7, TNFα, SCF, Flt3L, and anti-CD40 mAb yields an expansion of precursor cells with the majority having the morphological appearance of DC by day 4.
2. For precursor frequency analysis, low density cell cultures (up to 250 cells/well) are performed; 10 μl of the cell suspension is carefully placed into each well of a 20-well Terasaki tray (*see* **Note 14**).
3. To achieve even distribution of cells in the bottom of each well, the Terasaki tray is inverted on incubation at 37.5°C in a humidified 10% CO_2-in-air incubator.
4. After overnight incubation, the tray is carefully turned over and cells are cultured for 4 days.
5. For surface phenotype analysis of the cultured cells, a large yield is required. In this case, sorted precursor cells are diluted with culture medium containing cytokines to a concentration of 1×10^5 cells/ml, and 100 μl aliquots placed in a 96-well flat-bottomed culture tray.

3.3.2. Culture of Thymic Precursors with BM Feeder Cells in the Presence of Flt3L

This culture system can be used for studying the potential of precursors to generate various DC populations (11).

1. Sorted thymic precursor cells (from C57BL/6 CD45.2 mice) are counted and diluted in the culture medium containing the appropriate concentration of Flt3L (100–200 ng/ml).
2. BM feeder cell suspension from C57BL/6 CD45.1 mice is prepared and erythrocytes removed by a 30-s exposure to 0.168 M NH_4Cl at 4°C and washed three times immediately with BSS–3% FCS.

3. 1.5×10^5 Erythrocyte-depleted CD45.1 BM feeder cells in 100 μl are added to each well in a 96-well culture tray.
4. $1–5 \times 10^4$ Precursors in 100 μl medium containing Flt3L are added to each well containing the BM feeder cells.
5. Cells are cultured at 37°C in a 10% CO_2-in-air incubator for 7–9 days.

3.3.3. Analysis of Cultured Cells

1. To recover cells after culture, 1/10th volume of 0.1 M EDTA pH 7.2 (20 μl) is first added to each well and mixed well by repeated passage through a pipette tip. Cells are then passed through a 45-μm Nylon cell strainer to remove clumps (*see* **Note 15**).
2. For phenotypic analysis, combine the cells from multiple wells in a 10-ml conical tube and centrifuge to a pellet.
3. Resuspend cells in BSS–EDTA–3% FCS containing 1 mg/ml rat–Ig and encubate for 10–20 min, then stain cells at 4°C for DC surface molecules (at 10^6/10 μl and $1–2 \times 10^6$ cells/sample), e.g. APC-anti-CD45RA, Alexa-594-anti-CD11c, PE-anti-MHC-class-II and FITC-anti-Sirpα (*see* **Notes 16–18**).
4. For analysing cells cultured in the presence of BM feeder cells, cells are resuspended in BSS–EDTA–3% FCS containing 1 mg/ml rat-Ig (10 μl per 10^6 cells) and incubated for 10–20 min at 4°C (*see* **Note 17**).
5. The cells are then stained for various DC surface molecules, e.g. CD11c, MHC-class-II, CD45RA, and Sirpα (*see* **Note 18**), as well as fluorescent-conjugated anti-CD45.2 and CD45.1 to distinguish cells derived from the precursors and from BM feeders, respectively.
6. Wash stained cells with BSS–EDTA–3% FCS through a layer of FCS and resuspend cells in 200–300 μl BSS–EDTA–3% FCS containing PI (0.5 μg/ml). PI staining is used to exclude dead cells and autofluorescent cells (*see* **Note 19**).
7. Analyse the stained cells on FACSDiva (or on other flow cytometers available) and collect 50,000–100,000 cells/sample. The precursor-derived DC can be defined as $CD45.2^+CD11c^+$ (**Fig. 18.3A**).

3.4. Intrathymic and Intravenous Transfer of Thymic Precursors

To study the developmental potential of the thymic precursors in vivo, intrathymic or intravenous precursor transfer systems can be used.

3.4.1. Intrathymic (i.t.) Transfer of Thymic Precursors

1. Eight to twelve-week-old C57BL/6 CD45.1 recipient mice are sublethally irradiated (750R, 1Rad= 0.01 Gy) and used 1–3 h later.

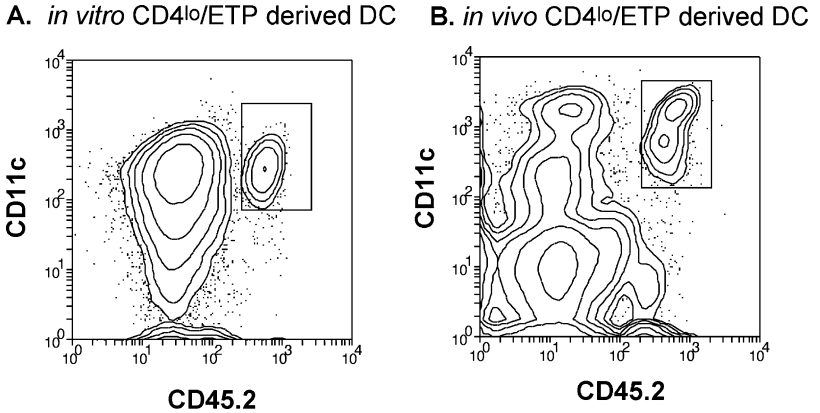

Fig. 18.3. Analysis of the CD4^{lo}/ETP-derived DC in vivo and in vitro. (**A**) DC derived from the CD4^{lo}/ETP precursors cultured with Flt3L and CD45.1 BM feeder cells for 9 days. The cells were collected from the cultures and stained with anti-CD45.2-FITC and anti-CD11c-APC. The precursor-derived DC are identified as CD45.2^{+}CD11c^{+} as indicated by the box. (**B**) Thymic DC derived from the CD4^{lo}/ETP precursors 7 days after intravenous transfer. Thymus was removed from each recipient and pooled. Thymic DC were prepared as described in **Section 3.4.3** (and in **Chapter 14**). The DC preparation was stained with anti-CD45.2-FITC and anti-CD11c-APC. The precursor-derived DC are identified as CD45.2^{+}CD11c^{+} as indicated by the *box*.

2. The irradiated mice are anaesthetized by intraperitoneal injection of a mixture of Ketave 100 and Rompun at an appropriate concentration.
3. Intrathymic injection is performed according to the procedures described previously (12). A midline incision is made in the skin overlying the lower cervical and upper thoracic region, and the upper third of the sternum is bisected longitudinally with fine scissors to expose the thymus. A suspension (10 μl) containing the appropriate number of purified precursor cells from C57BL/6 CD45.2 mice is injected directly into the anterior upper portion of each thymus lobe using a 50-μl Hamilton syringe with a 30-ga needle. The incision is then closed with wound clips. The mice are kept under a warm lamp till they recover. Usually 1–2 × 10^4 precursor cells/lobe can efficiently generate significant number of DC 2 weeks after injection.

3.4.2. Intravenous (i.v.) Transfer of Thymic Precursors

Eight to twelve-week-old C57BL/6 CD45.1 recipient mice are lethally irradiated with two doses of 550 Rad with a 3-h interval and used 1–3 h later. A suspension (200 μl) containing the appropriate number of purified precursor cells together with 5 × 10^4 recipient-type bone marrow cells is injected into the tail vein (*see* **Note 20**). Neomycin (sulphate at 1.1 g/l) is added to the drinking water for 2 weeks after irradiation to prevent infection. Usually 2 × 10^4 precursor cells/mouse will generate moderate number of DC in recipient thymus and spleen 2–3 weeks after injection.

3.4.3. Analysis of Progeny of Transferred Precursor Populations

At various times after precursor transfer, the recipients are sacrificed and the thymus and spleen removed for analysing the precursor-derived DC.

1. DC are prepared from the recipient thymus (and spleen in the case of i.v. transfer) as described in detail previously (13) and in **Chapter 14** of this book.
2. The DC preparations are stained in multi-colours with anti-CD45.2 for precursor-derived cells and together with antibodies to various DC surface molecules, e.g. FITC-anti-CD45.2, APC-anti-CD45RA, Alexa-594-anti-CD11c, PE-anti-CD8, and biotin-anti-Sirpα with PE.Cy7-Streptavidin as a second stage reagent.
3. The stained cells are analysed on a flow cytometer and 50,000–100,000 cells are collected for each sample. The precursor-derived DC are revealed by gating for $CD45.2^{+}CD11c^{+}$ cells (**Fig. 18.3B**) and the precursor-derived DC populations are determined by the expression of various DC surface molecules.

4. Notes

1. Density is measured using a pycnometer. The pycnometer is a glass flask with a close-fitting ground glass stopper with a capillary tube in it, so that air bubbles may escape from the apparatus. This enables the density of a fluid to be measured accurately, by reference to water, using an analytical balance. The flask is weighed empty, full of water, and full of Nycodenz, and the specific gravity of the Nycodenz is calculated. A correction will need to be made as the density of water will not be 1 g/cm^3 at 4°C. It is important to ensure that Nycodenz is mixed thoroughly and at 4°C for all density measurements and for use in experiments as temperature, pH, and osmolarity all affect the buoyant density of cells. To calculate the dilution of stock, use the formula:

   100×1.16 (stock density) $+ 1.0 \times A = (100 + A) \times 1.086$ (required density) where A = additional volume of BSS to be added to 100 ml stock.
2. The CO_2 asphyxiation is preferred in these experiments, because this procedure does not cause internal bleeding in the chest and can avoid blood cell contamination of the thymus.
3. Efficiency of separation will be lost and yields reduced if the density separation is overloaded. Do not load more than four organs ($\sim 10^9$ cells) per 10 ml of Nycodenz.

4. A sharp interface will reduce the efficiency of the density separation. Disrupt the interface slightly using a pipette, to create a density gradient rather than a sharp cutoff.
5. After centrifugation the light density fraction of cells will have formed a band at the interface zone between the FCS and Nycodenz while dense cells will have formed a pellet. Cells of intermediate density will be found in the gradient between these zones, so collect all cells down to the 7 ml mark while concentrating on the light density band at the interface.
6. Adequate dilution and mixing of the light density fraction of cells with BSS is essential to recover them as a pellet during centrifugation.
7. It is important to use warm but not cold medium to wash the dish, otherwise adherent cells will be released.
8. Centrifuging through a layer of FCS can efficiently separate cells from unbound (excess) mAb.
9. A large amount of beads are required for the first round magnetic beads depletion. For economic reasons, BioMag beads are used for this round of depletion to reduce the cost.
10. The efficiency of the depletion is greatly increased by maximizing contact between beads and cells in a concentrated slurry. The bead–cell slurry is kept concentrated at the bottom of the tube by rotating the tube at a 30° angle. We create the angle by fitting a wide ring around the top of the tube to elevate it from the horizontal position.
11. BioMag beads are very small and migrate very slowly to the magnet. Therefore, it is important to leave the tube on the magnet for at least 3–5 min to successfully remove unwanted cells that are attached to the beads.
12. Despite low levels of CD4 on the surface of these precursors, CD4 is generally not used for staining. The combination of Thy-1 and c-kit gives the best separation of this population.
13. The combination of fluorescent-conjugated anti-CD25 and anti-c-kit are used to stain and separate the DN thymic precursor populations. These precursor populations can be distinguished with a developmental sequence: $CD25^{-}$c-kit^{+} (DN1) → $CD25^{+}$c-kit^{+} (DN2)$^{-}$→ $CD25^{+}$c-kit^{-} (DN3) → $CD25^{-}$c-kit^{-} (DN4). The DN1 population contains some precursors with a phenotype overlapping with the $CD4^{lo}$/ETP except the low levels of surface CD4. The combination of anti-CD25 and anti-CD44, although was used in many published studies, should not be used

to identify DN1 precursors as the $CD25^{-}CD44^{+}$ population is heterogeneous and contains c-kit^{-} non-precursor cells (6).

14. To avoid evaporation from Terasaki wells during incubation, the edge wells of the tray are filled with 10 μl culture medium without cytokines.
15. This procedure is necessary to break up the DC clusters into single-cell suspension. It is important to include EDTA in all medium after culture for phenotype analysis. DC tend to form clumps which can cause nozzle blockages in the flow cytometer.
16. Multi-colour fluorescent staining can be performed using different combinations of conjugated antibodies. The selection of different fluorochrome-conjugated antibodies to be used is based on the model of flow cytometer to be used and the available lasers and detectors fitted on the machine.
17. The rat–Ig blocking step is important to reduce non-specific binding of the fluorescent-conjugated antibodies (most of these are rat-Ig) to the cells.
18. The blocking rat-Ig can be washed off after 20 min incubation, cells are then stained with appropriate conjugated antibodies. Alternatively, the blocking rat-Ig can be left in the tube for 10 min before adding an equal volume of the staining antibodies. In this case, the concentration of each antibody added should be twice of the final concentration to be used.
19. Remove autoflourescent cells (mainly macrophages) during fluorescence-activated cell sorting or analysis by gating out cells that have low levels of fluorescence in two or more fluorescent channels. Ideally, use a combination of the PI and an unused channel (preferentially APC or PE channel). During multicolour sorting or analysis, it may be necessary to combine autofluorescence in the PI channel with low fluorescence in a channel that is being used, but with most of cells staining strongly.
20. The recipient-type bone marrow cells are co-injected to ensure long-term survival of the irradiated mice.

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Chapter 19

Isolation of Rat Intestinal Lymph DC

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Abstract

Dendritic cells (DCs) migrate constitutively from the intestine via the lymph to the mesenteric lymph nodes. These migrating intestinal lymph DCs (ilDCs) carry antigens acquired in the intestine and play important roles in both the initiation of immune responses and the maintenance of oral tolerance. The ilDC population is made up of at least three functionally different DC subsets. Like many DC populations, ilDCs are exquisitely sensitive to their environment, changing their phenotype and maturing in response to the procedures associated with their extraction from solid tissues. We have developed and refined a method for collecting and purifying these DC subsets from rats, without inducing them to mature. This method involves two separate surgical procedures, separated by at least 6 weeks. Initially, mesenteric lymph nodes are removed. After the animals have fully recovered we cannulate the thoracic duct and collect the ilDCs on ice, minutes after they have left the lymph vessel. The DCs are then enriched using magnetic beads and purified by flow cytometric sorting. We describe this method here, including our recent refinements to limit the use of the restraining "Bollman" cage.

Key words: Dendritic cells, intestinal, migrating, rat, surgery, thoracic duct.

1. Introduction

DCs are exquisitely sensitive to changes in their environment. Subtle changes can induce maturation, leading to profound changes in their phenotype and functions (e.g. (1, 2)). Some populations of DCs are migratory, travelling from the peripheral tissues via the lymph to the draining lymph nodes. In the steady state, these migrating DCs are required for the development of oral tolerance (3). After stimulation, they are necessary (4) and sufficient (5) for the induction of immune responses against protein antigens. Migratory DCs can be purified and

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collected from lymph by cannulation of lymphatic vessels (6). This technique has two advantages for the investigation of DC biology. First, DCs collected in this way can be "metabolically frozen" on ice the instant they leave the animal and are therefore maintained in their physiological state. Second, these DCs are the actual migratory population involved in the transport of antigens from peripheral tissues to secondary lymphoid organs and the transfer of information about the peripheral microenvironment. These cells are very difficult to identify and purify by any other method.

The lymphatic vessels of many species have been cannulated to collect migrating dendritic cells (7). While it is possible to directly cannulate the afferent lymphatics that carry lymph from peripheral tissues to the draining lymph nodes, the small diameter of these vessels and the small volumes of lymph that can be collected (6) have largely restricted this technique to larger animals (7, 8). Sanders and Florey first showed that after surgical removal of the lymph nodes, afferent and efferent lymphatics reanastomosed to restore lymph flow from peripheral tissues to the thoracic duct. Lymph collected from animals prepared in this way is described as being "pseudo-afferent". Thoracic duct cannulation in rats was first developed by Bollman (9). The technique was first used to study DCs, "non-lymphoid" cells, in pseudo-afferent lymph in 1983 (6). The technique described here includes recent refinements we have made to reduce the time for which animals are restrained after surgery, adapting methods previously described elsewhere (10).

The use of rats for thoracic duct cannulation presents a number of challenges. Compared to mice and humans there are relatively few reagents available, including monoclonal antibodies. There are also very few genetically modified rats and techniques for generating gene-targeted knockout rats have only recently been developed (11). Although gene targeting in rats is in its infancy, rat microarrays are readily available. Several groups have recently collected thoracic duct lymph from mice (12, 13, 14), but only Joan Rhodes has combined this with mesenteric lymphadenectomy to isolate murine intestinal DCs (15–17). To our knowledge, this technique of collecting mouse DCs is currently not in use.

In summary, the techniques described here for cannulation of the thoracic duct after removal of the mesenteric lymph nodes enable the direct study of important populations of migrating DCs, with minimal ex vivo manipulation. They present the opportunity to study these DCs under steady-state conditions, after vaccination, and in animal models of intestinal pathology.

2. Materials

2.1. Mesenteric Lymphadenectomy

1. Male PVG rats are usually used, at a weight of 80–95 g (*see* **Note 1**).
2. Surgical hat, gown, and mask for operator (*see* **Note 2**).
3. Heating pad.
4. Hair clippers.
5. Fibre optic lamps for illumination.
6. Binocular headband magnifier for 3.5× optical magnification.
7. Sterile surgical instruments: scissors – small; needle holder; curved forceps; straight forceps; curved watchmaker's forceps; scalpel handle (no. 3); scalpel blades (no. 10).
8. Sterile surgical swabs, 4 and 8 ply.
9. Surgical drapes.
10. Cotton buds.
11. Sterile surgical gloves.
12. Suture material (Vicryl 4.0).
13. Equipment for the induction and maintenance of anaesthesia.
14. Isoflurane.
15. Chlorhexidine gluconate surgical scrub.
16. Buprenorphine hydrochloride analgesic (0.3 mg/ml). Dilute 1:10 in PBS to 0.03 mg/ml. For each animal, prepare 50 μl in an insulin syringe.
17. Bupivecaine hydrochloride analgesic (Marcaine 0.25%).
18. PBS (phosphate-buffered saline).
19. Cold sterilising solution.
20. Tissue adhesive.

2.2. Thoracic Duct Cannulation

1. All surgical equipment and reagents needed for mesenteric lymphadenectomy are also required for the thoracic duct cannulation, as well as the following.
2. Animals from which the mesenteric lymph nodes have been removed 6–12 weeks previously (*see* **Note 3**).
3. Intravenous catheter 24 ga × 3/4″.
4. Extension tube for catheter (0.76 m narrow bore (2 mm)).
5. Durapore tape in 1 cm and 2.5 cm widths.

6. Non-dissolving suture material, cut in 8-cm lengths and sterilised.
7. Thoracic duct catheter, polyurethane fine bore tubing 0.6 mm internal diameter and 0.9 mm outer diameter. The catheter must be treated to bond heparin to the internal bore and prepared for insertion. For a batch of six rats, a 4.5-m length of tubing is rinsed through with acetone and immersed in 5% v/v 3-aminopropyltriethoxysilane (APS). This improves the binding of heparin. It is then rinsed with acetone and cut into 75-cm lengths. A blob of vetbond adhesive (~2 mm diameter) is applied around the catheter, 5 cm from the end. This helps to secure the catheter in place. Finally, the tubing is heparinised by immersion in a solution of heparin at 5,000 U/ml (*see* **Note 4**).
8. 10-ml Syringes filled with PBS (2 per animal), attached to 21 gauge needles.
9. Collection flasks: T75 tissue culture flasks. Pierce 2–3 holes in the lid with a 19 gauge needle.
10. 0.5 M EDTA (ethylenediaminetetraacetic acid) in distilled water, pH 7.2.
11. Heparin: Heparin in used two buffers to help prevent blood clots trapping the DCs.
12. Collection buffer: PBS with 20 U/ml heparin, 10 mM EDTA. Add 25 ml to each collection flask.
13. Infusion buffer: 0.9% w/v sodium chloride and 5% w/v glucose mixed 50:50 with PBS and 2 U/ml heparin; 1 l is required for infusion of six rats over 48 h
14. Sterile surgical instruments: scissors – large; aneurysm needle; retractors; curved watchmaker's forceps; microsurgical scissors; smooth, pointed glass probe; 2 mm diameter (Made in-house); skin-tunnelling needles.
15. Bollman cages for overnight restraint of cannulated animals. These are not available commercially. Full specifications are available from the authors.
16. Multi-channel cassette pump.
17. Covance infusion harness.
18. Horses tail hairs, sterilised.
19. Modified rat cages, with a small hole at floor level to allow exit of thoracic duct cannula carried within the armoured tether.

2.3. Enrichment and Purification of DCs

1. Complete tissue culture medium (CM): RPMI supplemented with 5% heat-inactivated foetal calf serum; 50 μM 2-mercaptoethanol (2-ME, Sigma, Gillingham, Dorset, UK),

and with 2 mM MUTHU-glutamine, 100 U/ml penicillin, 100 μg/ml (Invitrogen, Paisley, UK).

2. Cell strainers (100 μm).
3. 50-ml Centrifuge tubes (BD Falcon) (*see* **Note 5**).
4. Lysis buffer: 8.29 g NH_4Cl, 1 g $KHCO_3$, 37.2 mg Na_2EDTA, 800 ml H_2O. pH 7.2–7.4 with 1 N HCl. Add H_2O to 1 l. Filter sterilise. Store at room temperature.
5. Haemocytometer.
6. OX62 Microbeads (Miltenyi Biotec).
7. Separation columns and magnetic devices, suitable for the number of cells to be obtained (Miltenyi Biotec).
8. Fluorescent antibodies for identification of rat lymph DCs, e.g. antibodies specific for rat MHC class II (OX6-PerCP), CD103 (OX62-PE), SIRPα (OX41), CD11b/c (OX42-FITC). The OX41 antibody is conjugated to fluorescent Alexa 647 using an antibody conjugation kit.

3. Methods

3.1. Mesenteric Lymphadenectomy

1. Use isoflurane in oxygen to induce and maintain anaesthesia and place the animal on its back on the warming pad.
2. The abdomen should be shaved using hair clippers, and the skin sterilised using Hibiscrub diluted 50:50 in warm water or a similar skin-sterilising solution.
3. Firmly fix the rat to the operating table by taping its feet, with its head away from the operator and administer systemic analgesic subcutaneously, e.g. 100 μl buprenorphine hydrochloride (0.03 mg/ml) or as advised by local veterinary specialist (*see* **Note 6**).
4. Starting at the xiphisternum, make a distal 2 cm mid-line skin incision. The location of this incision is indicated by the diagram in **Fig. 19.1A**. Then make a similar-sized mid-line incision along the linea alba to enter the peritoneal cavity.
5. Using cotton buds, gently manipulate the intestines out through the incision and lay them on saline-soaked gauze to the animal's left. The area of the inferior vena cava is then exposed by proximal manipulation of the loops of the small intestine.
6. The mesenteric lymph nodes must all be identified and removed (*see* **Note 7**). Once identified (see below), each

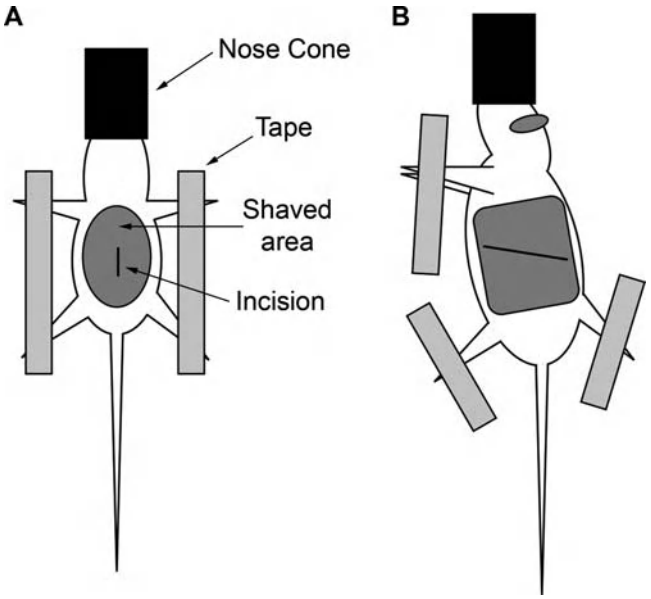

Fig.19.1. Positioning of animals for surgery and location of initial incisions. Animals are shaved and prepared for either (**A**) mesenteric lymphadenectomy or (**B**) thoracic duct cannulation. The location of the anaesthetic nose cone, the area to be shaved, the site of the initial incision, and the placement of the adhesive tape used to secure the animal are all shown.

node is exposed by careful removal of adipose tissue with forceps. The node is then grasped and teased from the surrounding connective and adipose tissues with a cotton bud, allowing its removal (*see* **Note 7**).

7. Identify and remove the two isolated lymph nodes visible as darker-coloured patches buried in the adipose tissue adjacent to the caecum. The caecum may need to be gently manipulated to expose these nodes (*see* **Note 8**).
8. Identify and remove the entire "chain" of mesenteric nodes running along the (anterior) mesenteric vein. These nodes are clearly visible at the root of the mesentery. Begin removing the nodes at the caecal end of the chain. With care, the entire series of nodes may be removed as one or two pieces. Use pressure from cotton buds to secure haemostasis.
9. Identify and remove the lymph nodes that effectively form a continuation of the mesenteric lymph node chain as it passes through a proximal loop of the small intestine. These nodes are most easily identified if this proximal intestinal loop is moved to the animals left.
10. Once all the lymph nodes have been removed, check haemostasis and replace the intestines.

11. Local analgesia should be provided to assist the animal's recovery. This can be achieved by subcutaneous injection of a total of 0.5 ml Marcaine (or similar, as recommended by local veterinarians) in several places around the surgical site.
12. Complete surgical closure of the wound, terminate anaesthesia, and allow the animal to recover from the surgery (*see* **Note 9**).

3.2. Thoracic Duct Cannulation

1. After the mesenteric lymphadenectomy procedure, animals must be allowed to recover for at least 6 weeks. This allows the afferent and efferent lymphatics to re-anastomose, enabling collection of DCs by thoracic duct cannulation.
2. Use isoflurane in oxygen to induce and maintain anaesthesia and place the animal on its back on the warming pad.
3. Use clippers to shave the animal's abdomen and flank, removing hair from a region between the mid-line and backbone and distally from mid-thorax to waist. Also shave the area on the animal's back between the shoulder blades. Sterilise the skin with Hibiscrub or alternative agent, as above.
4. Administer systemic analgesic subcutaneously, e.g. 100 μl buprenorphine hydrochloride (0.03 mg/ml) or as advised by local veterinary specialist.
5. First, an intravenous catheter must be introduced into the animal's tail. Infusion of PBS through this catheter causes the animal to become hypervolaemic, swelling the thoracic duct and facilitating the subsequent steps. The catheter is easier to introduce if the tail vein is dilated. Therefore, warm the tail in water for 30 s. The water temperature should not be uncomfortably hot to the touch, as excessive heat may damage the tail.
6. Fill the extension tube with PBS, ensuring that no bubbles are present. Introduce an i.v. catheter into a lateral tail vein and remove the metal introducer. If placed in the correct position blood will flow down the catheter into the luer hub. At this time, attach the extension tube and secure the catheter to the tail with Durapore tape in at least three places. Be sure to cover the area where the catheter enters the vein (*see* **Note 10**).
7. Slowly infuse a bolus of 2–3 ml PBS through the i.v. catheter.
8. To prepare for the cannulation procedure, position the animal on its right flank. Use adhesive tape to secure both forelimbs together on the left side. The rear limbs are

stretched to left and right and secured with tape, as indicated in **Fig. 19.1B**.

9. Cover the animal with a surgical drape. A hole of approximately 10 × 10 cm over the ribcage and subcostal region allows access for surgery.
10. Starting at the xiphisternum, make a subcostal skin incision 6–8 cm long towards the animal's left side, in the location shown in **Fig. 19.1B**. Next make a small incision through the double muscle layer, taking care not to damage the underlying organs. Expand the subcostal muscular incision to about 5–6 cm.
11. The kidney is tethered by a vascular stalk and embedded in adipose tissue. In order to expose the thoracic duct, using cotton buds carefully free the kidney from its fatty bed so that it is mobile on its vascular stalk.
12. Retract the abdominal organs, using two PBS-soaked 4-ply swabs. The liver, spleen, and stomach are retained to the anterior of the cavity. The kidney and intestines are retained to the posterior. Then position a retractor to hold the swabs and organs in place.
13. Expose the thoracic duct, which may be identified running adjacent to the abdominal aorta. It is often obscured as it lies underneath the edge of the psoas muscle. Separate the thoracic duct and aorta from the muscle by firmly pulling the muscle laterally with cotton buds, exposing the two vessels.
14. Use the glass rod to clear the duct of any overlying membranous and connective tissues. Take extreme care not to damage the duct and do not attempt to remove the blood vessels that cross the duct. These vessels delineate the upper limit of the usable duct. By applying the glass rod against the side of the aorta, separate the aorta from the duct just below the crossing blood vessels. Then separate the duct from the fascia on its other side, thus generating a channel around the duct through which an aneurysm needle may be pushed to introduce a 10 cm length of Mersilk behind the duct. The suture should be positioned 5 mm distal to the overlying blood vessel and tied with a single loose overhand knot (*see* **Note 11**).
15. Exteriorise the catheter by pushing a 16-ga disposable needle through the abdominal wall from the outside, proximal to the incision site, and close to the mid-line. Do not pierce the skin, and take care not to puncture any abdominal organs. Feed the catheter through the needle from the interior to the exterior, ensuring that the end with the adhesive blob remains inside the animal. Remove

the needle, and fill the catheter with PBS using a syringe attached to a 21 gauge needle. Keep the needle and syringe attached to the catheter.

16. Using a skin-tunnelling needle, create a channel beneath the skin from the location where the cannula emerges from the abdominal wall, to a point on the animal's back between the shoulders. Push the catheter along the needle and exteriorise it through the skin at the end of the channel. A small skin incision, made with scissors, may facilitate the exteriorisation of the tunnelling needle.
17. The duct must be cut to allow the insertion of the cannula. Holding microsurgical scissors vertically above the duct make a transverse incision in the upper wall of the duct at a location 2 mm proximal to the suture. Expand the hole slightly by placing the tips of the scissors in the hole and opening them.
18. The cannula must be trimmed to enable the correct length to be inserted into the duct. Therefore, offer the catheter up to the duct and determine its optimal length, remembering that the cannula will not penetrate more than 8–10 mm distal to the location of the suture around the duct. Use a scalpel to trim the cannula and cut a 45 degree bevel at the tip of the catheter. The bevel should face up when inserted in the duct.
19. Insert the tip of the catheter into the duct and gently push it along the duct, about 8–10 mm until resistance is encountered, as shown in **Fig. 19.2** (*see* **Note 12**). Use serrated forceps to tighten the loose suture around the duct. Do not knot the suture at this stage.
20. Remove the syringe and needle from the end of the cannula and check that drops form at the end of the cannula. To check that the cannula is inserted correctly, raise the loose end above the level of the animal. Fluid should only flow back a few millimetres into the cannula indicating that it fits snugly within the duct (*see* **Note 13**).
21. Secure the cannula firmly by knotting the suture around the duct. Do not over-tighten this knot, as this may crush the polyurethane cannula and prevent the flow of lymph.
22. Remove the retractor and swabs, and return all the organs to their original positions using cotton buds.
23. Local analgesia should be provided to assist the animal's recovery. This can be achieved by subcutaneous injection of a total of 0.5 ml Marcaine (or similar, as recommended by local veterinarians) in several places around the wound site.

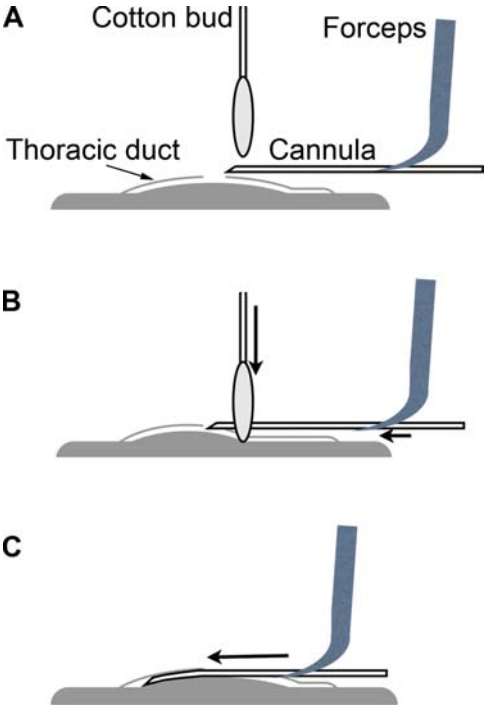

Fig.19.2. Detail of procedure for insertion of cannula into the thoracic duct. **(A)** The cannula, held with curved forceps, is aligned such that its tip is adjacent to the thoracic duct incision where it will be inserted. A cotton bud is readied and **(B)** used to apply downward pressure on the tissue immediately proximal to the insertion point. This positions the duct to allow the pointed tip of the cannula to be inserted into the incision. **(C)** The cannula is gently fed along the thoracic duct a distance of approximately 5 mm until resistance is encountered.

24. Close the thoracic wound with continuous sutures. Continuous sutures are suitable for both muscle and skin, as animals restrained in Bollman cages cannot access the wound area.
25. Place the animal in a thoracic harness, passing the exposed portion of the cannula through an armoured tether (*see* **Note 14**).
26. Before recovery from anaesthesia, place the animal into a Bollman restraining cage. Take care not to dislodge the tail vein catheter or thoracic duct cannula.
27. Infuse animals with infusion buffer at 2–3 ml/h through the intravenous catheter, using the pump.
28. Lymph should be collected into collection flasks, on ice. Each flask contains 25 ml of collection buffer (*see* **Note 15**). Ensure caps are not tightly fastened, as a build up of pressure can stop lymph flow.

29. It is common for clots to form in the cannula, preventing lymph flow, especially in the first 6–8 h after surgery. Lymph flow should therefore be checked at least every 2 h during this initial period (*see* **Note 16**).
30. After an overnight collection of lymph from animals housed in restraining cages, they are transferred to the modified normal cages. First, the i.v. catheter is removed from the tail, then animals are carefully lifted from the restraining cages and placed in the normal cages. The thoracic duct cannula in its armoured tether is pushed through the hole drilled in the cage, and the animal is provided with ample food and water.
31. Lymph may be harvested for a number of days, although we have not maintained animals for more than 3 days after cannulation.
32. At the end of the experiment, animals are humanely killed.

3.3. Enrichment and Purification of DCs

1. All steps are performed using ice-cold solutions, and cells are kept on ice at all time unless otherwise stated.
2. Lymph is strained through a 100-μM cell strainer into a number of 50-ml centrifuge tubes to remove any clots and debris. Lymph collection flasks are thoroughly rinsed with approximately 10 ml ice-cold CM, and this is also strained (*see* **Note 17**).
3. Lymph samples are spun at 380 × *g* for 5 min, at 4°C, to pellet cells.
4. Cell pellets are resuspended in approximately 20 ml CM per 200 ml lymph.
5. Samples are spun at 380 × *g* for 5 min, 4°C to pellet cells.
6. Each sample is thoroughly resuspended in 5 ml ACK lysis buffer and incubated at room temperature for 3 min without agitation.
7. 15 ml CM is added to each sample to prevent further cell lysis. A sample of cells is counted using a haemocytometer. Samples are again spun at 380 × *g* for 5 min, at 4°C, to pellet cells.
8. Samples are resuspended in CM at approximately 1×10^9 cells/ml, and 10 μl OX62 microbeads are added per 1×10^8 cells, prior to incubation on ice for 30 min. The OX62 antibody binds the rat CD103 molecule (*see* **Note 18**).
9. Cells are washed in 10 ml CM per sample, centrifuged at 380 × *g* for 5 min, at 4°C and resuspended at approximately 5×10^8 cells/ml.

10. Microbead-mediated enrichment of CD103-expressing cells is performed using the "depletes" programme on an AutoMACS machine (*see* **Note 19**).
11. A sample of the OX62-enriched DCs is counted using a haemocytometer.
12. If DCs are to be separated into the three subsets by MoFlo sorting, they are stained with antibodies specific for CD103 (OX62), MHC class II (OX6), CD172a (OX41), and CD11b/c (OX42). Each of the antibodies should be conjugated to a different fluorochrome; the specific choice of

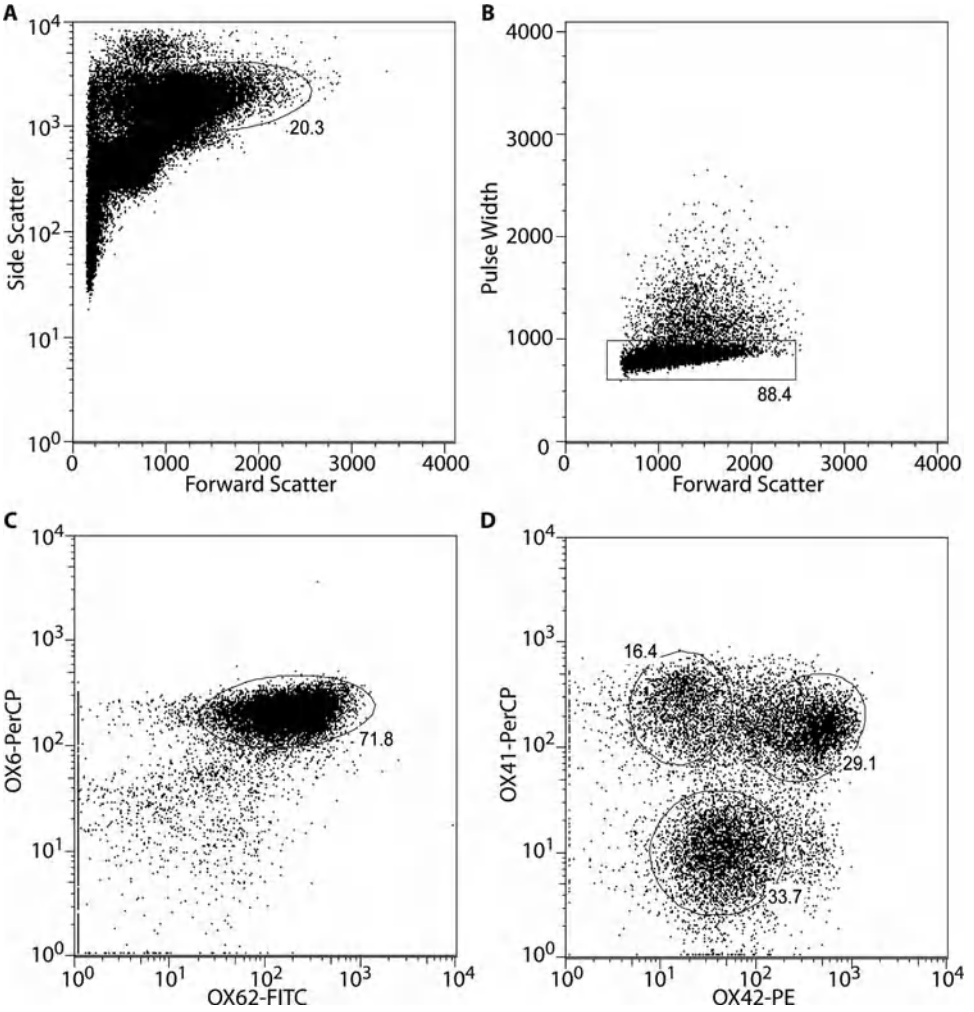

Fig.19.3. Gating strategy for flow cytometric sorting of rat intestinal lymph DCs. (**A**) A high side scatter, medium-to-high forward scatter gate is initially defined. (**B**) The pulse width parameter is used to remove doublets and larger clusters of cells from the sorted population. (**C**) Rat intestinal lymph DCs are defined by their high expression of MHC class II (OX6) and CD103 (OX62). They make up approximately 12% of this enriched sample and approximately 0.4% of total lymph cells. (**D**) Intestinal lymph subsets are defined by their expression of CD11b (OX42) and CD172a (SIRPα) (OX41)

fluorochromes will depend on the configuration of the cell sorter (*see* **Note 20**).

13. Following staining, DC subsets are sorted using the gating scheme shown in **Fig. 19.3**.
14. Sorted DC subsets may be used to investigate a broad range of important immunological phenomena, including the responses to oral adjuvants (5) and TLR ligands (18, 19), the maintenance of oral tolerance (20), and the origins of intestinal autoimmune disease (21).

4. Notes

1. Although PVG rats are generally used, we have also successfully cannulated Lewis animals. The surgery is less difficult if males are used, as they carry less abdominal fat. Investigators must ensure that they have received the necessary permissions from the relevant veterinary authorities to perform these surgical techniques on live animals. The nature of these permissions will depend on the country in which the experiments are performed.
2. All surgical procedures must be performed using sterile technique. This will have a number of benefits; increasing the frequency of complication-free procedures, minimising adverse effects on the animals, and maximising the reproducibility of the experiments.
3. Collection of DCs from the thoracic duct is a two-step procedure. First, mesenteric lymph nodes are removed from young animals. Afferent and efferent lymphatics are then allowed to re-anastomose over a period of at least 6 weeks. Then, in the second surgical procedure, a cannula is inserted into the thoracic duct to drain the DC-containing lymph. We do not usually perform cannulations on animals later than 12 weeks after mesenteric lymphadenectomy, as they become too large for their restraining cages. Careful management of animal stocks is therefore critical to prevent the performance of unnecessary procedures.
4. Cannulae are stored in heparin at 4°C for up to a week before use. APS is a corrosive agent. Appropriate precautions must be taken, as described in the literature supplied with the product.
5. Appropriately sized centrifuge tubes must be used with the cell strainers. We have found that tubes from suppliers

other than BD have fractionally smaller diameters, leading to spillage of valuable lymph when using BD cell strainers.

6. To obtain the maximum number of DCs after thoracic duct cannulation, it is important to remove as many as possible of the mesenteric lymph nodes. Any nodes not excised will trap DCs, reducing yield. However, nodes seem to be arranged "in parallel" rather than "in series" as we have found that intentionally leaving approximately half the nodes in place only reduces the final DC yield by half (SM, unpublished data).
7. It is important to avoid damage to large blood vessels. If necessary, haemostasis may be maintained by temporary application of direct pressure using a cotton bud.
8. The lymphatic system of the rat is described in detail by Tilney (22).
9. Surgical complications are rare but may include haemorrhage, infection, or post-operative ileus. Animals must be checked frequently after surgery.
10. If the tail is allowed to hang vertically during the procedure, blood may flow back into the catheter and a clot will block the tube. To prevent back flow, position the tail to the side of the animal, supported by the bench.

    On occasion, the catheter may not be introduced into a lateral vein at the first attempt, and blood fails to flow back into the introduced catheter. The catheter should then be removed, the tail re-warmed, and a new catheter re-inserted at a different point along the vein, at least 3 cm from the initial puncture. Alternatively, attempts may continue using the lateral vein on the other side of the tail.
11. Any damage to the duct at this stage will usually prevent a successful outcome of the procedure. Take extreme care not to tear the delicate thoracic duct when clearing the membranes and when inserting the aneurysm needle.
12. The orientation of the hole in the thoracic duct will often prevent insertion of the cannula, due to the shape of the underlying tissues. Application of firm pressure from a cotton bud at a location proximal to the insertion point will often improve this orientation, allowing the catheter to be inserted.
13. On occasion the cannula will not be properly positioned within the lumen of the thoracic duct – drops will either not form at the end of the catheter or fluid will continue to flow back when the catheter is raised. Sometimes the inserted end of the cannula may be pushing against a lymphatic valve and a very slight withdrawal of the cannula

will solve the problem. If such repositioning is unsuccessful, the cannula should be removed and attempts made at re-insertion, taking care not to push the catheter too deep, between the duct and the underlying muscle, or too shallow, between the duct and any remaining overlying connective tissue. This is the stage at which the procedure is most prone to failure, as repeated attempts at re-insertion can damage the duct, rendering cannulation impossible.

14. Animals remain in the restraining "Bollman" cages for only one night. After this period they are placed, singly, in their normal cages. These cages are modified with a small hole at floor level which allows the end of the cannula, protected within an armoured tether, to pass out of the cage. The harness and tether prevent the animal from damaging the cannula.
15. The volume of collection buffer used will depend on the volume of lymph to be collected. We aim to have a final concentration of no less than 10% collection buffer, and find that pooled lymph from 2–3 rats, collected overnight, reaches a volume of 150–250 ml. If lymph is not pooled, or a shorter interval is used, a smaller volume of collection buffer will be required and a smaller collection flask may be used.
16. If a clot should block the cannula, flow can often be restarted by attaching a 10-ml syringe and a 21-ga needle to the cannula to apply suction. If flow does not restart, clots can sometimes be removed using a sterile horse tail hair. The hair is inserted into the cannula and carefully pushed past the clot, then twisted to catch the clot, and slowly removed. Occasionally, it may prove impossible to remove a blockage. In this situation the investigator must decide whether to re-anaesthetise the animal and insert a replacement cannula.
17. We typically collect approximately 100 ml of lymph per 24 h from animals receiving i.v. infusions. The volume obtained from non-infused rats is substantially lower. If pooled, lymph from six rats should typically yield 2.5×10^9 cells (not including RBCs).
18. We use OX62 microbeads to enrich DCs prior to MoFlo sorting to generate pure subsets of cells. This enrichment is economically attractive because DCs comprise only approximately 0.4% of cells in steady-state thoracic duct lymph from rats after mesenteric lymphadenectomy. After enrichment, samples will contain 10–30% DCs. On occasion, DCs may be prepared in other ways. For instance, to determine

the absolute number of DCs per animal per unit time, non-enriched samples may be used for FACS analysis.

19. We commonly use an AutoMACS machine (Miltenyi), but LS or XS columns and an MACS Separator (Miltenyi) operate with similar efficiency and require less expense in environments where there might be fewer users of this technology.
20. The combination of fluorochromes described in the materials and methods is appropriate for use with a sorter with two lasers, at 488 nm and 633 nm. Other combinations may be required for other machines. If streptavidin-bound secondary antibodies are used, samples should not be washed in medium containing RPMI as the high levels of biotin therein will prevent binding of the secondary antibody. Under these circumstances we wash cells in PBS supplemented with 5% v/v foetal calf serum prior to addition of the streptavidin–fluorochrome complex.

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Chapter 20

Mouse Models of Viral Infection: Influenza Infection in the Lung

Adele M. Mount and Gabrielle T. Belz

Abstract

Respiratory viral infections are a major cause of morbidity and mortality. Protection of the respiratory tract from pathogen infections, such as influenza virus, requires the orchestrated activation and trafficking of pulmonary dendritic cells (DCs) from the lung to the lymph node (LN) in order to ensure optimized T-cell responses. Gaining a better understanding of the cellular and molecular processes that protect the lung during infection is essential for future advances in vaccine strategies and treatments. Influenza viral infection in mice offers a very well-defined immunological system in which the underlying parameters regulating the generation of protective immunity can be elucidated. In this chapter, we review methods for quantitative analysis of DC and T-cell responses in a murine model infection of influenza. Antigen-specific tracking and quantitation of viral immune responses have been greatly facilitated by the advent of MHC tetramers and intracellular cytokine analysis, together with gentle isolation procedures for dendritic cells allowing detection of viral and endogenous antigens.

Key words: Influenza virus, dendritic cell, antigen presentation, infection, T-cell activation.

1. Introduction

1.1. The Protective Armoury of the Lungs

Infections, particularly from viruses such as influenza virus and respiratory syncytial virus, are potent stimulants to the respiratory tract. The airways and lungs are constantly exposed to a multitude of infectious and non-infectious agents and mechanisms to protect these vital tissues are critical. This is achieved through (i) the physical barrier provided by the airway epithelium and (ii) immune cells that act as sentinels in the lung and lymph nodes (LNs) draining the lungs. The conducting airways of the lung are lined by a mucociliary blanket, which acts to exclude inhaled

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antigens and pathogens, while the epithelial cell layer acts as a molecular sieve to exclude inhaled antigens and pathogens based on their molecular weight.

1.2. Ferrying Antigen to the Lung

The dendritic cells (DCs) of the airways and alveoli play an integral role in initiating the immune response to pulmonary infections (*see* **Fig. 20.1**). During respiratory infection, DCs acquire virus or viral antigens and transport these to the mediastinal LNs draining the respiratory tract. These LNs also receive lymph from the peritoneal cavity. Within the mediastinal LNs, antigen-bearing DCs drive initial activation, proliferation, and differentiation of $CD4^+$ and $CD8^+$ T cells (1, 2). Following these events, $CD8^+$ T cells migrate from the mediastinal LNs to the lung where they can mediate protection by destroying infected cells.

1.3. DCs of the Lungs and Bronchial-Associated Lymphoid Tissue (BALT)

Mucosal-associated lymphoid tissues (MALT) represent a diffuse system of lymphoid tissue that is found underlying the epithelium of body sites such as the gastrointestinal tract, lungs, salivary gland, eye, and skin. In the lung, these organizations of

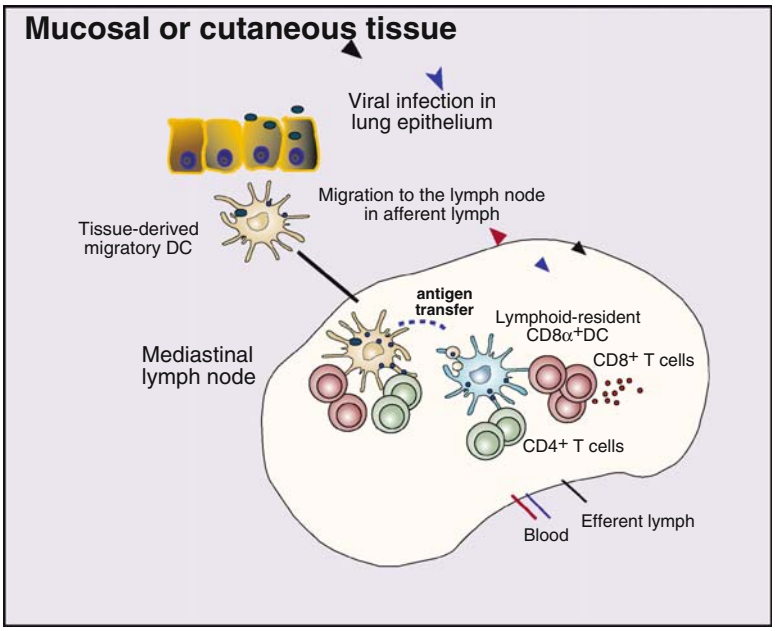

Fig. 20.1. Migratory route of DCs transporting virus and antigens from peripheral tissues to mediastinal LNs. Pathogen-derived antigens are transported by DC to the draining lymph nodes (*upper panel*) for presentation to T cells. Migration of antigen-laden DCs from periphery is required to initiate both the primary and secondary immune response. DCs can either (i) initiate the T-cell activation cascade themselves by presenting antigens derived from viral replication within the cell itself (direct or endogenous antigen presentation) to the T cell, or alternately (ii) pass their antigen over to lymphoid-resident DCs (cross-presentation to $CD8^+$ T cells and exogenous antigen presentation to $CD4^+$ T cells) that then present antigen to T cells.

lymphoid tissues are known as bronchial-associated lymphoid tissues or BALT. Although the primary immune response to respiratory viral infections is initiated in the mediastinal LNs, DCs within the lung and BALT may be important in driving the continued expansion of T cells following their migration to the lung (3). In the lung and gut, organized secondary lymphoid tissues may be induced by antigenic stimulation or localized inflammation. The formation of such tissues in lung is known as induced bronchus-associated lymphoid tissues or iBALT (4). These structures are formed several days (generally 5–6 days) after first infection and are composed of B-cell follicles, T cells, and DCs. Significantly, the formation of this tissue within the infected lung tissue may provide a front-line defence in respiratory immune responses.

1.4. DCs of the Mediastinal LNs

DC subsets found in the airways and lung parenchyma are composed of two populations of cells: $CD11c^+CD11b^+CD103^+$ and $CD11c^+CD11b^+CD103^-$ DCs. These DCs appear to be derived from blood monocytes and constitutively migrate in lymph to the mediastinal LNs even in the absence of infection or overt inflammation (5, 6). In addition to the migratory DCs, lymphoid-resident DCs, those that develop within the lymphoid tissue themselves, comprise at least half of DCs found in the LNs (7). The surface molecules CD11c, CD8α, and CD45RA allow the discrimination of three populations of DCs, namely the populations are $CD8\alpha^+CD4^-$ (CD8 DCs), $CD8\alpha^{+/-}CD45RA^+$ (plasmacytoid DCs, pDCs), and $CD8\alpha^-CD45RA^-$ (or double-negative) DCs. pDCs, noted for their production of interferon-α, appear to be relatively immature and express high levels of CD45RA, intermediate levels of CD11c, and only low levels of costimulatory and major histocompatibility complex (MHC) class II molecules (8, 9).

1.5. Influenza Virus: A Model of Primary and Recall Respiratory Infection

The mouse is a commonly used model for studying viral infections. Most strains of influenza virus can be used to infect mice although prior adaptation of the virus by multiple passages through mouse lung allows for selection of viral mutants that are able to replicate efficiently in the murine respiratory tract. Intranasal inoculation results predominantly in lower respiratory tract infection although all epithelial cells of the bronchi and alveoli are susceptible to fully adapted mouse strains.

A classical viral model of respiratory infection in mice has utilized different influenza viruses to elicit primary and secondary, or recall, immune responses. This approach depends on identifying heterologous viruses in which the surface glycoproteins (the hemagglutinin, HA and neuraminidase, NA), the main target

for antibody responses, differ between viruses while the internal proteins, those that are the main targets of cytotoxic $CD8^+$ T-cell responses, are conserved (10, 11). Sequential infection with influenza A HKx31 (X-31) and PR8 (A/Pueto Rico/8/34, PR8) viruses provides an elegant strategy to examine antigen presentation and T-cell responses in a model system. X-31 influenza is a genetic reassortant of the H3N2 A/Aichi/2/68 and H1N1 A/Puerto Rico/8/34 influenza strains such that X-31 shares the six internal genes with the PR8 virus and the coat proteins are derived from A/Aichi/2/68 (12). Therefore, X-31 and PR8 are serologically distinct viruses and cross-reactive neutralizing antibodies are largely avoided because X-31 and PR8 express different surface HA and NA proteins. Prime and challenge experiments can be conducted because most of the viral peptides generated during processing of viral proteins are recognized by influenza-specific $CD8^+$ T cells directed towards the conserved internal proteins (*see* **Fig. 20.2** and **Table 20.1**). Similar approaches are now used in other viral systems.

T-cell responses: T cells play an essential role in protection against a variety of infections. Influenza infection elicits a broad range of virus-specific T-cell responses mainly directed towards the internal genes of the virus. The best-defined model system for studying antigen presentation and T-cell responses to influenza has been described in C57BL/6 mice ($H\text{-}2^b$) in which the viral genome of X-31 and PR8 has been scanned several times for the presence of canonical and non-canonical T-cell epitopes (13–17). The primary $CD8^+$ T-cell response to either strain is dominated by naïve T-cell recognition of two determinants, the nucleoprotein ($NP_{366–374}$, $H2D^b$) (15) and the acid polymerase ($PA_{224–233}$, $H2D^b$) (16). A lower proportion of the other determinants are detected in the broncho-alveolar lavage (BAL), LNs, and spleen. The secondary influenza-specific cytotoxic T-cell response is only slightly more rapid (~1–2 days) than the primary immune response, in part restricted by the rate-limiting migration of DCs from the site of infection in the lung to the draining mediastinal LNs. In both primary and secondary lung infections, the mediastinal LN remains the site of initiation of the cellular immune response (1, 2); although late in infection, DCs within the lung and iBALT may drive continued proliferation and activation of T cells at the infection site (3, 4). In contrast to the similarity of the magnitude of the $D^bPA_{224–233}$ and $D^bNP_{366–374}$-specific $CD8^+$ T cells during the primary immune response (generally ~1–2% of $CD8^+$ T cells in spleen), $D^bNP_{366–374}$ response dominates the secondary response to X-31 →PR8 challenge (infection of i.p. PR8-primed mice with X-31intranasally >3 weeks after priming), accountin for up to 60–80% of influenza-specific $CD8^+$ T cells. Despite the dominance of $D^bNP_{366–374}$, the other

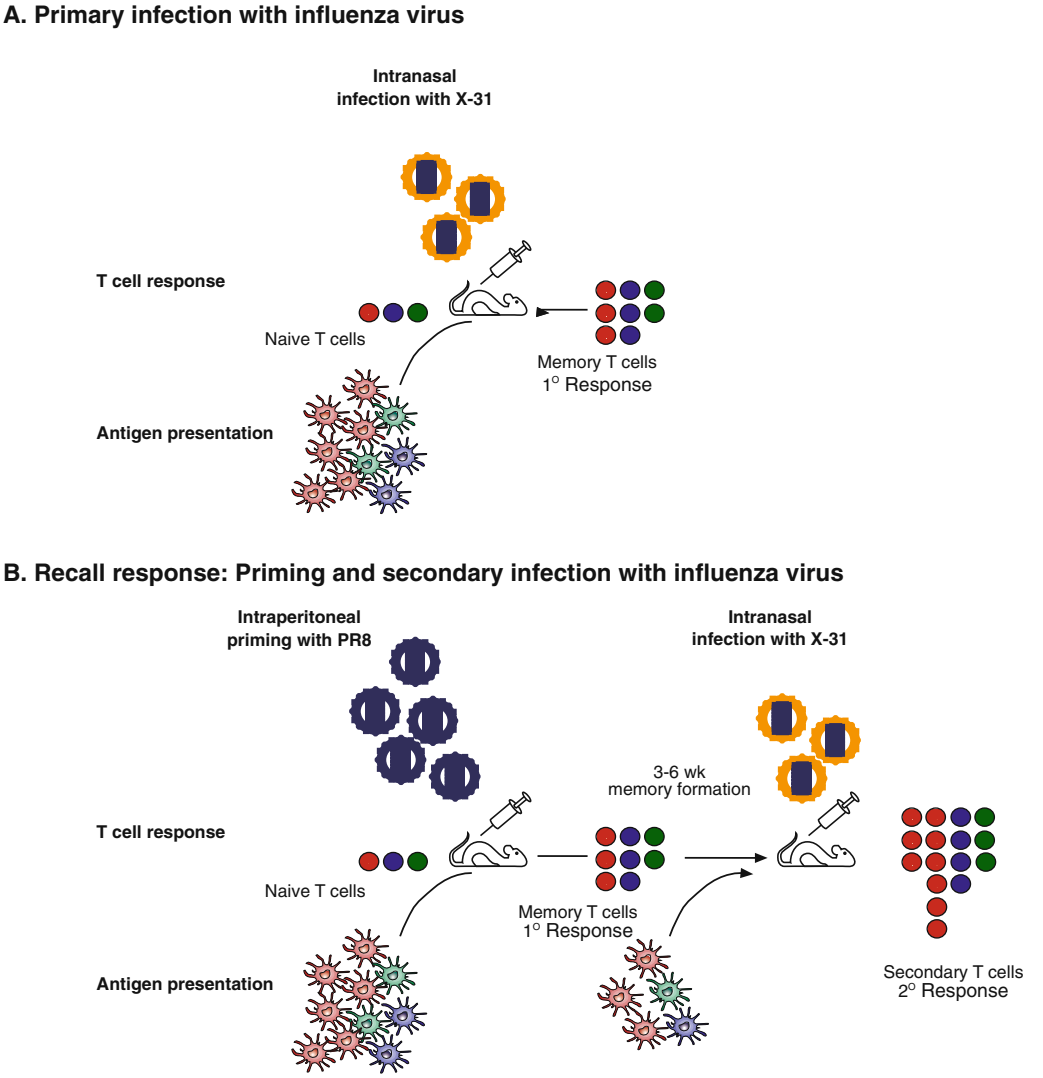

Fig. 20.2. Experimental approach to prime/boost regime for influenza infection in murine model. Mice are infected or primed with influenza X-31 intranasally, or PR8 intraperitoneally to elicit a primary immune response. Differing numbers of DCs carry antigens that can stimulate the naïve T cells specific for each viral antigen generated. This contributes to the differences in the frequency of different populations of influenza-specific T cells in the memory populations found in spleen and lung. A recall (or secondary) response is elicited by challenging mice with X-31 several weeks after their primary encounter with the related PR8 influenza virus.

determinants identified for influenza can still be isolated after secondary challenge, albeit at low frequency. Efforts to understand the factors driving the kinetics and hierarchy of these T-cell populations have underpinned careful approaches to map and quantify the contribution antigen-presenting cells (APCs) make in presenting different T-cell MHC/peptide complexes and the T-cell response (3, 16, 18–25).

Table 20.1
Major T-cell epitopes in H-2^b (C57BL/6) murine models of influenza infection

Epitope	MHC restriction	Peptide sequence	Viral protein	Original References
$NP_{366–374}$	D^b	ASNENMETM/ ASNENMDAM	Nucleoprotein	(15)
$PA_{224–233}$	D^b	SSLENFRAYV/ SCLENFRAYV	Acid polymerase	(16)
$PB1_{703–711}$	K^b	SSYRRPVGI	RNA-directed RNA polymerase subunit P1	(26)
$PB1\text{-}F2_{62–70}$	D^b	LKTRVLKRWRL	RNA-directed RNA polymerase subunit P1	(13)
$NS2_{114–121}$	K^b	RTFSFQLI	Non-structural protein 2/nuclear export protein	(14)
$M1_{128–135}$	K^b	MGLIYNRM	Matrix protein	(14)
$HA_{332–340}$	D^b	TGLRNIPSI	Hemagglutinin	(27)
$HA_{211–225}$	A^b	YVQASGRVTVSTRRS	Hemagglutinin	(17)

2. Materials

2.1. Infections

1. Penthrox® (active ingredient methoxyflurane).
2. Injectable anaesthetic: 80–100 mg/kg ketamine and 10 mg/kg Rompun® (active ingredient xylazine).
3. Avertin® (active ingredient 2,2,2-tribromoethanol; 50 mg/kg).

2.2. Media

All media were sterilized by filtration through a 0.22-μm membrane filter and stored in the dark at 4°C.

1. Complete medium: RPMI-1640 or DMEM supplemented with 10% foetal calf serum (FCS), 50 μM 2-mercaptoethanol, 2 mM L-glutamine (ICN Biomedicals), 100 U/ml penicillin, and 100 μg/ml streptomycin.
   RPMI-1640 (mouse tonicity): RPMI-1640 powder (Gibco BRL), 9 g NaCl, 20 g $NaHCO_3$, 1.1 g sodium pyruvate, 2 mM L-glutamine (Gibco BRL), 1.0 g penicillin, and 1.0 g streptomycin in 10 l of Milli-Q water. Final pH 7.2. This medium has been modified to mouse osmolarity (308 mOsm/kg) and additional Hepes buffering is included to reduce dependence on CO_2. Cells are generally cultured in 10% CO_2 atmosphere for this media.

2.3. Antibody Depletion Cocktails

1. $CD8^+$ T-cell-enrichment cocktail: Optimally titrated rat anti-mouse monoclonal antibodies for use at 10 µl/10^6 cells: anti-CD11b (M1/70), anti-F4/80 (F4/80), anti-erythrocyte (Ter-119), anti-Gr-1 (RB6-8C5), anti-MHC class II (M5/114), and anti-CD4 (GK1.5).
2. $CD4^+$ T-cell-enrichment cocktail: Optimally titrated rat anti-mouse monoclonal antibodies for use at 10 µl/10^6 cells: anti-CD11b (M1/70), anti-F4/80 (F4/80), anti-erythrocyte (Ter-119), anti-Gr-1 (RB6-8C5), anti-MHC class II (M5/114), and anti-CD8 (53-6.7) (*see* **Notes 1** and **2**).

2.4. Intracellular Cytokine Staining of Lymphocytes

1. Brefeldin A: Dissolve 1 mg Brefeldin A (Epicentre Biotechnologies, #B901MG) in 200 µl 100% ethanol to create a stock solution of 5 mg/ml. Aliquots can be stored at −70°C. Working dilution is 1/1,000 to give a final concentration of 5 µg/ml. Brefeldin A should be included in all solutions until fixation is complete (*see* **Note 3**).
2. Flow buffer: Dulbecco's phosphate-buffered saline (PBS without Ca^{++} and Mg^{++}) containing 1% bovine serum albumin (BSA) and 0.02% (w/v) sodium azide (NaN_3)(2% FCS can be substituted for BSA).
3. Flow buffer/Brefeldin A: Add 5 µg/ml Brefeldin A to flow buffer.
4. PBS/Brefeldin A: Add 5 µg/ml Brefeldin A to PBS buffer.
5. Fixation buffer (2X): 4% Paraformaldehyde dissolved in PBS, pH 7.0. Store freshly made aliquots at −20°C and thaw immediately before use.
6. Permeabilization buffer: Dissolve 0.5% w/v saponin (Sigma S-7900) in flow buffer (*see* **Note 4**).
7. Stimulating peptide: Stock peptides (1 µM) should be titrated to determine the optimal concentration for stimulation.
8. Antibodies: Fluorescently conjugated rat anti-mouse interferon-γ (IFN-γ), tumour necrosis factor, interleukins (e.g. IL-2, 4, 10, and 17) (IFN-γ-PE, Pharmingen; isotype for IFN-γ intracellular staining, PE rat IgG_1, Pharmingen).

2.5. Tracking Lung Migrating DCs via In Vivo CFSE Labelling

1. CFSE (5- and 6-carboxyfluorescein diacetate succinimidyl ester) (Molecular Probes, Eugene, OR, USA): Dissolve CFSE powder in dimethyl sulphoxide (DMSO) to a final concentration of 25 mM [25 mg CFSE (molar mass of 557.47 g/mol) desiccated powder is equivalent to 44.8 µM]. Dissolve 25 mg CFSE in 1,790 µl DMSO to obtain

a 25 mM solution. This solution can be stored in 50–200 μl aliquots at −20°C and is stable following several rounds of freeze/thawing (28, 29).

2. Flow buffer: Dulbecco's phosphate-buffered saline (PBS without Ca^{++} and Mg^{++}) containing 1% bovine serum albumin (BSA) and 0.02% (w/v) sodium azide (NaN_3) (2% FCS can be substituted for BSA).

2.6. Miscellaneous Reagents

1. 96-Well cell culture cluster: MICROTEST™ U-bottom with lid, tissue culture treated (Becton Dickinson).
2. Dynal beads: Sheep anti-rat M-450 IgG-coated Dynal beads (Dynal, Oslo, Norway). Dynal beads are used at a ratio of 3–5 beads per cell. An alternative to Dynal beads is Qiagen BioMag goat anti-rat IgG magnetic separation particles (Qiagen). BioMag beads are used at a ratio of 8–10 beads per cell.
3. Dynal magnet: MPC-L magnet or DynaMag™-15.
4. Spiral rotator: Siramix 10 (Denley, Billingshurst, England).
5. Propidium iodide (PI): Prepare a stock solution at 200 μg/ml in PBS. A working solution is made by diluting the stock to 2 μg/ml in PBS. Aliquot and store at 4°C protected from light.
6. MHC tetramers: Reagents for MHC classes I and II tetramers are available from various facilities including the NIH Tetramer Facility (http://www.niaid.nih.gov/reposit/TETRAMER/genguide.html), regional tetramer core facilities, and commercial suppliers (e.g. Proimmune, Pro5® MHC class I pentamers).

3. Methods

3.1. Priming Influenza-Specific Responses

3.1.1. Intranasal Inoculation of Mice

1. Anaesthetize mice by inhalation anaesthetic such as methoxyflurane or using intraperitoneal injection of avertin (2,2,2-tribromoethanol) or ketamine/xylazine (*see* **Note 5**).
2. Gently hold mouse in palm of hand and administer 25–50 μl of diluted virus by placing droplets alternately over the nostrils of the mouse. Allow the fluid to be fully inhaled (*see* **Notes 6** and **7**).
3. Allow mice to recover in their cage on a heated pad (*see* **Note 8**).

3.1.2. Primary and Secondary Infection

This approach is utilized to inoculate naïve mice or those that have been "primed" to generate memory populations of lymphocytes at least 3 weeks previously.

Method

1. Dilute X-31 influenza virus in PBS/BSA such that the dose of administration is 25–50 μl (*see* **Note 5**). Place on ice.
2. Anaesthetize mice by inhalation anaesthetic such as methoxyflurane or using intraperitoneal injection of avertin (2,2,2-tribromoethanol) (*see* **Note 6**).
3. Gently place droplets over the nostrils of the mouse and allow the fluid to be fully inhaled (*see* **Note 8**).
4. Allow mice to recover in their cage on a heated pad (*see* **Note 9**).

3.1.3. Priming Memory Lymphocyte Responses

To study how DC behaviour, antigen presentation, and lymphocyte responses are regulated during a secondary infection, populations of antigen-experienced cells (memory) are generated by "priming" with an antigen or virus prior to secondary challenge.

Method

1. Dilute PR8 influenza virus in PBS/BSA such that each dose of administration is 500 μl (*see* **Note 5**). Place on ice (*see* **Note 10**).
2. Restrain the mouse and inoculate one dose of diluted virus to the mid-to-lower quadrant of the abdomen (*see* **Note 11**).
3. Allow mice to develop memory cells during the following weeks prior to challenge with X-31 (*see* **Notes 12** and **13**).

3.2. Collecting Tissues

3.2.1. Broncho-alveolar Lavage (BAL)

The BAL cells provide a very useful way of tracking recruitment of virus-specific T lymphocytes into the inflammatory site of the lung and a method by which to evaluate the efficiency of labelling of APCs (by tracking macrophage uptake of label) when introduced to the respiratory tract.

Method

1. Euthanize the mouse immediately prior to lavage.
2. Dampen the fur of the animal with 70% ethanol.
3. Using pins secure the animal on styrofoam panel and with scissors, make a small incision in the animal skin overlying the abdomen. Peal skin up towards the head of the animal to expose thoracic cage and neck.
4. Carefully dissect the tissue from the neck to expose trachea.
5. Make a small incision in the trachea between the tracheal cartilages to allow passage of 20-ga flexible cannula into trachea.
6. Gently introduce the catheter into the trachea (*see* **Note 14**).
7. Attach the catheter to a 1-ml syringe containing complete media or PBS.

8. Gently inject media or PBS into the lungs and withdraw the plunger of the syringe.
9. Remove syringe from needle, inject recovered lavage fluid into 15-ml collection tube kept on ice.
10. Repeat procedure such that three washes are performed per animal.
11. Pool experimental lavages from group and place on ice.
12. Wash the BAL once in complete medium and resuspend in 5–10 ml complete medium.
13. To remove macrophages incubate BAL on tissue culture treated 10 cc dishes at 37°C for 1 h (*see* **Note 15**).
14. Collect non-adherent and loosely adherent cells, wash, and prepare for flow cytometric staining and analysis.

3.2.2. Spleen and LNs

1. Remove spleen or appropriate LNs from mice and place into complete medium. Care should be taken to minimize contamination of the organs with fat or connective tissue (*see***Note 16**).

3.3. Purification of T Cells

Partial purification of T cells is generally required for a primary respiratory infection with viruses such as influenza and some herpes viruses. In these infections, the frequency of each population of antigen-specific $CD8^+$ T cell is often < 1–2% of $CD8^+$ T cells limiting the capacity to accurately detect these cells in an unenriched population of cells (26, 30, 31).

Method

1. Single-cell suspensions are made from LNs or spleen by gently passing tissues through a sieve.
2. Wash cells once with complete medium and centrifuge at 4°C at 400 × *g* for 5 min.
3. Resuspend in 10 μl $CD4^+$ or $CD8^+$ depletion cocktail/1×10^6 cells and incubate for 30 min on ice (*see* **Note 17**).
4. Wash cells once by adding 5 ml complete medium underlay with 1 ml EDTA–FCS. Centrifuge at 4°C at 400 × *g* for 5 min.
5. Carefully remove supernatant by aspiration.
6. Resuspend pelleted cells in 500 μl complete medium and transfer to prewashed sheep anti-rat IgG magnetic beads (Dynal) with the required beads calculated at 3 beads/cell (*see* **Note 18**).
7. Place cells/beads mix on a rotator angled at 45° for 20 min at 4°C (*see* **Note 19**).
8. Following incubation, dilute the cells and beads with 2–3 ml complete medium and place against a magnet (Dynal) to bind bead/antibody-coated cell complexes.

9. Recover the supernatant containing the enriched T-cell population and discard bound bead/antibody-coated cell complexes containing non-T cells.
10. The purity of T cells is determined by staining a small sample of enriched cells with antibodies towards CD4 or CD8. These cells are analyzed by flow cytometry to determine the percentage of cells positive for coreceptor markers, should routinely be between 60 and 80% pure after enrichment.

3.4. Analysis of CD8+ T-Cell Responses

The availability of peptide/MHC class I tetramer and the development of intracellular cytokine staining assays have greatly facilitated the investigation of the response characteristics of T cells during a viral infection (18, 32, 33).

3.4.1. Peptide/MHC Class I Tetramer Staining

1. Resuspend enriched $CD8^+$ T-cell populations in flow buffer at $1–2 \times 10^7$ cells/ml.
2. Add 100 μl of cells to each well of a 96-well U-bottomed plate and wash once, remove supernatant.
3. Resuspend cells in 50 μl MHC tetramer staining reagent and incubate for 40 min in the dark (*see* **Notes 20** and **21**).
4. Wash cells once and stain with 50 μl surface antibody such as fluorescently labelled anti-CD8 (*see* **Note 22**).
5. Wash cells twice with flow buffer and analyze on a flow cytometer. Select lymphocytes on the basis of forward and side light scatter, excluding dead cells with high PI (FL3) fluorescence.

3.4.2. Intracellular Cytokine Staining of Lymphocytes

Intracellular cytokine analysis is routinely used to both quantitate the number and the functional properties of antigen-specific T cells present in lymphocyte populations isolated from tissues. This approach is very robust and can be used when MHC/peptide tetramers are not available but depends on prior knowledge of the fine epitope specificity of the T-cell populations to be investigated. Where such information is unavailable or has not been determined, alternate approaches may be useful (34).

1. Plate $5 \times 10^5 - 2 \times 10^6$ cells in 200 μl complete medium containing 5 μg/ml Brefeldin A and 10 IU interleukin-2 ± 0.1–1 μM stimulating peptide into a 96-well U-bottomed plate (*see* **Notes 23–26**).
2. Incubate at 37°C, 5% CO_2 for 5 h (*see* **Note 27**).
3. Spin plate and resuspend cells in directly conjugated monoclonal antibodies to surface molecules + Fc block (anti-CD16/32, Pharmingen) diluted in 0.1% PBS/BSA + 5 μg/ml Brefeldin A.
4. Incubate at 4°C for 30 min.

5. Wash with 100 μl PBS + 5 μg/ml Brefeldin A (no BSA).
6. Resuspend cells in 100 μl PBS to ensure they are a single-cell suspension (*see* **Note 28**).
7. Add 100 μl of 4% paraformaldehyde and resuspend cells.
8. Incubate at 4°C for 15 min.
9. Wash once in PBS (*see* **Note 29**).
10. Resuspend in 100 μl/well permeabilization buffer for 10 min at room temperature (~20–22°C).
11. Spin plate and resuspend cells in cytokine fluorescently labelled monoclonal antibody diluted in permeabilization buffer.
12. Incubate at 4°C for 30 min.
13. Wash with flow buffer twice and analyze samples on a flow cytometer.

3.5. Understanding DC kinetics and Antigen Presentation in Influenza Infection

Initiation of the immune response to respiratory infections depends on the migration of lung DCs to the mediastinal LNs (1, 2, 24). Several studies have endeavoured to understand the mechanisms of migration of DCs from the lung to LNs using direct labelling of DCs with CFSE (20, 24). At this stage, ideal methods to label migrating cells in the lung (or skin) are not available; however, the currently used approaches have provided a first insight into the kinetics of DC migration and allowed a more complete understanding of the number and types of APCs presenting antigens that shape the T-cell immune response.

3.5.1. Tracking Lung Migrating DCs via In Vivo CFSE Labelling

Method

1. Dilute 25 mM CFSE stock by diluting to an 8 mM solution with PBS (16 μl of CFSE + 34 μl media/mouse; *see* **Notes 30** and **31**).
2. Anaesthetize mice by inhalation anaesthetic such as methoxyflurane or using intraperitoneal injection of avertin (2,2,2-tribromoethanol) or ketamine/xylazine (*see* **Note 5**).
3. Gently hold mouse in palm of hand and administer 50 μl of CFSE by placing droplets alternately over the nostrils of the mouse. Allow the fluid to be fully inhaled (*see* **Notes 6, 32**, and **33**).
4. Allow mice to recover in their cage on a heated pad (*see* **Note 7**).
5. Six hours after CFSE administration, additional DC stimulus may be applied (*see* **Note 34**).
6. At appropriate times after CFSE labelling (e.g. 6, 12, 24, and 48 h) remove the lung draining LNs.

7. Prepare DCs from LNs as described in Chapter 23, Section **3.1**.
8. Analyze cells by flow cytometry. Calculate the number and percentage of recruited DC (i.e. $CFSE^+CD11c^+$ cells) in the LNs.

3.5.2. Isolation and Direct Ex Vivo Analysis of Antigen Presentation by DCs of the Mediastinal LN Draining the Lung

Procedures for isolation and direct ex vivo analysis of antigen presentation of lung-derived antigens are described in detail in Chapter 23, Section **3.1**.

Tracking viral antigen presentation has been greatly facilitated by the development of a library of antigen-specific *lac-Z* hybridomas that allow quantitative assessment of the number of APCs in an LN-presenting antigen (24, 35), bone marrow chimeric mice expressing antigen-specific T-cell receptors (TCR) (retrogenic mice (36, 37)), and recombinant viruses (38) that encode T-cell epitopes specific for currently available TCR transgenic mice. Despite these advances, comparisons largely revolve around tracing peptides generated at different stages of the viral life cycle resulting in different numbers of peptides generated for loading of MHC molecules. In addition, studies are often limited to detailed analysis of either $CD4^+$ or $CD8^+$ T-cell responses. An alternate library of reagents available for BALB/c mice (*see* **Table 20.2**) in which both $CD4^+$ and $CD8^+$ specificities are known for a single protein – the hemagglutinin – allows concurrent evaluation of viral antigen presentation of T-cell responses. This has made it possible to start to reconcile the functions of different DC subsets presenting antigens in related systems (39) (*see* **Note 35**).

Table 20.2
Major T-cell epitopes in H-2^d (BALB/c) murine models of influenza infection

Epitope	MHC restriction	Peptide sequence	Viral protein	Transgenic Mouse line	References
$HA_{512–528}$	K^d	IYSTVASSL	Hemagglutinin	CL4	(40, 41)
$HA_{126–139}$	A^d	HNTNGVTAACSHE	Hemagglutinin	HNT	(42, 43)

4. Notes

1. All antibodies are derived from rat, which enables depletion with anti-rat IgG-coated magnetic beads.
2. We use negative selection for non-DC lineage cells using depletion cocktails and immunomagnetic beads to minimize modifications to DC function that could occur from

direct antibody binding to ligands on DCs. As a number of common surface markers are shared between DCs and other lymphoid cells, care must be taken to avoid loss of DC subtypes that may express markers more commonly on T cells, B cells, NK cells, and macrophages.

3. Brefeldin A and monensin are the cytokine secretion inhibitors most commonly used in intracellular cytokine analysis. When peptides are used for stimulation, either Brefeldin A or monensin may be added at the time of initial stimulation because peptide processing through the endosome is not required. Despite this, each of these inhibitors has distinct characteristics that should be kept in mind when choosing an inhibitor particularly when considering the surface markers and types of cytokines to be analyzed (44).
4. Fixation and permeabilization solutions can be substituted by commercially available Cytofix–Cytoperm reagent kit (Becton Dickinson, PharMingen).
5. Confirm the level of anaesthesia by using the blink and pedal reflex method. If the animal is to be anaesthetized for more than a few minutes, apply lubricant to the eyes to prevent them drying out.
6. Care should be taken to ensure PBS diluent for virus is from a source that is free of lipopolysaccharide (LPS), which may mediate activation of DCs and interferes with subsequent uptake, migration, and presentation of antigens (e.g. GIBCO DPBS).
7. Care should be taken in assessing the depth of anaesthetic provided to animals. If mice are very deeply anaesthetized, introduction of the inoculation fluid may cause respiratory distress. In general, mice handle 25–50 μl well and show a mild transient increase in rate and depth of breathing before returning to the normal pattern of respiration.
8. It is important to ensure that the mouth is gently closed and the nasal passages not obstructed during this procedure to facilitate inhalation/inoculation of the virus into the respiratory tract and lungs rather than the gastrointestinal tract. Very lightly anaesthetiszd mice will be prone to endeavouring to swallow the fluid rather than inhaling the inoculum leading to a lower dose of inoculation or variation between mice in the dose delivered by intranasal administration. For experimental purposes, it is critical that all mice receive the full inoculating dose to ensure infection and consistency within and between experiments.

9. Placing mice on a heat pad following inoculation facilitates their capacity to maintain body warmth and recover well.
10. In this approach to infection, large doses of PR8 virus are generally used ($\sim 10^7$) as the virus principally undergoes an abortive infection, with little or no virus reaching the lung epithelial cells that possess the molecular machinery for efficient replication.
11. A very small amount of inoculation fluid may appear on the fur of the mouse. Ensure all fluid/virus is removed by blotting with a tissue, as this virus could be licked/inhaled from the fur of the mouse resulting in a potent inoculation of the animal via the lung.
12. At least 3 weeks should be allowed for fully differentiated memory T-cell populations to develop. Genomic analysis suggests that progressive differentiation of T cells occurs during the first 3 weeks after challenge with an antigen, after which a stable memory phenotype is maintained (45).
13. PR8 virus is quite virulent even at low doses (<100 PFU) via the intranasal route and thus is generally given via intraperitoneal inoculation. This results in an abortive infection with little localization of virus to the lung. An alternative approach is to infect mice first with X-31 intranasally, then 3–6 weeks later inoculate mice intranasally with PR8 virus. A complete comparative analysis of these two approaches has not been done, but limited evaluation of the two systems does not show differences in recruitment of memory T cells although peak lung viral titres differ (Kallies and Belz, unpublished).
14. The incision should be made between the upper cartilage rings of the trachea. Take care not to cut entirely through the trachea –this will make it quite difficult to insert the cannula. The cannula should be inserted a short distance into the trachea towards the lung, stopping just before the bifurcation of the trachea (at this point pressure will be detected). Perforation of the trachea will result in the flushing fluid filling the pleural cavity.
15. Macrophages can interfere significantly with analysis of BAL samples particularly at the peak of the immune response. These cells can be substantially depleted by a brief period of adherence on tissue culture plates. However, activated T cells may also be somewhat adherent and careful washing of plates is necessary to ensure as many cytotoxic T cells are recovered from plates while the macrophages, which adhere to plates quite firmly, are depleted from the BAL.

16. Removal of residual fat on LN is critical. The fat is toxic to the cells, will not degrade during preparation for T-cell-enrichment digestion for DCs, and predisposes cells to clumping and dying. All visible fat should be removed before commencing the experiment.
17. Red blood cell lysis may be performed prior to depletion of non-T-cell fraction. The antibody depletion cocktail contains Ter119 that depletes red blood cells so this is not usually necessary. Cells should be counted to calculate the optimal amount of mAb cocktail required for depletion. This cell count should include erythrocytes, which will subsequently be depleted by Ter119 antibody.
18. Reduced volumes for incubation of the magnetic bead/cell slurry will optimize the bead/cell contacts allowing for reduced incubation times for binding. Care should be taken to avoid evaporation when small volumes are used.
19. Mixing the bead/cell slurry on an angled rotor (30° angle) is designed to ensure that the mixture remains at the bottom of the tube during the incubation period.
20. Each batch of fluorescently conjugated MHC class I tetramer should be titrated to determine the optimal dilution for staining. The recommended working dilution is 1:100 but often the optimal dilution for different tetramers and batches may vary. Naïve cells or an irrelevant tetramer recognizing the same MHC molecule but different T-cell epitopes should be used as negative controls for specific tetramer staining.
21. Staining is generally done at room temperature (20–22°C), however, staining can also be performed at 4°C or 37°C. Higher intensity tetramer stains may be obtained using temperatures above 4°C but some surface markers are sensitive to higher temperatures.
22. In some cases, the binding of antigen-specific T lymphocytes is influenced by the CD8 coreceptor molecule. This is particularly applicable to H-2 K^b tetramers. In some cases, the CD8-mediated non-specific tetramer binding has been observed where the tetramer may bind to all $CD8^+$ T cells. This can occur when the 53-6.7 antibody is used. Conversely, other CD8 monoclonal antibodies (such as CT-CD8α from Caltag) can significantly block tetramer binding. To eliminate these confounding possibilities from experiments, the optimal MHC tetramer/CD8 dilution should be determined by performing a cross-titration experiment with the tetramer and the desired CD8 antibody especially when H-2 K^b tetramers are used.

23. Additional controls to ensure specific cytokine due to stimulation are being measured. (i) To gain a good measure of the quality of the cells enriched from virally infected mice, enriched cells to which peptide is not added provide an optimum negative control. Cells damaged during the isolation process are more prone to die during culture or alternately produce levels of cytokine above background (usually ~0.2%). The % cytokine$^+$ cells incubated without peptide was subtracted from the % cytokine$^+$ cells incubated with peptide. (ii) Add a molar excess of recombinant cytokine to anti-cytokine antibody prior to staining to abrogate specific staining. (iii) Incubate control cells with unlabelled anti-cytokine antibody followed by incubation of cells with a directly conjugated anti-cytokine antibody.
24. The optimal concentration of each stimulating peptide should be determined by examining optimal cytokine production over a range of concentrations.
25. If IL-2 is added to cultures, and it is anticipated that staining will be performed for IL-2, then human IL-2 should be used such that the endogenously produced IL-2 can be readily discriminated during staining.
26. APCs infected with a virus or pathogen can be used in place of peptide alone as the stimulus for T cells. In this case, the response of the T cells will reflect an overall response to multiple T-cell specificities encoded by the virus.
27. Shorter times for stimulation can be used; however, maximal cytokine production occurs between 5 and 6 h of stimulus (46).
28. Carefully resuspending cells in PBS prior to the addition of 2X concentration of paraformaldehyde will minimize the formation of cell aggregates.
29. After fixing, cells may be stored for up to 2 days at 4°C in PBS in the dark or processed immediately. Although storage for several days is possible, optimal surface staining will be detected if processing is continuous. Fluorochromes lose some intensity during storage.
30. Dilution of 25 mM CFSE stock solution to 8 mM solution will often become turbid with some white precipitate. Despite this the solution will label APCs and DCs.
31. Labelling approaches should be evaluated for their "cleanliness" prior to evaluation of the physiological implications of results. Many reagents contain trace amounts of the Toll-like ligand LPS which may mediate activation of DCs and interferes with subsequent uptake, migration, and presentation of antigens.

32. Care must be taken to ensure that CFSE solution is only applied to nostrils. Inadvertent application of CFSE to skin or other areas of the mouse will result in labelling DCs of that area and the migration of labelled cells to the regional LNs.
33. Control treatment of mice with DMSO carrier is critical to assess the level of inflammation induced by the carrier itself. Inflammation is induced and can be monitored by determining the expansion of LNs with DMSO carrier alone in the absence of CFSE. It should be noted that effects such as these can potentially modify the behaviour and function of DCs. Care must be taken to assess that these parameters of DC behaviour have not been affected when using such labelling techniques.
34. CFSE labelling can be applied either before or after infection or treatment with an inflammatory stimulus.
35. Differences in specific viral tropisms, dose, and levels of inflammation induced during infection, together with altered representation of dendritic cell populations in different mouse strains, can impact on which dendritic cell populations present pathogen and model antigens (Belz and Mount, unpublished).

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Chapter 21

DCs in Mouse Models of Intracellular Bacterial Infection

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Abstract

Direct infection of antigen-presenting cells (APCs) with living intracellular bacteria may influence the early innate immune response as well as the following T-cell response. Revealing the identity of primarily targeted cells during infection is therefore an important task, which requires sensitive methods for the intracellular detection of bacteria before their extensive replication.

Determination of colony-forming units (cfu) combines – in contrast to microscopy-based methods – a high sensitivity with the specific recognition of viable multiplying organisms. We recently explored an FACS-based ex vivo cell isolation protocol followed by cfu quantification of lysate-derived bacteria in order to make even very few organisms visible. With this approach, the evaluation of cell-subsets during the earliest phase of bacterial infection became feasible. In general, the assay should be transferable to the ex vivo detection of other intracellular (viral or parasitic) pathogens.

Key words: Splenic DCs, *Listeria monocytogenes*, multi-color cell sorting, intracellular bacteria, ex vivo isolation.

1. Introduction

In the last decade, dendritic cells (DCs) have been described as the "initiators" of $CD8^+$ T-cell responses against intracellular pathogens (1). Recent work has provided evidence that intracellular bacteria (2) as well as other pathogens (viruses, parasites, and even extracellular bacteria) can be detected in DCs (3, 4). The potential consequences of the presence of replicating pathogens in those central APCs for the dynamics of infection and the initiation of specific immune responses are not clear. It has been speculated that pathogens could use the migratory capacity of DCs in order to spread systemically (5). Active uptake of pathogens by DCs, however, could be also beneficial to the host; the

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intracellular presence of microbes could lead to strong activation of DCs and induction of direct $CD8^+$ T-cell priming (6).

Infection with *Listeria monocytogenes* (*L.m.*), a facultative intracellular bacterium, is a broadly used experimental mouse model (7). After intravenous application, a systemic infection is rapidly established in liver and spleen. Detection of intracellular *L.m.* during the early infection phase requires highly sensitive methods, because only a minor part of the injected bacteria reaches the spleen or liver and manages to survive in intracellular niches (8). As conventional microscopy is limited by its moderate sensitivity and additionally lacks live/dead discrimination, we extended a recently described flow cytometry-based ex vivo isolation approach (9) to the early phase of *L.m.* infection (2). This method combines the advantages of highly selective multi-color cell sorting with the well-known sensitivity of traditional bacteria cultures and provides novel insights into the early distribution patterns of living bacteria.

2. Materials

2.1. L.m. Strains and Mice

1. *L. monocytogenes* stock solution (store at −80°C): strain 10403 (10) (alternatives *see* **Note 1**).
2. 6–8-week-old female C57BL/6 (B6) mice (obtained from Harlan-Winkelmann, Borchen, Germany).
3. Brain heart infusion (BHI) medium: 37 g BHI CM225 (Oxoid, Basingstoke, UK) dissolved in 1,000 ml ddH_2O; store at 4°C.
4. Bio-photometer.

2.2. Collagenase Digestion

1. Cell culture medium: supplement 500 ml RPMI (with 1.1915 HEPES, 0.4 g L-glutamine, 0.1 ml 2-mercaptoethanol and 50 ml heat-inactivated FCS); store at 4°C.
2. Gentamicin: store at room temperature.
3. Collagenase type IV (Sigma Aldrich, Taufkirchen, Germany): store at −20°C and always dilute it freshly in cell culture medium before use.
4. DNase type 1 (Sigma Aldrich, Taufkirchen, Germany): dissolve 1 mg in 10 ml cell culture medium and immediately freeze in single-use aliquots (200 µl) at −20°C.
5. EDTA buffer: 0.1 M EDTA in PBS, pH 7.2. Store at 4°C.
6. 6-Multi-well plates.
7. Cell strainers (100 µm).

2.3. Staining

1. Buffer for erythrocyte lysis: 0.17 M NH_4Cl in 0.3 M Tris (pH 7.5); store at 4°C.
2. Washing buffer: PBS supplemented with 0.5% bovine serum albumin (BSA), pH 7.45; store at 4°C.
3. F_C block: rat anti-mouse CD16/32 (2.4 G2).
4. Antibodies:
   (a) APC-conjugated anti-mouse CD11c (HL3).
   (b) FITC-conjugated anti-mouse CD49b/Pan-NK (DX5).
   (c) PE-conjugated anti-mouse CD8 alpha (5H10).
   (d) PE-Cy7-conjugated anti-mouse CD11b (M1/70).

   Store all antibodies at 4°C.
5. Propidium iodide (PI): Dissolve 5 mg in 2.5 ml PBS and freeze aliquots at −20°C. On the day of the experiment, thaw an aliquot, dilute 16 μl of the stock solution in 4 ml washing buffer and keep it dark on ice until use (NB: PI is a potential mutagen and should be handled with care: always wear nitrile gloves during dilution).

2.4. Gating and Sorting

1. MoFlo™ XDP (Beckman Coulter, Inc., Fort Collins, CO, USA).
2. Software: Summit v5.1 (Beckman Coulter, Inc., Fort Collins, CO, USA).
3. FACS Aria (BD Biosciences, San Jose, CA, USA).
4. Software: FACS DIVA v6.1 (BD Biosciences, San Jose, CA, USA).

2.5. cfu Determination

1. BHI agar plates: 26 g BHI agar is dissolved in 500 ml ddH_2O at 85°C for 30 min. Solution is then autoclaved (121°C, 20 min), cooled down, and plates are immediately prepared, dried, and stored at 4°C.
2. 0.1% Triton X solution: 100 μl Triton X-100 is dissolved in 100 ml ddH_2O for 1 h with a magnetic stirrer, sterile-filtrated (0.45 μm), and subsequently stored at 4°C.
3. PBS: 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.45).

3. Methods

The following method was primarily used to isolate intracellular *L.m.* from murine spleens shortly after infection. As *L.m.* show rapid proliferation and spreading from the initially infected target

cells into other cellular compartments, the chosen time-point of the ex vivo isolation is crucial. Deeper insights into the kinetics of systemic infection can be gained by comparing the distribution of *L.m.* at consecutive time-points (e.g., 1, 3, 15, and 72 h). In principle, the method can be easily adapted to other intracellular pathogens (e.g., *Salmonella* ssp.; *see* (2)), but potentially different kinetics should then be taken into account.

Using the MoFlo^{TM} XDP, up to four different cell populations can be sorted in parallel. In the following example, we focused on conventional dendritic cells (CD8α^+ and CD8α^- DCs), Pan NK$^+$ NK-cells, and CD11b$^+$CD11c$^-$ myeloid cells (macrophages, granulocytes). Using alternative antibody combinations, myeloid cells could be more specifically addressed (e.g., CD11b^{hi} Ly6G^{hi} granulocytes) and additional splenic DC cell subsets (e.g., plasmacytoid DCs) could be analyzed.

Caution: *L.m.* is a pathogenic organism that can cause severe disease in humans. Pregnant women as well as immunocompromised people are at particular risk and should not work with *L.m.* All work with viable *L.m.* has to be performed under biosafety level 2 conditions. Investigators working with *L.m.* should contact their institutional biological safety department for more specific guidance and approval.

3.1. Systemic L.m. Infection of Mice

1. *L.m.* stock solution (~1.1 × 10^8 cfu/ml) is thawed and 20 µl dissolved in 10 ml 4°C cold BHI medium and subsequently vortexed. The bacteria are grown overnight in a shaking incubator. As it usually takes ~5 h until the bacteria reach the early log growth phase, the shaking program (90 rpm at 37°C) should be started with a time delay; during the first hours, uncontrolled growth can be minimized by reducing the temperature below 10°C (if an integrated cooling device is available; alternatives *see* **Note 2**).
2. The following morning, bacterial growth can be quantified by measuring the light absorbance of the BHI broth at 600 nm using a standard photometer. Early log phase cultures are typically obtained at A_{600} of 0.05–0.1 (0.65–1.1 × 10^8 bacteria/ml) (*see* **Note 3**).
   The concentration is then adjusted to 2.5 × 10^6 bacteria/ml by diluting the BHI broth with ice-cold PBS.
3. Four B6 mice (*see* **Note 4**) are exposed in a BSL-2 working bench to an infrared heating lamp for 5 min in order to dilate their tail veins (distance approximately 30 cm).
4. The warmed mice are then placed into a mouse restraining device and 200 µl (5 × 10^5 bacteria) is injected into the lateral tail vein using a 1-ml syringe with a 27 gauge needle.
5. Mice are placed into a safety cage for 60 min in a well-tempered and quiet place.

6. The exact bacterial dose is documented by plating a diluted aliquot (5,000 bacteria in 10 μl are expected) in triplets on fresh but dry BHI plates. The culture plates are then incubated for 24 h in a 37°C incubator.

3.2. Collagenase Digestion

1. In order to isolate maximal numbers of infected APCs, spleens have to be pre-treated with freshly prepared collagenase/DNase mix: lyophilized collagenase (0.7 mg/ml) is dissolved in antibiotics-free RPMI medium (*see* **Note 5**), a DNase aliquot (final concentration 1 μg/ml) is added after thawing and the final mix is supplemented with gentamicin (final concentration 5 μg/ml, *see* **Note 6**). The mix is stored on ice until use.
2. One hour after infection, mice are sacrificed and spleens are taken out. The collagenase mix is added to a 6-well plate (4 ml/well) and cell strainers (100 μm) are placed in the prepared wells. The intact organ is laid into the cell strainer and an additional amount of 1 ml collagenase mix is carefully injected into the organ using a 1-ml syringe with a 27-ga needle. The bloated spleens are then incubated for 30 min at 37°C and 5% CO_2 in a BSL-2 incubator.
3. After this first incubation step, the partially digested spleens are completely meshed through the cell strainer using a rubber plunger of a 5-ml syringe. The cell suspension is then incubated for another 30 min. During the last 5 min, 500 μl EDTA buffer is added and mixed with the splenocytes.

3.3. Staining

1. After incubation, the splenocyte suspensions are harvested and the wells are repeatedly washed with fresh antibiotic-free cell medium (5 ml/well) in order to avoid any cell loss. The cell suspension is centrifuged at 300 × *g* and the pellet is dissolved in 5 ml erythrocyte lysis buffer at room temperature; 7 min later, the reaction is stopped by adding 5 ml ice-cold RPMI medium. After centrifugation (300 × *g*, 7 min), cells are resuspended in 10 ml RPMI medium, filtered once again through a cell strainer, and quantified using a counting chamber.
2. In order to block F_C-specific receptors, cells are resuspended in washing buffer containing anti-CD16/CD32 (0.5 μg/ml) after another centrifugation step. The sample is adjusted to a final concentration of 10^8 cells/ml and is stored on ice for the following 15 min.
3. For proper compensation of multi-color analyses, single-color control stainings are indispensable. Therefore, a small part of the cells is incubated in a round-bottomed 96-well plate (2–5 × 10^6 cells/well; for pipetting scheme *see*

Table 21.1
Staining scheme Single-color controls are stained in a 96-well round-bottomed plate. Cells are distributed into five wells and 50 μl staining buffer containing the indicated concentration of antibodies is added. Cell samples are stained with the indicated complete mix of antibodies (for concentrations see singlecolor stainings)

	1	2	3	4	5
Single-color stains	Unstained	PanNK-FITC 5 μg/ml	CD8α-PE 1 μg/ml	CD11b-PE-Cy7 2 μg/ml	CD11c-APC 2 μg/ml
Cell sample	Pan NK-FITC CD8α-PE CD11b-PE-Cy7 CD11c-APC				

Table 21.1). Staining buffer is added (150–180 μl), cells are centrifuged (300 × *g*, 2 min), and the pellets are resolved in 50 μl containing the indicated amount of the respective fluorescence-conjugated antibody. Cells are kept dark on ice for 20 min. The remaining cells are filled up with staining buffer, spun down (300 × *g*, 7 min), and subsequently incubated with a staining mix (*see* **Note 7**) under the same conditions as the single-color stains (*see also* **Table 21.1**). Optimized staining of the cell sample can be achieved by continuous rolling on a roller rack at 4°C (wrapping the tube with aluminum foil prevents light exposure).

4. Washing buffer is added to the cell sample (end volume 10 ml) and the single-color controls (end volume 200 μl), centrifuged (300 × *g*, 7 min and 2 min, respectively), and the probes are washed again twice under the same conditions. Finally, the pellet is resuspended in washing buffer (final concentration 2×10^8/ml). For live/dead discrimination, the probes are diluted 1:1 with freshly made PI buffer and kept dark on ice for 2 min (*see* **Note 8**). Cells are then washed for the last time to remove unbound PI and diluted in washing buffer (final concentration: 2×10^8/ml).

3.4. Gating and Sorting

1. Shortly before cell sorting, the cell suspension is filtrated over a membrane (30 μm) to remove aggregates. A final cell concentration of 1×10^8 cells per ml is optimal for high-speed cell sorting.
2. In the presented example, splenocytes were sorted by a MoFlo™ XDP (Beckman Coulter, Inc., CO, USA). This instrument allows cell sorting with high speed, highest

purity, and continuous cooling of the sample (*see* **Note 9**). The instrument used for this experiment was equipped with a 488-nm laser (Coherent, setup with 100 mW out of 200 mW) and a 642-nm laser (25 mW).

The cell sorter was setup with the following conditions:

Frequency (Hz)	98,974
Number of streams	4-Way sort
Deflection (%)	74, 49, 51, 71
Sort mode	Purity 1 drop
Nozzle (μm)	70
Pressure (psi)	60

3. The performance of the instrument can be guaranteed by a daily QC control with SpetralAlign- and 8-peak-beads. Furthermore, the PMT gain is determined during the QC routine. The CV and the minimum PMT voltage for the used parameters should be

Channel	Fluorochrome	CV	$PMT\text{-}Voltage_{min}$(V)
FL1	FITC	<1.5	>520
FL2	PE	<1.5	>530
FL3	PI	<2.5	>550
FL5	PECy7	<3.0	>610
FL6	APC	<3.0	>620

4. To evaluate the experimental setup and to allow optimal compensation from the single-color controls, at least 25,000 cell counts per single-color sample are acquired and stored. The compensation values are determined with the autocompensation tool of Summit v5.1.
5. To setup the sort decisions we used fluorescence minus one (FMO) controls. The experimental sort setup is shown in **Fig. 21.1A**.
6. The sort speed should not exceed 40,000 events per second. After sorting of 10^7 cells, the sort sample is usually shortly taken out of the sample station and vortexed twice for 2 s to avoid cell aggregation.
7. Sorted cells are collected in 5-ml Falcon tubes that are precharged with 1 ml FCS (the inner surface of the tubes should be completely coated with FCS by rolling the closed tubes).
8. Upon completion of the sort, the collected cells are stored on ice in the dark and checked for purity on a different cell

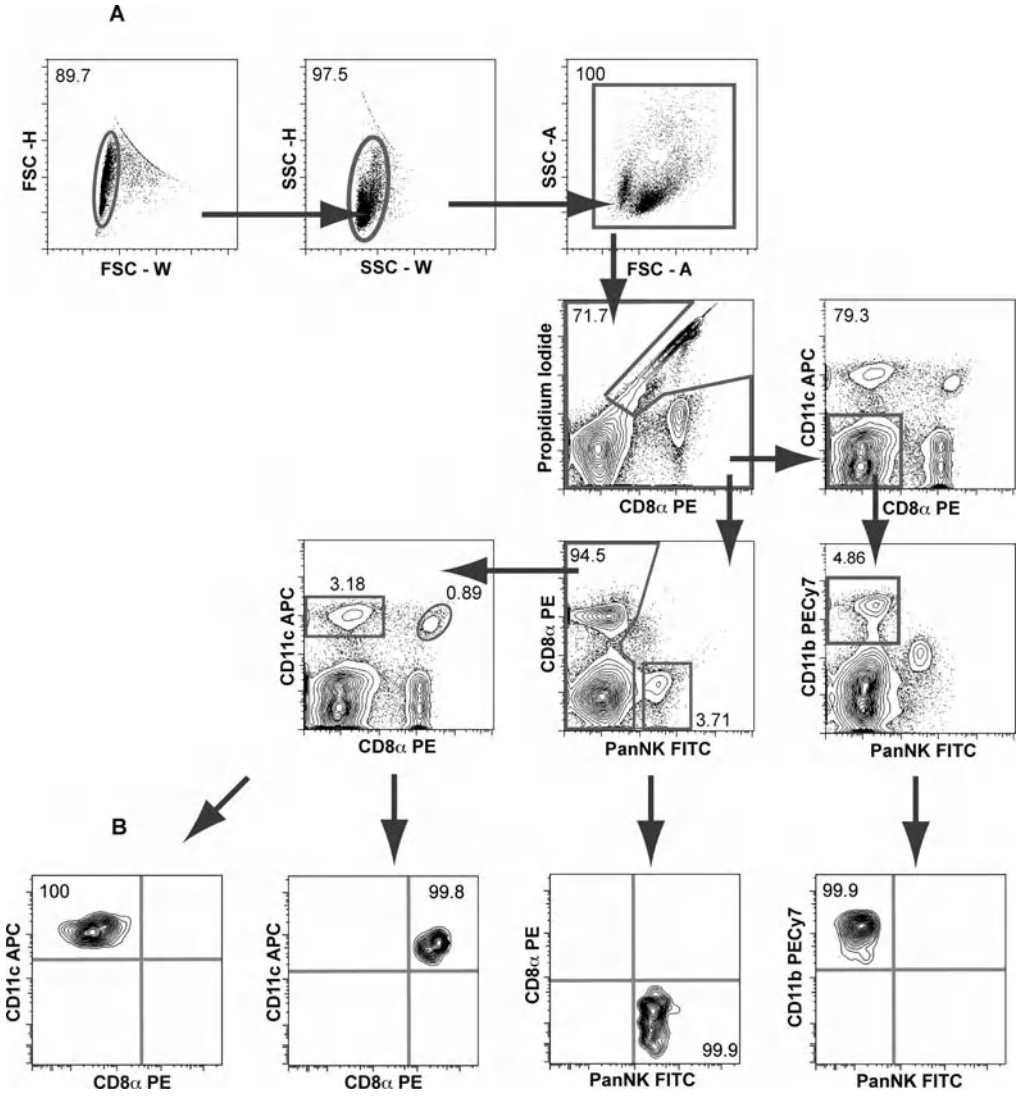

Fig. 21.1. (**A**) Gating strategy for five-color sorting. *First row:* sorting gate on scattered light conditions using pulse width to define singlets and exclude doublets and aggregates. *Second row:* left panel shows live cell definition (PI negative); *right panel* defines CD11c$^-$ CD8α^- pre-gate for macrophages and granulocytes. *Third row: middle panel* allows segregation of PanNK$^+$ NK cells; *left panel* shows the discrimination of CD11c$^+$ PanNK$^-$ into CD8α^+ and CD8α^- DCs; *right panel* shows the sort decision for the CD11c$^-$ pre-gated CD11b$^+$ PanNK$^-$ combined population of macrophages and granulocytes. (**B**) The purity controls for all 4-way sorted populations are shown; left panel: CD8α^-DCs; *middle left panel*: CD8α^+ DCs; middle right panel: NK cells; right panel: macrophages and granulocytes (*see* **Note 11**).

sorter (here: FACS Aria). For each sorted population, at least 1,000 cells are acquired (*see* **Note 10**). The results are shown in **Fig. 21.1B**(*see* **Note 11**).

3.5. cfu Determination

The sorted cell subpopulations are recounted using a Neubauer cell chamber. Cell suspensions are then centrifuged (300 × *g*, 5 min) and resuspended in 300 µl PBS; 300 µl Triton X

solution (*see* **Note 12**) is added and cells are mechanically disrupted by shaking cells for 10 s on a vortexer. Cell lysates are then further diluted 1:10 in Triton X followed by extensive vortexing. Dried BHI agar plates are then plated in triplets (1:6: 100 μl undiluted cell lysate, 1:60: 100 μl diluted solution, and 1:600: 10 μl diluted solution, alternative *see* **Note 13**). Plates are then incubated for 12–24 h in order to quantify colony-forming units by counting macrocolonies (*see* **Note 14**). The enrichment of bacteria in the subpopulations can be quantified by the division of the absolute number of isolated bacteria per subpopulation through the number of sorted subset cells (*see* **Fig. 21.2**).

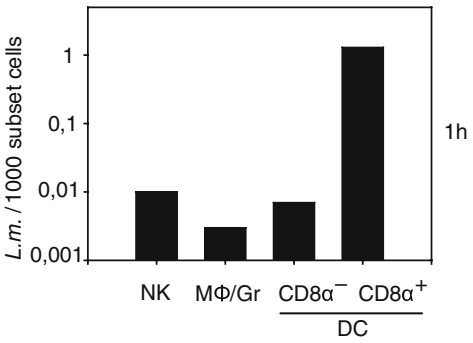

Fig. 21.2. *L.m.* is quantified in ex vivo isolated splenocytes. $CD8\alpha^+$ DCs and $CD8\alpha^-$ DCs were sorted from $DX5^-CD11c^{hi}$ splenocytes. Granulocytes and macrophages/monocytes (MΦ/Gr) were pre-gated on $CD11c^{lo}$ and $CD8^{lo}$ cells and sorted together as $CD11b^+$ cells. NK cells are defined as Pan NK^+ (DX5) cells. The number of bacteria per 1,000 subset cells is shown.

4. Notes

1. Other available wild-type (e.g., EGD) as well as recombinant *L.m.* strains (e.g., ovalbumin expressing *L.m.*) show a similar distribution pattern and can be used alternatively.
2. Isolation of *L.m.*-infected splenocytes is a time-consuming method, but it usually can be well integrated in a working day. The infection of mice early in the morning requires growing *L.m.* overnight. In the absence of a cooling device, *L.m.* can be grown at 20°C over night. As the expression of some virulence factors is temperature-dependent (11), *L.m.* should be finally incubated for at least 1 h at 37°C to reach the log phase.
3. In order to determine the cfu equivalent of the measured light absorbance, standard curves have to be established. This can be done by determining at hourly intervals for

6–8 h the light absorbance (A_{600}) and correlate it with the respective bacterial colony counts.

4. Direct comparisons of mouse strains (e.g., gene-deficient vs. littermate controls) should be done the same day under identical isolation and staining conditions. Using two mice per group allows the integration of two consecutive cell sortings into the described working schedule. The overall reduction of the sorted cell numbers leads to a 50% lower sensitivity.
5. Collagenase has to be prepared freshly at the day of use, as dissolved frozen aliquots show reduced activity! Every new batch should be carefully titrated in order to achieve an optimal digestion. Too high concentrations may lead to tissue clumping and cell loss.
6. At the used concentration, gentamicin inhibits growth of extracellular bacteria but does not pass the cellular membrane. This leaves intracellular bacteria unaffected. Higher concentrations can kill even intracellular bacteria.
7. Function of the antibodies should be tested in the planned combination in advance and the required concentrations should be carefully titrated. Take into account that substantial amounts of antibodies are needed (e.g., for 4×10^8 cells, 40 μl antibody stock solution (dilution 1:100)). Therefore, check their availability in time! Keep antibodies always cool and dark. Use filter tips to avoid any contamination of the antibody vials.
8. The last wash step, shortly before cell sorting, is necessary to remove unbound PI, which could cause a general shift of all cells (including the living ones) during sorting.
9. Regarding the temperature influence on the viability of cells, all cells (sample and sorted cells) should always be kept at 4°C.
10. For purity controls, an aliquot of the cells of interest should be sorted into pure PBS and not FCS-coated tubes in order to avoid background noise from FCS-derived protein aggregates.
11. High purity can be achieved by strict gating. However, this strategy can lead to a substantial cell loss of the target population and has to be taken into account for the accurate determination of recovery rates (*L.m.* in sorted cell subsets/*L.m.* in the pre-sort sample).
12. Triton X solution is light-sensitive and should be used within 48 h upon preparation.
13. For maximal sensitivity, cell subsets can be resuspended in 200 μl PBS, and subsequently mixed with another 200 μl

Triton X. In order to quantify accurately the amount of *L.m.*, two plates should be plated with 100 μl of the highest concentration. The 1:10 and 1:100 dilutions can be plated in conventional triplets again.

14. *L.m.* colonies are clearly identified as smooth opaque colonies. If potential contaminations have to be excluded, microscopical analysis (*L.m.* presents as Gram-positive rods) can easily be performed.

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Chapter 22

Studying the Function of Dendritic Cells in Mouse Models of Asthma

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Abstract

Dendritic cells (DCs) are known to play a crucial role in the induction of allergic asthma in mouse models. Their antigen presentation capacity, linked to their capacity to prime naïve T cells and polarize them towards a Th1, Th2, Th17 or Treg profile, allows them to efficiently initiate an immune response to allergens. Airway dendritic cells also play a crucial role in the local restimulation of circulating effector T cells upon allergen challenge. Given their important implication in pathogenesis of asthma in mice models, the study of environmental and pharmacologic effects on DCs function is now a blooming field. There is therefore a critical need for a stable, yet flexible animal model to investigate the effects of various environmental factors (endotoxins, pollutants, etc.) or pharmacologic molecules on DCs and subsequently on their role in asthma pathogenesis. This chapter presents an approach using a reliable animal model of asthma that has the advantage to allow interventions on DCs before their use to induce allergic asthma. We also cover some of the endpoint techniques used to assess asthma and the immune reactions involved in its pathogenesis.

Key words: Dendritic cells, mouse model of asthma, ovalbumin model, bone marrow-derived dendritic cells, adoptive transfer, ovalbumin-pulsed dendritic cells.

1. Introduction

Dendritic cells (DCs) have important antigen uptake and processing capacities, along with a capacity to express co-stimulatory molecules. These features confer them the capacity to efficiently prime naïve T cells, a feature not achieved by other antigen-presenting cells (APCs) (1–3). DCs are situated in the epithelium of the airways, which puts them in close proximity to the

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lumen. Extended dendrites actually reach out into the mucus layer covering the airway epithelium in order to sample and capture environmental antigens (4, 5). After this antigen uptake, DCs express higher levels of co-stimulatory molecules and migrate to the draining lymph nodes of the lungs, where they reach the T-cell zone in order to interact with naïve T cells (6, 7).

Since these original observations, the role of DCs in the induction of the allergic response has been well documented (4, 8). But their role in asthma pathogenesis is not restricted to the sensitization phase and our team previously reported that DCs are needed for the response to the allergen challenge. Indeed, depletion of DCs before allergen challenge prevented the apparition of classical allergic asthma features (9–11). The picture is not so simple, as it is also known that all subsets of DCs do not harbour the same function in immunity. In this regard, we previously reported that the plasmacytoid DCs are necessary for induction of tolerance towards allergens (12, 13).

It is now clear that developmental lineage of DCs is not a predetermining factor in control of immune function. A crucial factor is the microenvironment where the DCs find itself during antigen encounter. For example, the load of endotoxin that is co-administered with the antigen affects the outcome of the DC response in the airways differentially compared with the gut (14). It was observed that a high level of endotoxins was able to induce a Th1 response, while a low level was inducing a Th2 response towards the allergen. This effect was traced to be DC-mediated (15).

Given the importance of this cell type in the regulation of allergic asthma development, the need for reliable, yet flexible animal models and protocols is obvious. To prove that a particular compound has direct effects on DCs, one can also purify lung DCs and co-culture these in the presence of lung-derived Th2 cells and study T cell–DC interaction ex vivo thus studying the effect of the compound. Therefore, it is also handy to study the effect of a certain compound on primary sensitization induced via the inhaled route, such as induced by Der p1 (16), OVA + LPS (14), OVA in the absence of tolerogenic DCs (13) or OVA in the context of GM-CSF overexpression (17). To finely dissect the mechanisms involved in Th2 sensitization one can combine these models with injection of a CFSE-labelled cohort of naïve Ag-specific T cells to study the effects on T-cell division and polarization (18, 19). To definitively prove that the compound works on DCs, it is important then to turn to models in which Th2 sensitization is exclusively induced by mDCs, preferably with a step where one can expose the compound to DCs in vitro. This is made possible by the very efficient culture protocols that are now available to generate large amounts of mDCs and pDCs from the bone marrow (20, 21). These cultured DCs can be exposed to

OVA or another allergen and (economically small amounts of) compound in vitro and subsequently injected intratracheally or intranasally into naïve mice, followed by a period of aerosol exposure. Injection of OVA-pulsed DCs i.t. leads to Th2 sensitization and subsequently all features of asthma or allergic rhinitis develop (22–24). If a compound can inhibit this protocol it is likely to affect DC functions in the lungs as well. This model can even be adapted to the humanized SCID model of asthma: This model can be set up in such a way that monocyte-derived DCs from HDM-allergic donors are injected intratracheally into mice that received a cohort of T and B cells from the same allergic donor. Injection of allergen-pulsed DCs leads to all features of asthma. As mo-DCs are cultured for 7 days from monocytes, these cells can be exposed to compounds in vitro, prior to adoptive transfer in vivo(25).

Here, we describe a set of protocols that allow the investigation of DC's capacity to induce allergic asthma in mice. This model employs DCs derived from the bone marrow (BMDCs) as the sole source of antigen, with which they are pulsed before intratracheal (i.t.) transfer to a recipient mice. This model is therefore reliable, as one can be specific of lung sensitization compounds (one allergen only), but more importantly, whether it is mediated by the DCs, in a manner far more physiological than with a systemic immunization. This is also a flexible approach as many allergens can be used (although this protocol will describe the use of ovalbumin). It is also flexible as many treatments can be applied to DCs specifically before their adoptive transfer to the recipient. The presented set of protocols will therefore cover the whole process, from BMDC generation to their Ag-pulsing, maturation, i.t. administration and endpoint assessment of allergic asthma development.

2. Materials

2.1. Mice

1. C57BL/6 or BALB/c mice can be obtained from certified providers such as Harlan (www.harlan.com) or Jackson Labs (www.jax.org).
2. Mice should be used in experiments at 6–8 weeks of age (*see* **Note 1** for the use of gene-deficient mice).

2.2. Bone Marrow Dendritic Cell Generation and Culture

1. PBS: phosphate-buffered saline (Invitrogen-Gibco) (20).
2. HBSS: Hanks' balanced salt solution (Invitrogen-Gibco).
3. TCM: tissue culture medium. Constitution: RPMI-1640 + Glutamax-I, 5% foetal calf serum (FCS), 0.05 mg/ml gentamicin, 5×10^{-5} M β-mercaptoethanol.

4. RBC lysis buffer: 0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA. This solution must be filter-sterilized before use.
5. Nylon cell strainer (100 μM) (BD Biosciences, Falcon).
6. 50-ml Tube (BD Biosciences, Falcon).
7. Desired dissection tools (forceps, scissors...).
8. 70% ethanol (EtOH).
9. Ice bucket to keep HBSS cold.
10. Cell culture grade 100-mm Petri dishes (BD Biosciences, Falcon).
11. Recombinant GM-CSF (Bioceros).
12. Trypan Blue (Invitrogen-Gibco).
13. Ovalbumin (Worthington Biochemical).

2.3. Immunophenotyping of Mouse Bone Marrow DCs

1. RPMI-1640, Ice-cold (Gibco-Invitrogen).
2. 50-ml Tube (Falcon, BD Biosciences).
3. FACS buffer: 0.5% BSA, 0.05% NaN_3, PBS, filter-sterilized.
4. 96-Wells Flexiplate (Falcon, BD Biosciences).
5. FACS tubes (Falcon, BD Biosciences).
6. 2.4G2 Blocking antibody (Bioceros).
7. Antibodies:
   a. α-I-Ad/I-Ed FITC (MHC-II) FITC Ab (eBiosciences).
   b. α-CD11c APC (eBiosciences).
   c. α-CD80 PE (BD Biosciences).
   d. α-CD86 PE (BD Biosciences).
   e. α-CD40 PE (eBiosciences).
   f. α-CD24 (HSA) PE (BD Biosciences).
   g. α-CD54 (ICAM-1) PE (BD Biosciences).
   h. α-CD11b (Mac-1) PE (BD Biosciences).

2.4. Sensitization Protocol

1. Ovalbumin grade III (Sigma–Aldrich).
2. PBS (Invitrogen-Gibco).
3. Anaesthetic: isofurane or injectable anaesthetic, according to local animal care regulations.
4. Micropipette, sterile micropipette tips.
5. Antigen-pulsed DCs prepared as per the 'Bone Marrow Dendritic Cell generation and culture' protocol.
6. Apparatus to suspend the mice by the upper front teeth.

2.5. Collecting and Processing of Murine BAL

1. PBS–EDTA: PBS, 0.1 mM EDTA, filter-sterilized.
2. 23-ga Catheter.
3. Desired dissection tools (forceps, scissors…).
4. Suture thread (Johnson & Johnson Medical).
5. 15-ml Tube (BD Biosciences, Falcon).
6. RBC lysis buffer: 0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA. This solution must be filter-sterilized before use.
7. FACS buffer: 0.5% BSA, 0.05% NaN_3, PBS, 0.1 mM EDTA, filter-sterilized.
8. OCT (Sakura).
9. Aluminium cryovials (Sanbio).

2.6. Preparation of Cytospins from Murine BAL Cells

1. Starfrost microscopic slides (Klinipath).
2. Cytospin paper (Shandon).
3. Cytospin centrifuge (Shandon).

2.7. Intracellular Staining for Cytokines in Broncho-alveolar Cells

1. RPMI-5% FCS.
2. Paraformaldehyde 4% in PBS.
3. Permeabilization buffer: PBS, saponin (0.5%), FCS (2%), sodium azide (0.1%).
4. Anti-CD3 antibody (Bioceros).
5. Anti-CD28 antibody (Bioceros).
6. 96-Well Flexiplate.
7. RBC lysis buffer: 0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA. This solution must be filter-sterilized before use.
8. Golgistop (BD Pharmingen).

2.8. Periodic Acid Schiff (PAS) Staining

1. Periodic acid (Sigma–Aldrich).
2. Schiff's reagent (Sigma–Aldrich).
3. Mayer haematoxylin (Sigma–Aldrich).
4. Entellan (Merck).

2.9. In Vitro Restimulation of Lymph Node Cells

1. TCM: tissue culture medium. Constitution: RPMI-1640 + Glutamax-I, 5% foetal calf serum (FCS), 0.05 mg/ml gentamicin, 5×10^{-5} M β-mercaptoethanol (Invitrogen-Gibco).
2. HBSS: Hanks' balanced salt solution (Invitrogen-Gibco).
3. Nylon cell strainer (100 μM) (BD Biosciences, Falcon).
4. 96-Well plate, U-bottom (BD Biosciences, Falcon).
5. Ovalbumin (Worthington).
6. 1-ml Syringe.

7. 50-ml Tube (BD Biosciences, Falcon).

2.10. Antigen Presentation Assay with DO11.10 T Cells

1. CFSE: Make a stock solution and keep it at −20°C: 5 mM in DMSO (Invitrogen).
2. PBS (Gibco-Invitrogen).
3. RPMI-1640 (Gibco-Invitrogen).
4. RPMI-1640 with 10% FCS (Gibco-Invitrogen).
5. HBBS: Hanks' balanced salt solution (Gibco-Invitrogen).
6. TCM: tissue culture medium. Constitution: RPMI-1640 + Glutamax-I, 5% foetal calf serum (FCS), 0.05 mg/ml gentamicin, 5×10^{-5} M β-mercaptoethanol (Invitrogen-Gibco).
7. RBC lysis buffer: 0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA. This solution must be filter-sterilized before use.
8. FACS buffer (PBS pH 7.8, 0.5% BSA, 0.05% Na-azide).
9. Ovalbumin (Worthington).
10. Monoclonal antibodies:
    a. KJ1.26-PE (Caltag).
    b. CD4-APC (BD Biosciences).
11. Antibody receptor FcγRII and FcγRIII blocking antibody (2.4.G2 clone) (Bioceros).
12. 7AAD, dead cell marker (BD Pharmingen).
13. 100-μm Cell strainer (BD Biosciences, Falcon).
14. 96-Well U-bottomed plate (BD Biosciences Falcon).
15. 15-ml Tubes (BD Biosciences, Falcon).

3. Methods

3.1. Bone Marrow Dendritic Cell Generation and Culture

Day 0 (20)

Sacrifice mice according to local animal guidelines.

1. Isolate rear legs from mice, by carefully separating them from the hips.
2. Remove adhering tissue from femurs and tibias.
3. Collect bones in cold HBSS.
4. Sterilize the outside of intact bones by rinsing them in 70% EtOH.
5. Take care of manipulating the sterilized bones in a sterile environment to avoid eventual contamination of the culture.

6. Wash bones twice with cold HBSS.
7. Smash bones with mortar in a small volume of HBSS (use 50 ml in total for rinsing and smashing).
8. Filter cells through nylon cell strainer (100 μm) into 50-ml tube.
9. Spin cells down: 400×*g*, 7 min, 4°C, use these settings unless stated otherwise.
10. Resuspend cells in RBC lysis buffer (1+# of mice×1 ml = # ml of RBC lysis buffer needed).
11. Incubate for 4 min on ice, shake occasionally.
12. Fill the 50-ml tube with ice-cold HBSS and spin down again.
13. Discard supernatant and resuspend pellet in TCM, take count of sample (1:10 diluted with Trypan Blue).
14. Put cells in culture dishes at a concentration of $2–3\times10^6$ cells/Petri dish in 10 ml TCM with the addition of 20 ng/ml GM-CSF (1 μl GM-CSF/10 ml of TCM). Incubate at 37°C, 5% CO_2 in a humidified atmosphere.

Day 3

15. Add 10 ml of fresh TCM with GM-CSF (20 ng/ml) to the plates.

Day 6

16. To refresh the media (half of it): Collect 10 ml of the plates and spin at RT.
17. Discard the media and resuspend the pellet in 10 ml fresh TCM + 20 ng/ml GM-CSF per plate and put it back in the original plates.

Day 8

18. Repeat the procedure of day 6.

Day 9

19. Pulsing the DCs: Add 100μl of OVA Worthington (20 mg/ml in PBS) to the plates (*see* **Note 2** on the modification of the protocol to stuffy pharmacologic molecules or other stimulations of DCs).

Day 10

20. Harvest the cells by vigorous pipetting and wash Petri dish once with cold PBS.
21. Make a cellular suspension of 1×10^6 cells/80 μl (12.5×10^6cells/ml suspension) in PBS for instillation to recipient mice.

The phenotype of the BMDCs can be analyzed by their surface marker. This can allow their classification into the major phenotypes (CD24, CD54 and CD11b) and can reveal their maturation state after experimental treatments (CD80/CD86, CD40).

3.2. Immunophenotyping of Mouse Bone Marrow DCs

1. Harvest non-adherent DCs by pipetting up and down (check for complete retrieval under microscope).
2. Spin cells: 7 min, 400×*g* at 4°C.
3. Resuspend cells in PBS if they are to be used sterile or in FACS buffer and count them.
4. Place approximately 1×10^6cells/well in Flexiplate 96-well plate.
5. Spin plate for 3 min, 400×*g* at 4°C.
6. Wash cells using 200 μl of FACS buffer.
7. Spin plate for 3 min, 400×*g* at 4°C.
8. Incubate cells with 40 μl of antibody mixes for 30 min at 4°C in the dark.
   1. 2.4G2 (1/100) + CD11c APC (1/800) + MHC-II-FITC (1/200) + isotype PE (1/20).
   2. 2.4G2 (1/100) + CD11c APC (1/800) + MHC-II-FITC (1/200) + CD80 PE (1/800).
   3. 2.4G2 (1/100) + CD11c APC (1/800) + MHC-II-FITC (1/200) + CD86 PE (1/200).
   4. 2.4G2 (1/100) + CD11c APC (1/800) + MHC-II-FITC (1/200) + CD40 PE (1/80).
   5. 2.4G2 (1/100) + CD11c APC (1/800) + MHC-II-FITC (1/200) + CD24 PE (1/100).
   6. 2.4G2 (1/100) + CD11c APC (1/800) + MHC-II-FITC (1/200) + CD54 PE (1/200).
   7. 2.4G2 (1/100) + CD11c APC (1/800) + MHC-II-FITC (1/200) + CD11b PE (1/300).
9. Wash cells twice in FACS buffer.
10. Analyze marker expression by gating on $CD11c^+MHC\text{-}II^+$. After stimulation, CD80/86 CD40, CD24 (HSA) and CD54 (ICAM-1) expression should increase, reflecting an increase in the co-stimulation capacity of the DCs and their maturation. $CD11b^{hi}$ expression is used to identify conventional (myeloid) DCs as $MHC\text{-}II^{hi}$, $CD11c^{hi}$ and $CD11b^{hi}$ cells.

3.3. Sensitization Protocol

After completing the generation of BMDCs, the sensitization protocol can begin (22). Here, the pulsed DCs will induce the allergen sensitization into allergen-naïve recipient mice.

1. On the first day of the experimental protocol (day 0), the Ag-pulsed DCs are administered to the recipient by i.t. instillation.

1. The mouse must first be anaesthetized (according to local guidelines, preferably by injectable anaesthetics, because inhaled anaesthetics can kill DCs).
2. From now on, manipulations should be performed in a class II biosafety cabinet to ensure sterility during procedure.
3. Anaesthetized mice can now be suspended by the upper front teeth. This puts the mouse in a position where the rear legs are horizontal, touching the work surface and the head is vertical, suspended to the apparatus. It is an optimal position for the trachea to be vertical and then the liquid will more easily reach the airways.
4. Then the tongue is pulled out of the mouth of the animal and held on one side of the mouth, always maintained in extension. This extension is important, as it ensures the closing of the oesophagus and allows the trachea to be reached. Anaesthetized mice should not exhibit swallowing activity, further ensuring that i.t. injection will effectively reach the airways.
5. While the tongue is kept pulled, the liquid containing DCs (1×10^6 cells/80 µl) is dropped far in the mouth of the animal behind the tongue, as close to the vocal chords as possible. The instillation must be done according to the speed at which the liquid is dragged to the airways in order to ensure that it all reaches the trachea.
6. Keeping the animal in such a position as long as possible will ensure the proper instillation of the liquid to the airways. Mice should breathe normally before being returned to the cage.
7. Mice should then be monitored during the period of wake up after the anaesthetic, preferably on a heating mat.

2. On days 10, 11 and 12, ovalbumin 1% in PBS is nebulized for 30 min to the mice.
3. On day 13, 24 h after the last challenge, mice are sacrificed for the analysis of the various parameters of interest (*see* **Note 3** for modifications of this protocol).

3.4. Collecting and Processing of Murine BAL

3.4.1. Collect BAL

1. Dissect the dead mouse in order to access the trachea.
2. Insert the suture thread between the oesophagus and the trachea.

3. With scissors, cut the trachea between the cartilage rings enough in order to insert the catheter (be careful NOT TO cut the trachea completely).
4. Insert the catheter through the orifice towards the lungs, but maintain it in the trachea.
5. Secure the catheter in place with the suture thread.
6. Using PBS–EDTA at RT, wash the lung three times with 1 ml, putting retrieved broncho-alveolar lavage fluid (BALF) in the 15-ml tube. The whole 1 ml must be injected, then slowly retrieved. The first 1-ml lavage will most of the time result in limited retrieval (0.5–0.7 ml) of the liquid, but following lavages should be retrieved almost entirely.
7. Keep the BAL fluid on ice during the procedure then at 4°C if necessary.
8. If histology is to be performed on the lungs, 1 ml of PBS–OCT (1:1 solution, mixed, at least 6 h before use) is injected in the lungs via the catheter. The lung is cut out carefully, not to damage it, and transferred in an upright position, by holding the trachea up with forceps, to the aluminium cryovial. The cap is screwed on the container and snap-frozen in liquid nitrogen.

3.4.2. Processing BALF

1. Centrifuge 15-ml tubes for 3 min, 400×*g*, 4°C.
2. Discard supernatant, then resuspend cells in 1 ml of red blood cell (RBC) lysis buffer, incubate for 4 min on ice, then add 11 ml FACS–EDTA buffer.
3. Centrifuge 15-ml tubes for 3 min, 400×*g*, 4°C.
4. Wash in FACS–EDTA, 3 min, 400×*g*, 4°C.
5. Resuspend in 200 μl of FACS–EDTA buffer and count the number of cells.
6. Keep apart what you will need for cytospins and use everything that remains to apply for FACS staining.

3.5. Preparation of Cytospins from Murine BAL Cells

1. Identify your slides.
2. Count the total number of cells available in your BALF.
3. Use 50,000 cells from the cells isolated by BAL.
4. Spin down for 5 min at 400×*g* and resuspend in 50 μl PBS.
5. Assemble microscopic slides with the cytospin papers and the sample funnel in their cast for the cytospin centrifuge. Place the assembly in the rotor of the cytospin in their numbered positions.
6. First apply 50 μl of PBS and run a quick-spin to pre-wet the slides.

7. Apply 50 μl of samples to their respective assembled cast. Always use 12 slides to avoid imbalance, which could result in poor-quality sample impaction on the slide.
8. Close the rotor with its cap, and then close the cytospin centrifuge.
9. Spin the samples for 5 min at 300 rpm.
10. When the spin is finished, remove your slides, separate them carefully from their cytospin paper and let them dry in horizontal position.
11. The slides are now ready to be stained using various dyes, such as the Diff-Quik staining.
12. It is possible to conserve these slides before staining procedures. If so, it is better to store them in a sealed slidebox at −80°C. Remember to let slides reach RT before using them as condensation that would form if you open the sealed box while it is cold could damage your samples.

3.6. Determination of Cell Differential Counts on BAL Fluid Using Flow Cytometry

The degree and type of airway inflammation are often studied by determination of differential cell counts on cytospins of broncho-alveolar lavage fluid cells stained with May-Grünwald Giemsa (***see* Section 3.5**), in which the separation of eosinophils from neutrophils and of monocytes from activated T cells can be quite problematic (26). To avoid this problem, we have developed a very convenient 4-colour flow cytometry assay to differentiate different cell types in the BAL fluid. Using this protocol, we compared differential cell counts based on morphological criteria on May-Grünwald Giemsa-stained cytospins with the flow cytometric method. BAL fluid cells were discriminated based on forward and side-scatter characteristics, autofluorescence of macrophages and simultaneous one-step staining with antibodies for T cells (CD3-PECy5), B cells (CD45R-PECy5), eosinophils (CCR3-PE) and dendritic cells (MHC-II-FITC, CD11c-APC). The validity of our flow cytometric determination was tested by morphological analysis of flow-sorted cellular subsets. In an animal model of ovalbumin-induced asthma, this new method correlated very well to the differential counts based on cytospins. Flow cytometric determination of cellular composition of BAL fluid in mouse models of asthma is a rapid and easy method that can replace differential cell counts based on morphology. For a detailed protocol we refer to another paper by our group (26).

3.7. Intracellular Staining for Cytokines in Broncho-alveolar Cells

Day 1

1. Coat a 96-well plate with 5 μg/ml of anti-CD3 antibody in PBS at a ratio of 100 μl/well of an antibody dilution of 1/200. Incubate overnight at 4°C. Plan 3 wells/mouse (calculate 10 ml of anti-CD3 solution per plate).

Experiment Day

1. Perform the broncho-alveolar lavage (as previously described, 3×1 ml) with sterile PBS at room temperature.
2. Spin cells for 7 min at 400×*g*, at 4°C.
3. If necessary, lyse red blood cells as described previously with the RBC lysis buffer: Resuspend cells in RBC lysis buffer (1 + # of mice×1 ml = # ml of RBC lysis buffer needed).
4. Incubate for 4 min on ice, shake occasionally.
5. Fill the 50-ml tube with ice-cold media and spin down again.
6. Spin cells for 7 min at 400×g, at 4°C.
7. Resuspend cells in 150 µl RPMI-5% FBS/mice BAL.
8. Add 150 µl of a solution containing 1/125 dilution of anti-CD28 (final dilution of 1/250) in RPMI 5% and pre-warmed to 37°C
9. Meanwhile, empty the plate coated with anti-CD3 and wash twice with sterile PBS 250 µl/ well and make sure they are empty by blotting the plate on a paper tissue.
10. Add 100 µl of cell suspension prepared in '8' to the wells of the plate coated with anti-CD3. Incubate for 2 h at 37°C.
11. Add 100 µl of a solution of Golgistop containing 1/250 dilution of anti-CD28, 1/750 dilution of Golgistop in RPMI 5%
12. Incubate for 4 h at 37°C.
13. Transfer cells into a 96-well Flexiplate.
14. Spin the Flexiplate for 3 min at 400×*g*, 4°C.
15. Wash cells with FACS buffer and spin again.
16. Incubate cells for 15 min with 40 µl of anti-CD4 FITC (1/200) for 30 min at 4°C. (Do not forget your single stains controls and the isotype controls.)
17. Wash twice with FACS buffer.
18. Incubate cells with 40 µl of anti-cytokines mAb in permeabilization buffer, 30 min, 4°C:
    - Mix 1: α-IL-4 PE (1/30) and α-IFN-g APC (1/100).
    - Mix 2: α-IL-5 PE (1/60) and α-IFN-g APC (1/100).
    - Mix 3: α-IL-10 PE (1/30) and α-IFN-g APC (1/100).
    - Do not forget your single-stain controls and the isotype controls.
19. Wash cells twice in FACS buffer.
20. Resuspend cells in 200 µl FACS buffer for FACS analysis.

3.8. Periodic Acid Schiff (PAS) Staining

This staining allows the visualization of mucus production from goblet cells and therefore provides a method to visually assess the extent of mucus hypersecretion in the airways. The mucus will stain pink on the mounted slide.

This procedure can be performed on tissues mounted on microscopic slides coming from paraffin sections or from cryosections. Unless stated otherwise, steps are performed at room temperature.

Protective gloves should be worn for this procedure.

1. Hydration of slides:
   a. Paraffin sections:
      i. Put slides in xylene for 2 min. Repeat once.
      ii. Put slides in ethanol 100% for 2 min.
      iii. Put slides in ethanol 96% for 2 min.
      iv. Put slides in ethanol 70% for 2 min.
      v. Put slides in ddH_2O for 2 min. Repeat once.
   b. Cryosections:
      i. Defrost slides in cold formalin 4% (in ddH_2O), for 10 min, at 4°C.
2. Staining (now both types of slides are treated the same way):
   a. Incubate for 5 min in 0.5% periodic acid.
   b. Rinse in ddH_2O.
   c. Incubate for 10 min in Schiff's reagent, in the dark.
   d. Rinse for 30 min in running tap-water (make sure the flow does not detach your tissue sample by keeping it slow and far from the tissue).
   e. Incubate for 30 s in Gill's haematoxylin (filtrate before use).
   f. Rinse 10 min in running tap-water.
3. Dehydration.
   a. Put slides for 30 s in 70% ethanol.
   b. Put slides for 30 s in 96% ethanol.
   c. Put slides for 30 s in 100% ethanol, repeat.
   d. Put slides in xylene.
   e. Mount sections in Entellan.

3.9. In Vitro Restimulation of Lymph Node Cells

In order to assess the immune response induced by transferred DCs and the polarization of this reaction, it is possible to restimulate the cells of the lymph nodes (therefore containing antigen-presenting cells and effector T cells) with their Ag (ovalbumin in our protocol) and assess the production of cytokines by this lymph node cells population.

1. Collect the LN of interest in HBSS on ice. Of notice, the mediastinal LNs are the draining LNs for the lung while axillary LNs are non-draining LNs and therefore of particular relevance for control.
2. Smash the LNs through a 100-μm cell strainer using the plunger of a sterile syringe.
3. Spin down the cells at 400×*g* and 4°C for 7 min (use these parameters unless specified otherwise).
4. Resuspend the pellet in 1 ml TCM.
5. Count cells with Trypan Blue.
6. Add 9 ml TCM and spin down.
7. Resuspend the cells in TCM, so that the final concentration will be 2×10^6c/ml.
8. Pipette 100 μl cell suspension/well (6 wells/condition or group) in a U-bottomed 96-well plate.
9. Add 100 μl of 20 μg/ml ovalbumin in TCM.
10. Culture for 96 h in a humidified incubator at 37°C and 5% CO_2.
11. Spin the plate for 3 min at 400×*g*, 4°C and transfer the supernatant to a new plate. Store supernatant at −20°C for the measurement of cytokines in the few next weeks, −80°C for longer storage.
12. Perform an ELISA on the supernatant for IL-4, IL-5, IL-10 and IFN-γ, using commercially available ELISA kits.

Our experience with such a protocol shows that supernatant from peripheral LNs can be used undiluted while the supernatant from mediastinal LNs can be diluted by 10.

3.10. Antigen Presentation Assay with DO11.10 T Cells

Days 0–9

1. Production of BMDCs as described previously.

Day 10

1. Collect the spleen and cervical, mediastinal, brachial, axillary, inguinal and mesenteric LN from a DO11.10 mouse and put them in HBSS on ice (*see* **Note 4**).
2. Smash the LNs through a 100-μm filter and collect cells in HBSS in a 15-ml tube.
3. Spin down the cells at 400×*g*, 4°C for 7 min.
4. Resuspend the pellet in 1 ml PBS.
5. Count the living cells.
6. Resuspend the cells in PBS, so that the final concentration will be 5×10^7cells/ml.
7. Immediately before use, dilute CFSE (1/10 from stock mentioned in material) to 0.5 mM in PBS

8. Warm cells to 37°C in water bath and add 20 μl CFSE (0.5 mM)/ml of cell suspension.
9. Incubate for 10 min at 37°C and invert tubes every 2 min.
10. Wash cells three times by adding an excess of RPMI-10% FCS and spinning the cells to block the reaction.
11. Count living cells and resuspend the cells in TCM, so that the final concentration will be 3.3×10^5 cells/ml.
12. Pipet 150 μl cell suspension/well (12 wells/condition or group) in a 96-well U-bottomed plate.
13. Store in a humidified incubator at 37°C, 5% CO_2 until APCs are added.
14. Resuspend APCs in 50 μl at the concentrations to test.
15. Incubate in a humidified incubator at 37°C and 5% CO_2 for 72 h.

Day 13

1. Collect cell suspensions for each condition in a 5-ml FACS tube (12 wells/condition, pooled in one tube).
2. Spin the cells down
3. Freeze (−20°C) 1 ml of the supernatant of each condition for cytokine concentration determination by ELISA.
4. Resuspend pellet in FACS buffer (200 μl/condition) and stain with kj1.26-PE (1/30), CD4-APC (1/200), 2.4.G2 (1/30) for 30 min in dark at 4°C, not forgetting to use some cells for single stain and unstained controls.
5. Wash once with FACS buffer.
6. Resuspend cells in 250 μl FACS buffer. Add 7AAD just before measuring FACS sample.
7. Start FACS analysis (*see* **Note** 5).

3.11. Conclusion

We previously used this model for the investigation of the necessity of DC CD80/CD86 co-stimulation during allergen sensitization, by using DCs from K/O mouse, and for determining the capacity of DC subsets to induce immunity in the lung (10, 27). This allowed us to observe the effect of CD80 or CD86 co-stimulation on the induction of naïve T-cell proliferation during sensitization. We have recently validated that all Th2 priming in this model is due to direct antigen presentation by transferred DCs and not due to transfer of antigen-MHC complexes to recipient DCs (28). We also used this model to investigate the effect of DC treatment with various drugs, among them, agonists of the proliferator-activated receptor-gamma (29) or in different studies, a sphingosine-1-phosphate receptor agonist (FTY720) (30), compounds of the prostaglandin family (31, 32) and purinergic receptor agonists (33). In this context, we treated DCs with the drugs

in order to specifically assess what is the effect of these drugs on DCs as opposed to the in vivo treatment. It is also possible to use this model to address the immunomodulating effect of microbial compounds on the potential of DCs to prime for Th2 responses in the airways (34).

4. Notes

1. Gene-deficient mice can be used to assess the role of individual proteins in the DC-mediated functions. In such cases, care must be taken to select matching strains between the donor and the recipients to avoid adverse immune reactions against the transferred cells. Care must also be taken to ensure that the mutation of your protein of interest does not hamper DC development that would preclude their generation from bone marrow precursors. Thorough screening of DC phenotype and functions should be made in parallel or previous to your experiments.
2. It is at this step that it is possible to treat DCs before their pulsing. While the DCs are in culture, they can be exposed to various inhibitors, drug compounds or environmental stimuli. Evaluation of the appropriate timing is to be performed for each individual compounds. As an example, various inhibitors of signalling pathways are usually preferentially administered 1 h before addition of the OVA.
3. If the role of DCs in the allergen challenge is to be studied, it is possible to avoid the nebulization of ovalbumin and replace it by a second i.t. instillation of Ag-pulsed BMDCs on day 11 and sacrifice the animals on day 13. For such a protocol, two cultures of BMDCs will have to be initiated 11 days apart to have them ready for adoptive transfer at both sensitization and challenge phases.
4. PBS and everything used to manipulate and resuspend cells before CFSE staining must be serum-free, as CFSE binds proteins, which are abundant in serum and this would dramatically reduce the efficacy of the labelling.
5. Many other parameters can be evaluated on mice in order to get relevant information on the asthmatic status of experimental mice. Here, we described approaches of immunological interest routinely used in our laboratory, but this could be complemented by other approaches. One of these approaches would be the evaluation of the bronchial hyperresponsiveness of the mice in order to see if treatment of DCs resulted in modification of this lung physiological

parameter. Methods to assess this parameter should be considered carefully before the experiments as some controversy exists on the use of whole-body plethysmography and the value of pause enhancement (Penh) provided by this technique. This value is seen as reflexive of the lung resistance (R_L), a measure usually obtained by invasive techniques, but there exists controversy on the use of Penh values in this context. Readers are advised to get proper information on the subject before choosing their analysis method. The method paper of Dr Umetsu team is a good resource on the use of both techniques (35).

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Chapter 23

Direct Ex Vivo Activation of T cells for Analysis of Dendritic Cells Antigen Presentation

Gabrielle T. Belz

Abstract

Dendritic cells (DCs) are a heterogenous population of professional antigen-presenting cells (APCs) that play a major role in the initiation of immune responses. DC subsets differ in their anatomical locations together with their intrinsic abilities to capture, process, and present antigens on their major histocompatibility (MHC) class I and class II molecules. These features enable each DC subset to have distinct roles in immunity to infection and in the maintenance of self-tolerance. The discrete features of DC subpopulations have largely been defined by cell surface phenotype and anatomical location, rather than function. We have developed direct ex vivo methods to efficiently isolate small numbers of DCs from lymph node (LN) draining tissues and infectious sites to allow fine probing of their function using very sensitive antigen-specific LacZ hybridomas and in vitro proliferation of CFSE-labeled T cells. These approaches are particularly sensitive for detecting endogenous antigens derived from pathogens and self-tissues. Understanding these interactions has begun to allow us to understand how integration of different populations in the DC network responds to multiple scenarios of infection.

Key words: Dendritic cell, antigen presentation, DC subsets, infection, T-cell activation.

1. Introduction

Antigen-presenting cells (APCs) are recognized as key initiators of adaptive immunity, particularly to pathogens, by eliciting a rapid and potent immune attack on infected cells (1). Dendritic cells (DCs) act as sentinels for pathogen invasion and are specially equipped to initiate and regulate immune responses in a manner that depends on signals they receive from microbes and their cellular environment (2). To achieve this, they are equipped with highly efficient mechanisms that allow them to detect pathogens,

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to capture, process, and present antigens, and to activate and guide the differentiation of T cells into effector and memory cells. The migratory DCs traffic from tissues to LNs where they mature. They are important in ferrying antigens from the body surfaces to the lymph nodes (LNs) where they encounter resident DCs. Resident DCs spend their entire lifespan in the lymphoid organs in an immature state until activated by signals reaching these organs (3). These resident DCs can be directly infected with pathogens or take up antigens from the migratory DCs for presentation to lymphoid cells (4, 5). Both migratory and resident DCs can be subdivided into several subtypes with different roles in antigen presentation (6–8). Elucidating the antigen-presenting abilities of distinct DC subsets and their capacity to present pathogen antigens has been a critical step in defining the fundamentally distinct and non-overlapping functions of DC subsets. We have developed methods of isolating DCs from murine LNs (and other lymphoid tissues) that allows purification for analysis of surface phenotype using immunofluorescent labeling and sorting for functional analysis (9, 10). This approach has allowed direct ex vivo interrogation of very small subpopulations of DCs for antigen presentation and has facilitated our understanding of the collaboration that occurs within the network of DC subtypes in driving $CD4^+$ and $CD8^+$ T-cell activation and differentiation.

2. Materials

2.1. Media

All media are sterilized by filtration through a 0.22-μm membrane filter and stored in the dark at 4°C.

1. Fetal calf serum (FCS): Heat-inactivate for 30 min at 56°C, aliquot, and store at −20°C.
2. Ethylenediamine tetra acetic acid (EDTA) pH 7.2. Mouse osmolarity: 0.01 M solution of EDTA was made by dissolving disodium salt (Sigma) in Milli-Q water and neutralized with NaOH to pH 7.2. Filter to sterilize and store at 4°C.
3. Balanced salt solution: EDTA (BSS–EDTA) (HEPES buffered, mouse tonicity) contains 150 mM NaCl and 3.75 mM Ca^{2+} and Mg^{2+} free KCl and 5 mM EDTA adjusted to a final pH 7.2 and mouse osmolarity 308 mOsm/kg.
4. BSS–EDTA–FCS: Add 2% fetal calf serum (FCS) to BSS–EDTA.
5. EDTA–FCS: 10 ml FCS supplemented with 1 ml of 0.099 M EDTA.

6. Complete medium (complete medium, complete RPMI) contained RPMI-1640 or DMEM supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine (ICN Biomedicals), 100 U/ml penicillin, and 100 μg/ml streptomycin.
   RPMI-1640 (mouse tonicity): RMPI-1640 powder (Gibco BRL), 9 g NaCl, 20 g $NaHCO_3$, 1.1 g sodium pyruvate, 2 mM L-glutamine (Gibco BRL), 1.0 g penicillin, and 1.0 g streptomycin in 10 l of Milli-Q water. Final pH 7.2. This medium has been modified to mouse osmolarity (308 mOsm) and additional pH 7.2 HEPES buffer is included to reduce dependence on CO_2. Cells are generally cultured in 10% CO_2 atmosphere for this medium.
   Dulbecco's Modified Eagle Medium 10 (complete *DMEM*) (mouse tonicity): 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1.2 mg/ml HEPES (Gibco BRL) in 100 ml of DMEM containing 10% FCS.
7. Red cell removal buffer: 8.99 g NH_4Cl (0.168 M) in 1 l Milli-Q water.
8. Flow buffer: Dulbecco's phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}) containing 1% bovine serum albumin (BSA) and 0.02% (w/v) sodium azide (NaN_3) (2% FCS can be substituted for BSA).
9. Propidium iodide (PI): Prepare a stock solution at 200 μg/ml in PBS. A working solution is made by diluting stock to 2 μg/ml in PBS. Aliquot and store at 4°C protected from light.
10. Collagenase/DNase: For 7X stock solutions, dissolve 7 mg collagenase (121 U/mg – Type III, Worthington Biochemicals) and 7 μg of DNase (Roche) in 1 ml complete medium (final concentrations for digestion are 1 mg/ml collagenase and 1 μg/ml of DNase; for working solution add 1 ml of stock solution to 6 ml complete medium, total volume 7 ml). Preparation is filtered through a 0.2-μm Millipore filter and aliquoted. May be kept in individual 1 ml aliquots and stored at −20°C.

2.2. Antibody Depletion Cocktails

1. DC-enrichment cocktail – conventional DCs (cDCs): Optimally titrated rat anti-mouse monoclonal antibodies for use at 10 μl/10^6 cells: anti-CD3 (KTR-1.1), anti-CD90 (T24/31.7), anti-CD19 (ID3), anti-Gr-1 (RB6-8C5), anti-B220/CD45R (RA36B2), and anti-erythrocyte (Ter-119) monoclonal antibodies.
2. DC-enrichment cocktail – plasmacytoid DCs (pDCs): Optimally titrated rat anti-mouse monoclonal antibodies for

use at 10 μl/10^6 cells: anti-CD3 (KTR-1.1), anti-CD90 (T24/31.7), anti-CD19 (ID3), anti-Gr-1 (RB6-8C5), and anti-erythrocyte (Ter-119) monoclonal antibodies.

3. $CD8^+$ T-cell-enrichment cocktail: Optimally titrated rat anti-mouse monoclonal antibodies for use at 10 μl/10^6 cells: anti-CD11b (M1/70), anti-F4/80 (F4/80), anti-erythrocyte (Ter-119), anti-Gr-1 (RB6-8C5), anti-MHC class II (M5/114), and anti-CD4 (GK1.5).
4. $CD4^+$ T-cell-enrichment cocktail: Optimally titrated rat anti-mouse monoclonal antibodies for use at 10 μl/10^6 cells: anti-CD11b (M1/70), anti-F4/80 (F4/80), anti-erythrocyte (Ter-119), anti-Gr-1 (RB6-8C5), anti-MHC class II (M5/114), and anti-CD8 (53-6.7) (*see* **Notes 1** and **2**).

2.3. Culture Plates

1. 96-Well cell culture cluster, V-bottom with lid, tissue culture treated, Costar®.
2. 96-Well cell culture cluster, MICROTEST™ U-bottom with lid, tissue culture treated, Becton Dickinson.
3. MICROTEST™ 96-well cell culture cluster, flat-bottom with lid, tissue culture treated, Becton Dickinson.

Choice of plates: A variety of 96-well tissue culture plates are available to perform direct ex vivo or in vitro assays. These include V-bottomed, U-bottomed, and flat-bottomed plates. The choice of plate depends on the number and type of cells to be analyzed. Small numbers of APCs can be brought into close proximity to readily present antigen to T cells when placed into V-bottomed 96-well plates. While this is beneficial in detecting antigen presentation by small numbers of cells, larger numbers of APCs or high levels of antigen presentation can result in significant proliferation in the well, followed by substantial cell death by 60 h culture leading to the appearance of a small response. In such cases, it is preferable to coculture cells in U-bottomed plates. Analysis of antigen presentation using hybridomas is performed in flat-bottomed plates.

2.4. Analysis of Antigen Presentation Using LacZ T-Cell Hybridoma

1. Fixation solution: 2% formaldehyde/0.2% glutaraldehyde in PBS (*see* **Note 3**). Store at 4°C.
2. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Progen #200-0191): 20 mg/ml X-gal diluted in *N*,*N*′-dimethylformamide. Store at −20°C. Potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$: Sigma–Aldrich, #31254). Potassium ferricyanide ($K_3Fe(CN)_6$: Sigma–Aldrich, #702587). These chemicals are highly toxic and should only be handled in a fume hood with appropriate protection for the handler.

3. Development solution: Dilute X-gal to 1 mg/ml in 5 mM potassium ferrocyanide/5 mM potassium ferricyanide/2 mM $MgCl_2$ in PBS (*see* **Note 4**). Store at 4°C.

2.5. Miscellaneous Reagents

1. CFSE (5- and 6-carboxyfluorescein diacetate succinimidyl ester): Dissolve CFSE powder (Molecular Probes) in DMSO to a final concentration of 5 mM [25 mg CFSE (molar mass of 557.47 g/mol) dessicated powder is equivalent to 44.8 μM]. Dissolve 25 mg CFSE in 8.96 ml DMSO to obtain a 5 mM solution. This solution can be stored in 50–200 μl aliquots at −20°C and is stable following several rounds of freezing/thawing (11, 12).
2. Dynal beads: Sheep anti-rat M-450 IgG-coated Dynal beads (Dynal, Oslo, Norway, #110.35). Dynal beads are used at a ratio of 3–5 beads per cell. An alternative to Dynal bead is Qiagen BioMag goat anti-rat IgG magnetic separation particles (Qiagen). BioMag beads are used at a ratio of 8–10 beads per cell.
3. Dynal magnet: MPC-L magnet or DynaMag^{TM}-15.
4. Spiral rotator: Siramix 10 (Denley, Billingshurst, England).

3. Methods

3.1. Isolation of DCs for Direct Ex Vivo Analysis of Antigen Presentation

This protocol allows the isolation of DCs that have been loaded with model antigens (such as ovalbumin) or pathogen-derived antigens (e.g., endogenously processed antigens from viral infection) in vivo and probing their antigen presentation abilities in vitro (*see* **Note 5**).

3.1.1. Method

1. Collect LNs or spleen in complete medium. Care should be taken to minimize contamination of the organs with fat or connective tissue (*see* **Note 6**).
2. Tip LNs or spleen into a Petri dish and remove all media with a pipette.
3. Gently dissociate LN or spleen with sterile curved scissors or scalpel blade.
4. Transfer into a tube containing 1 ml of 7 mg collagenase/DNase and 6 ml complete medium (*see* **Note** 7).
5. Digest by pipetting up and down with plastic transfer pipette for 20 min.
6. Add 630 μl 10% EDTA and pipette for another 5 min.
7. Underlay with 1 ml EDTA–FCS (*see* **Note 8**).

8. Pellet cells by centrifuging cells for 5 min at 400 × *g* (*see* **Notes 9** and **10**) and resuspend in antibody depletion cocktail (10 μl/10^6 cells) (*see* **Note 11**).
9. Incubate cells at 4°C for 30 min (*see* **Note 12**).
10. Wash cells with BSS–EDTA–FCS with an EDTA–FCS underlay.
11. Resuspend cells in 500 μl BSS–EDTA–FCS in a 5-ml polypropylene round-bottomed tube (Falcon #35-2003) and add appropriate number of Dynal beads that have been washed three times with BSS–EDTA–FCS (3:1 bead to cell ratio) (*see* **Note 13**).
12. Sit on angled rotor for exactly 20 min at 4°C (*see* **Note 14**).
13. Harvest cells by topping tube up with BSS–EDTA–FCS and placing on magnet for 2 min and transfer the supernatant to a new tube.
14. Add 5 ml BSS–EDTA–FCS and underlay with EDTA–FCS and wash by centrifugation for 5 min at 400 × *g*.
15. Resuspend cells for flow cytometric analysis or high speed flow cytometric sorting (*see* **Notes 15** and **16**).

3.2. Analysis of Individual LN Lymphoid Cells and APCs

Although individual LNs contain a small number of cells relative to the spleen, it is possible to individually dissect the behavior of different lymphoid cells even when those cell types represent a small fraction of cells within the LN. This approach allows analysis of large numbers of LNs as individuals in a relatively simple format (*see* **Note 17**).

1. Individual LNs are collected into a 0.5-ml eppendorf tube containing 200 μl complete medium. During the collection process very fine forceps are used to gently tear the LN into two or three pieces to allow the collagenase/DNase mixture access to the cells (*see* **Note 18**).
2. Add 200 μl of 2X solution of collagenase/DNase (resulting in 400 μl of collagenase/DNase/complete medium).
3. Digest by pipetting up and down with a p200 pipette in which the pipette tip has been trimmed down to allow the LN fragments to pass through. Multiple samples can be digested at once by aligning 0.5-ml eppendorf tubes in a p1000 tip box. An 8-well or 12-well multi-channel loaded with "trimmed" tips can then be used to proceed with the digestion.
4. Add 36 μl of 10% EDTA and pipette the mixture for a further 5 min.
5. Underlay digestion mixture with 100 μl EDTA–FCS and wash by centrifugation for 5 min at 1,500 × *g*.

6. Carefully aspirate supernatant and resuspend cells in 200–400 μl complete media and count cells (*see* **Notes 19** and **20**).

3.3. Purification of T Cells

1. Single-cell suspensions are made from pooled subcutaneous and mesenteric LNs by gently passing tissues through a sieve.
2. Wash cells once with complete RPMI media and centrifuge at 4°C at 400 × *g* for 5 min.
3. Resuspended in 10 μl depletion cocktail/1 × 10^6 cells and incubate for 30 min on ice.
4. Wash cells once with complete RPMI media underlaid with EDTA–FCS and centrifuge at 4°C at 400 × *g* for 5 min.
5. Resuspended in 500 μl complete media and transfer to a pellet of pre-washed sheep anti-rat IgG-coated magnetic beads (Dynal) at 3 beads/cell.
6. Place cells/beads mix on a spiral rotator angled at 45° angle for 20 min at 4°C.
7. Following incubation, dilute the cells and beads with 2–3 ml complete media and place against a magnet (Dynal) to bind bead/antibody-coated cell complexes.
8. Recover the supernatant containing the enriched T-cell population and discard bound bead/antibody-coated cell complexes containing the T-cell depleted fraction.
9. The purity of T cells is determined by staining a small sample of enriched cells with antibodies toward CD4 or CD8 and the relevant T-cell receptor (TCR). These cells are analyzed by flow cytometry to determine the percentage of cells positive for coreceptor and TCR markers. Transgenic T cells should routinely be between 85 and 98% pure after enrichment.

3.4. CFSE Labeling of Responder T-Cell Populations

CFSE labeling was originally described by Lyons and Parish (12) to provide a quantitative means of tracking lymphocyte cell division. The approach relies on the ability of CFSE to stably and covalently label intracellular molecules with the fluorescent dye, carboxyfluorescein. Following each cell division, the daughter cells inherit half of the fluorescent dyes and with each further cell division there is a sequential halving of the dye intensity. The biological and functional behavior of lymphocytes can be traced for up to eight divisions using this approach. Indeed, sophisticated approaches to modeling CFSE data have been developed to allow a broader understanding of how lymphocyte proliferation and survival times are regulated by different signals (13).

3.4.1. Method

1. Wash cells twice in PBS/BSA. Resuspend cells in a fresh tube at 1×10^7/ml (*see* **Note 21**) taking care to place the solution at the bottom of the tube without wetting the tube sides (*see* **Note 22**); 1–3 ml of cells can be labeled efficiently in a 15-ml tube while larger numbers of cells should be labeled in a 50-ml tube to ensure that the CFSE can be rapidly and homogenously mixed into the solution of cells.
2. 0.5 μM CFSE (*see* **Note 23**) is applied by pipetting a droplet onto the side of the tube (this is equivalent to 1 μl of 5 mM CFSE stock/ml cell suspension).
3. Cap the tube and vortex immediately for several seconds to mix thoroughly and ensure consistent and even labeling.
4. Incubate the cells in the presence of CFSE at 37°C for up to 10 min (*see* **Note 24**).
5. Wash the cells by diluting them in complete medium, centrifuge the cells to pellet them, and then discard the supernatant.
6. Wash the cells a further two times in complete medium prior to counting (*see* **Note 25**).

3.5. In Vitro Stimulation of T Lymphocytes

3.5.1. Direct Ex Vivo Analysis of Endogenously Loaded APCs

Endogenous loading of APCs occurs following infection of a mouse with a pathogen or inoculation with a surrogate model antigen (such as ovalbumin). At various times after exposure to the pathogen or antigen, DCs or APC subsets can be purified by high-speed flow cytometric sorting from the LNs or spleen of mice exposed to the antigen. Tracking antigen presentation in this way depends on the pathogen expressing a defined natural or surrogate antigen for which a responder T-cell population is available or can be generated. These responder populations may be purified CFSE-labeled naïve TCR transgenic T- cells or, alternately, an antigen-specific T-cell line generated in vitro and generally rested prior to use.

1. Prepare CFSE-labeled cells at 5×10^5/ml in complete medium for stimulation with and without antigens.
2. Titrate purified APCs: Add 100 μl of complete medium to all wells except the top row. In the top row, add equivalent numbers of each APC subset to be tested in 200 μl complete medium for dilution in each column of the plate (*see* **Note 26**). Perform a twofold dilution down the plate by transferring 100 μl of APCs to the next row of wells and mix thoroughly. Continue this process down the plate until the dilution series is complete. Discard the extra 100 μl of media from the last row containing APCs to leave 100 μl in each well of the plate. Place 100 μl complete media alone in the final row of the plate – to these wells T cells alone will be

added to provide a negative unstimulated T-cell control (*see* **Note 27**).

3. Add 100 µl of CFSE-labeled T cells (5×10^4) to each well of a 96-well V-bottomed tissue culture plate already containing 100 µl of diluted stimulator APCs.
4. Coculture CFSE-labeled T cells and titrating numbers of purified APCs for 60 h at 37°C and 10% CO_2.
5. Analyze the proliferation induced in the T-cell population by flow cytometric analysis (*see* **Fig. 23.1** and **Note 28**).

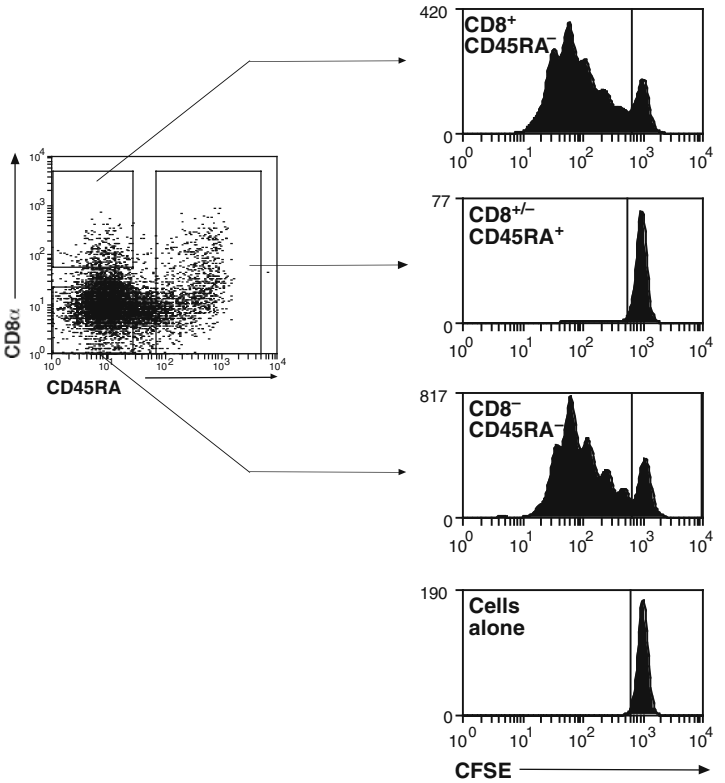

Fig. 23.1. Direct ex vivo analysis of antigen presentation. Different subsets of flow cytometrically purified DCs isolated from the draining LNs of pathogen-infected mice are cultured with small numbers of CFSE-labeled antigen-specific transgenic T cells for 60 h. Proliferation is assessed by the dilution of CFSE as T cells stimulated by DCs progressively divide.

3.5.2. Analysis of Cross-Presentation by Different Subsets of APCs

To determine the intrinsic capacity of different APCs to cross-present antigens, purified DCs are isolated from secondary lymphoid tissues of mice (*see* **Chapter 14**).

1. Resuspend DCs at 5×10^4/ml in complete medium. Add 100 µl of APCs to each well of a 96-well V-bottomed tissue culture plate.

2. Pulse DCs with titrating amounts of an antigen (e.g., ovalbumin) for 1 h at 37°C and 10% CO_2.
3. Prepare CFSE-labeled cells at 5×10^5/ml in complete medium for stimulation with and without antigens.
4. Wash antigen-pulsed APCs at least twice in complete medium, then add 100 µl of CFSE-labeled T cells (5×10^4, e.g., OT-I $CD8^+$ T cells). In separate wells, ovalbumin-pulsed DCs should be cocultured with $CD4^+$ OT-II cells to provide a positive control to demonstrate that exogenous antigens can be presented via their natural pathway of loading onto MHC class II molecules for $CD4^+$ T cells. Additional positive controls include the stimulation of T cells with peptide-pulsed DCs (0.5 µg/ml synthetic $OVA_{323–339}$ peptide, sequence ISQAVHAAHAEINEAGR, for $CD4^+$ OT-II or 0.01–0.001 µg/ml synthetic peptide $OVA_{257–264}$ peptide, sequence SIINFEKL, for $CD8^+$ OT-I cells).
5. Coculture CFSE-labeled T cells and APCs for 60 h at 37°C and 10% CO_2.
6. Analyze the proliferation induced in the T-cell population by flow cytometric analysis.

3.6. Harvesting Cells and Data Collection

Harvesting cells can be performed at various time-points after initiation of the coculture. The optimum time for analysis of direct ex vivo analyses is at 60 h.

1. Immediately prior to cell harvest and flow cytometric analysis add counting beads (*see* **Note 29**).
2. Prepare a stock of PI at 200 µg/ml in PBS and use solution at 2 µg/ml.
3. Mix the counting beads and cells thoroughly and transfer samples to polystyrene round-bottomed tubes, add 10 µl working solution PI/sample (*see* **Note 30**).

3.6.1. Data Collection

The flow cytometer should be set up to enable clear distinction between dead cells, counting beads, and live cells. This is established by first setting up forward scatter (FSC) and side scatter (SSC). This will allow the use of a gate for counting the number of beads analyzed. Next an FSC/PI (FL-3) dot plot should be set up to discriminate between live and dead cells. A region should be set using live cells which can then be applied to an FL-1 histogram to show CFSE peaks.

3.7. LacZ-Inducible T-Cell Hybridoma Assay

LacZ analysis of antigen presentation provides a reliable, rapid, and sensitive approach to probe the antigen-presenting capacity of small numbers of APCs on a single-cell basis. Panels of

lacZ-inducible $CD4^+$ and $CD8^+$ T-cell hybridomas can be generated toward specific antigens using the BWZ.36 fusion partner (14). The *lacZ* T-cell activation assay depends on engagement of the TCR with its cognate peptide resulting in the expression of nuclear factor of activated T cells (NFAT). This in turn drives the transcription of both the endogenous interleukin-2 gene, together with transcription of a heterologous reporter gene (15, 16). TCR induction of *lacZ* activity can be measured using a variety of *lacZ* substrates (17). The method described below allows sensitive and specific enumeration of APCs contributing to an immune response (*see* **Fig. 23.2**). More conventional measurement of absorbance of enzymatic products of lysed cells does not permit the detailed quantification of antigen presentation on a single-cell basis but can provide a broad overview of relative levels of antigen presentation.

1. Titrate LN cells or purified APCs: Add 100 µl of complete DMEM to all wells except the top row of a flat-bottomed 96-well plate. In the top row, add equivalent numbers of each APC subset to be tested to columns in 200 µl complete

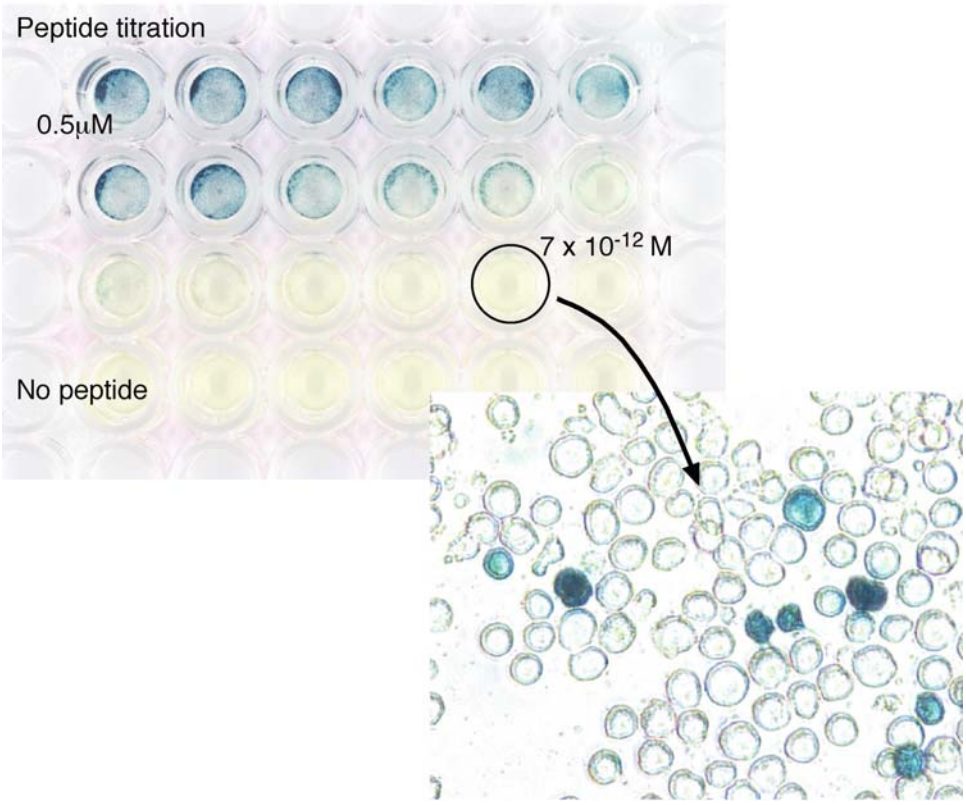

Fig. 23.2. Single-cell analysis of antigen presentation. Coculture of purified APCs/DCs allows fine single-cell probing and quantitation of cells presenting antigen at low levels of peptide/MHC complexes on the surface of the APC.

DMEM (*see* **Note 31**). Perform a twofold dilution down the plate by transferring 100 μl of APCs to the next row of wells and mix thoroughly. Continue this process down the plate until the dilution series is complete. Discard the extra 100 μl of media from the last row containing APCs to leave 100 μl in each well of the plate. Place 100 μl complete media alone in the final row of the plate – to these wells will be added hybridoma cells alone (*see* **Note 32**).

2. Resuspend antigen-specific *lacZ*-hybrids (*see* **Note 33**) at 1×10^6/ml and add 100 μl (1×10^5 hybrids) to each well including rows that contain media alone (no stimulators).
3. Coculture cells for 12–18 h.
4. Wash cultures once with PBS.
5. Fix for 5 min in 100 μl/well fixation (formaldehyde/glutaraldehyde) solution.
6. Wash once with PBS.
7. Add 100 μl X-gal development solution to each well.
8. Incubate at 37°C for 8–12 h.
9. Examine wells microscopically for the presence of blue cells and count blue cell frequency.

3.8. In Vivo Analysis of DC Activation of Antigen-Specific T-Cell Proliferation

Direct ex vivo assessment of the capacity of DC subsets to present endogenously loaded antigens (either by inoculation of animals with proteins or peptides, or by pathogen infection) has provided valuable clues to the specialist capacity of different DC subsets to contribute to an immune response. Translation of this information into the in vivo physiological setting has provided critical steps forward in our understanding of the interplay between DC subsets in the whole animal. Transfer of antigen-specific CFSE-labeled T cells into pathogen-infected mice provides an important method by which to probe in vivo antigen presentation. In general, this approach provides a global approach to evaluating the kinetics of antigen-presenting potential by multiple cell types. In an extension of this assay, an elegant approach to a more detailed analysis of the contributions of individual DC populations in vivo is possible where the lifespan, presence, or antigen-presenting capacity of different DC populations is temporally distinct (*see* **Fig. 23.3**) (18).

1. Infect mice with a pathogen or antigen to allow kinetic analysis of antigen presentation.
2. Prepare CFSE-labeled TCR receptor transgenic T cells specific for the antigen to be probed as described in **Sections 3.3** and **3.4** (*see* **Note 34**).

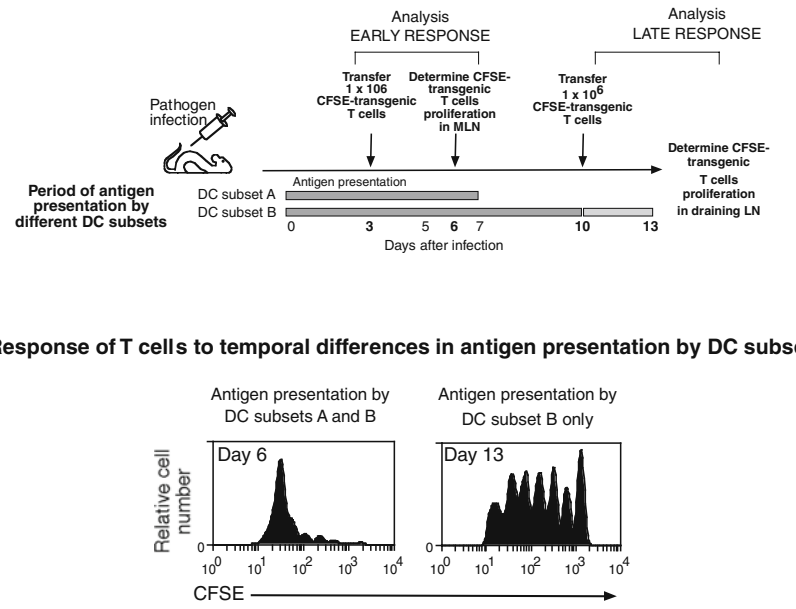

Fig. 23.3. In vivo analysis of the contribution of different DC subsets to an immune response. Differential antigen presentation by DC subsets provides an opportunity to probe their function in the in vivo setting. (**A**) The experimental approach for transfer and assessment of T-cell response to differences in the period of antigen presentation as determined from direct ex vivo analysis. (**B**) Proliferation induced in adoptively transferred T cells localized to the draining LNs 72 h after transfer. The response on day 6 after infection captures antigen presentation by all DC subsets found on day 3, while on day 13 some subsets of DC (e.g., DC subset A) have stopped presenting antigen allowing specific probing of the influence of DC subset B on the immune response. (Adapted from (18).)

3. Adoptively transfer 1–2 $\times$ 10^6 purified CFSE-labeled cells (*see* **Note 35**) via tail vein injection.
4. 60–72 h after transfer, Harvest spleen and draining and non-draining LNs into flow buffer (*see* **Note 36**).
5. Prepare a single-cell suspension of splenocytes or LN cells by gently teasing the tissues apart using forceps.
6. Pellet by centrifugation at 400 $\times$ *g* for 5 min.
7. Remove supernatant by aspiration taking care not to disturb the pellet.
8. Gently lyse red blood cells by adding 1 ml of red cell removal buffer for 1 min.
9. Add 10 ml flow buffer and wash twice.
10. Stain cells with antibodies to congenic marker to allow discrimination from endogenous cells (*see* **Note 37**).
11. Analyze by flow cytometry (*see* **Note 28**).

4. Notes

1. All antibodies are derived from rat, which enables depletion with anti-rat IgG-coated magnetic beads.
2. We use negative selection for non-DC lineage cells using depletion cocktails and immunomagnetic beads to minimize modifications to DC function that could occur from direct antibody binding to ligands on DCs. As a number of common surface markers are shared between DCs and other lymphoid cells, care must be taken to avoid loss of DC subtypes that may express markers more commonly on T cells, B cells, NK cells, and macrophages.
3. Glutaraldehyde solution for microscopy, BDH Laboratory Supplies; 36.5% formaldehyde solution for molecular biology, Sigma.
4. A 10X solution of potassium ferrocyanide/potassium ferricyanide and 100X solution of $MgCl_2$ can be stored at $-20^{\circ}C$ and is stable for long periods of time.
5. Isolation of DCs that maintain their antigen-presenting function depends on accomplishing the isolation process within a 3–4-h period.
6. Removal of residual fat on LNs is critical. The fat is toxic to the cells, will not degrade during digestion, and predisposes cells to clumping and dying. All visible fat should be removed before commencing the digestion process.
7. Individual batches of collagenase should be selected to ensure the absence of trypsin-like proteases that can strip phenotypic markers from the surface of cells and thus altered the apparent phenotype of cells that have been subjected to digestion. To test tryptic activity, thymocytes are incubated in collagenase for 30 min at $37^{\circ}C$ and then tested for any loss of trypsin-sensitive cell surface markers CD4 or CD8 by flow cytometry.
8. Ensuring adequate washing of DCs should be achieved using an EDTA–FCS underlay. Repeated or undue washing of cells will reduce their capacity to present loaded antigen.
9. Recovery of the maximal number of cells is facilitated by use of V-bottomed tubes or wells for centrifugation or analysis.
10. Isolation of DCs where the starting population is very small can be facilitated by adapting the protocol to use 1.5-ml or 0.5-ml eppendorf tubes.

11. Cells should be counted to calculate the optimal amount of mAb cocktail required for depletion. This cell count should include erythrocytes, which will subsequently be depleted by Ter119 antibody.
12. Placing the DCs/APCs on ice for any protracted period of time will reduce antigen-presenting capacity.
13. Reduced volumes for incubation of the magnetic bead/cell slurry optimizes bead/cell contacts allowing for reduced incubation times for binding. Care should be taken to avoid evaporation when small volumes are used.
14. Mixing the bead/cell slurry on an angled rotor (30° angle) is designed to ensure that the mixture remains at the bottom of the tube during the incubation period.
15. Purities are generally greater than 50–70% $CD11c^+$ cells, but even relatively poorly enriched samples (5–10%) greatly facilitate purification of DCs by high-speed sorting. More purified cells can be obtained by incubating antibody-coated cells with beads for longer periods of time. However, increased purity of cells can result in substantial loss of cell number and function prior to sorting. Cells are sorted at 35–55 psi and 6,000–8,000 cells/s on a MoFlo or FACSAria flow cytometric cell sorter using a 100-μm nozzle.
16. A "minimal" purification approach is utilized by omitting strategies to obtain highly purified populations such as a Nycodenz density gradient. Purification over such gradients results in enriched populations of DCs that are more amenable to analysis by flow cytometry, but frequently lose substantial capacity to present endogenously loaded antigens.
17. This approach allows the complete analysis of the populations resident in the LNs without the complications of potentially losing or overlooking cell types that could be important in a response.
18. In general, good numbers of total cells are recovered using this approach – individual LNs ranging in size from 3×10^5 (e.g., pancreatic LN) to $1–2 \times 10^7$ (e.g., mediastinal LNs from virally infected mouse).
19. Total cell numbers can be enumerated by direct counting of a small aliquot of cells (e.g., 10–15 μl) or, alternately, by the addition of $2–3 \times 10^4$ counting beads (Sphero™ Blank Calibration Particles 6.3 μm, BD Biosciences).
20. DCs can be quantified by staining for the expression of the integrin CD11c.

21. An optimum concentration for cell labeling is 1×10^7/ml. Cells at low concentration ($<1 \times 10^7$/ml) require the addition of a protein carrier such as bovine serum albumin or fetal calf serum (2–5%) to buffer the toxic effects of CFSE. For cells at high concentrations (5×10^7/ml), the presence of added protein or culture medium can reduce the extent of CFSE labeling. In such circumstances it is recommended that cells be prepared in PBS without the inclusion of protein.
22. A non-wetted tube is important to ensure that premature mixing of the CFSE concentrate does not occur. CFSE will react rapidly to aqueous solutions, therefore it is important to avoid such exposure until immediately prior to cell labeling.
23. Overlabeling cells with concentrations of CFSE > 0.5 mM can lead to toxicity and reduced proliferation. The optimum concentration of CFSE may need to be determined for each batch prepared to ensure that an effective ideal concentration is used.
24. Optimum labeling of cells is complete in just a few minutes. Increasing the labeling period can result in toxicity to the cells resulting in reduced proliferation.
25. At this point much of the CFSE is not stably incorporated into the cell and some CFSE staining will be lost during the first 24–48 h. Immediately after labeling the fluorescence of the cells will be extremely high and needs to be taken into consideration if flow cytometric analysis is required soon after labeling.
26. The maximal number of APCs is added to the top well in the assay. This number of APCs should be equivalent among the different populations of APCs to be compared. This number may result in subsequent crowding of the first well and reduce the overall proliferation in this well. The optimum range of APCs for good responses is generally from 5,000 to 13,000 APCs but will be dependent on the number of APCs within any purified population that actually carry antigen. Where possible, duplicate wells should be performed. Control T cells cultured without stimulus often survive relatively poorly compared with wells containing control APCs or APCs loaded with antigen.
27. Where possible, APC subsets should be purified from naïve animals and assayed in parallel. This approach controls for the possibility that stimulation could be induced in a non-antigen-specific manner, or self-antigens, expressed by the APCs themselves. In addition, it is important to have CFSE-labeled unstimulated cells together with stimulated

cells as controls. These cells provide a measure of autofluorescence (which is higher in stimulated cells than in unstimulated cells) and provides a measure of non-divided lymphocytes.

28. Freeware allowing analysis of lymphocytes labeled using fluorescent dye technologies (particularly CFSE) is available at: http://www.wehi.edu.au/WEHI_Groups/workgrp/cyton/website/pages/cc.html.
29. The number of beads added to a culture should be in the range of 5–10% minimum of the total events measured by flow cytometry. Beads should be added in a volume of 10–50 μl of PBS. To ensure consistency in the number of beads added a stock of beads should be prepared that can be used for an entire experimental set, and beads should be added with the same pipette and pipette tip to reduce error.
30. To ensure accuracy during data collection, cells and beads must be thoroughly mixed immediately prior to analysis.
31. In a typical experiment, 2×10^6 total LN or spleen cells or 10,000–50,000 purified DCs would be used as the starting population for the dilution series.
32. The inclusion of hybridomas cultured without stimulators or cultured with stimulators expressing an unrelated antigen not specific for triggering the T-cell hybrids provides a baseline background reactivity of the hybrids. This is important as over time background levels can increase and these need to be monitored carefully.
33. Attention to the condition of the hybridomas is critical to ensure they maintain sensitivity and reactivity. Hybrids frequently require subcloning to ensure high-quality cell lines are used. Continual selection and maintenance of reactivity require inclusion of geneticin (G418) and hygromycin in culture media.
34. To ensure precise discrimination of the adoptively transferred T cells from endogenous populations, it is preferable to use congenically marked cells (Ly or Thy antigens) to permit surface staining of samples rather than relying on the level of dilution of CFSE among transferred cells. If the antigenic stimulus is great, significant proliferation of T cells may occur making a proportion of the cells indistinguishable from unlabeled cells.
35. Different numbers of CFSE-labeled cells may be transferred into animals depending on the precise experimental question to be answered. It was found that the transfer of “large” numbers of transgenic T cells resulted in differences in surface phenotype and differentiation fate of the T cell in becoming a memory T cell when com-

pared with the transfer of "small" numbers (1–1,000) of T cells (19). To probe in vivo antigen presentation using CFSE-labeled cells as a sensitive measure of the capacity of expressed antigen to induce proliferation (or "priming") of T cells, ideally $>5 \times 10^5$ cells should be transferred to facilitate later isolation, identification, and analysis of these cells.

36. At ~60 h after adoptive transfer optimal proliferation of $CD8^+$ T cells will be evident only in the regional draining LNs with little or no proliferation in non-draining LNs. By 72 h significant release of proliferating cells from the draining LNs into the circulatory system may have occurred such that the non-draining LNs also harbor a large number of recirculating proliferating cells. TCR transgenic CSFE-labeled $CD4^+$ T cells may take 1–2 days longer to undergo the same level of proliferation. The precise timing of harvest should be determined for each antigen/infection/T cell specificity.
37. In many circumstances the antigenic stimulus will be significant and drive strong proliferation of the transferred T cells. In some instances, however, the stimulus will be weaker making it much more difficult to identify the transferred cells from among the endogenous lymphocytes within the preparation. In these circumstances, it is preferable to enrich the preparations for T cells (*see* **Section 3.3**) prior to staining for congenic surface markers and analysis by flow cytometry.

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Chapter 24

Methods to Study Pulmonary Dendritic Cell Migration

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Abstract

Dendritic cell migration from the airway to the lymph nodes is a key event in the development of airway immunity during infection, allergy, and vaccination. With judicial selection of materials, there are two approaches to study dendritic cell migration to the mediastinal lymph nodes without the induction of inflammation: airway administration of fluorescent OVA and latex beads. Our protocol describes how to label and track pulmonary dendritic cells from the airway and lung to the mediastinal lymph nodes and reveals how to avoid pitfalls and suboptimal assays.

Key words: Lymphatic, lung, mediastinal lymph node (MLN), dendritic cell (DC), pulmonary.

Abbreviations: BAL, bronchoalveolar lavage; DC, dendritic cell; MLN, mediastinal lymph node; IN, Intranasal; IT, Intratracheal.

1. Introduction[1]

Pulmonary dendritic cells (DCs) acquire antigen in the airway and lung and then migrate through lymphatic vessels to the mediastinal lymph node (MLN), where they present antigen and stimulate an immune response (1, 2). Because the airway frequently encounters foreign substances, the antigen-presenting role of DCs is critical for the maintenance of pulmonary health. Ideally, harmless antigens induce tolerance, whereas harmful antigens promote effector immune responses (3). One component

[1]Most of the introduction was reprinted from ***J Immunol Methods***. Sep 15;337(2):121–31, Jakubzick C, Helft J, Kaplan TJ, and Randolph GJ, Optimization of methods to study pulmonary DC migration reveals distinct capacities of DC subsets to acquire soluble versus particulate antigen. 2008 with permission from Elsevier.

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that influences tolerance versus effector responses is the state of DC maturation (4). In addition, other cellular mechanisms affect the immunological outcome in response to airway antigens (5).

Besides a small number of plasmacytoid DCs, there are two major DC subsets found in the alveolar space and lung parenchyma: $CD11c^{+}CD11b^{lo}CD103^{+}$ ($CD103^{+}$DC) DC and $CD11c^{+}CD11b^{hi}CD103^{-}$ ($CD11b^{hi}$ DC) DC (6, 7). Recent studies are beginning to shed light on their functional roles. Suggesting that the two populations possess markedly distinct functional roles, one group found that $CD103^{+}$ DCs nearly exclusively promote the proliferation of naïve $CD8^{+}$ T cells (8, 9), whereas $CD11b^{hi}$ DCs preferentially seem to induce proliferation of $CD4^{+}$ T cells (8, 10). $CD11b^{hi}$ DCs secrete a substantial number of chemokines during homeostatic and inflammatory conditions, whereas $CD103^{+}$ DCs mainly secrete chemokines associated with Th2 responses (11). Furthermore, the distribution of these two cell subtypes differs across the pulmonary parenchyma. $CD103^{+}$ DCs are located along the mucosal lining and vascular wall, while $CD11b^{hi}$DCs are mainly within perivascular regions (12). $CD103^{+}$ DCs express mRNA for langerin, produce IL-12 upon TLR stimulation, and express tight junction proteins that help them traverse the epithelium and acquire antigen (12).

There are three published labeling methods to track DC migration from the lungs to the draining lymph nodes: airway administration of carboxyfluorescein diacetate succinimidyl ester (CFSE), OVA-FITC, or latex particles (10, 13–16). However, in one of those studies, a qualitative and quantitative comparison of these approaches was performed (10). The three methods label DCs in a completely different manner. Since it was found that CFSE labels cells non-selectively, as it spontaneously penetrates cell membranes and irreversibly couples to cellular proteins and induces extreme inflammation and injury to the airways and lung, this method will not be discussed (10). OVA-FITC is a soluble protein taken up by pinocytosis, and fluorescent latex particles (0.5 μm diameter) are taken up by macropinocytosis or phagocytosis. The advantageous and disadvantageous features of each labeling method are discussed in Jakubzick et al. (10).

2. Materials

2.1. Anesthesia Solution

1. Avertin stock solution: Mix 15.5 ml *tert*-amyl alcohol (Sigma) in a dark bottle containing 25 g of 2, 2, 2-tribromoethanol (TCI America) at room temperature overnight. The stock solution is kept at room temperature, and it can be stored for up to 6 months in a dark bottle.

2. 2X Avertin: Mix 1 ml of the avertin stock solution into 39.5 ml of PBS overnight at room temperature. Filter the solution using a 40–70-μm strainer. Protect the solution from light exposure. The light-sensitive 2X avertin solution can be stored for up to 4 months at 4°C.

2.2. Commercial Tracers

Perform the following steps under sterile conditions.

1. Dilute OVA-FITC (Molecular Probes) with PBS to 5 mg/ml in its original vial (1). Then make 100 μl aliquots and store at –20°C. Purchased OVA-FITC from commercial suppliers might contain contaminates so consider using the protocol outlined in Section **2.3** (10).
2. Make fresh fluorescent latex particles on the same day as tracer delivery. Plain yellow-green fluorescent 0.5-μm latex particles (Polysciences) are diluted 1:25 in PBS (15), for a final administration of approximately 3.64×10^8 particles per mouse.

2.3. Contamination-Free OVA-FITC Tracer

1. Germ-free OVA is extracted from chicken eggs (4).
2. Sterile OVA is then conjugated to FITC using the Fluo-Reporter FITC protein labeling kit from Invitrogen as per manufacturer's instructions.

2.4. Labeling DCs in Mice

Take the following items to the animal facility.

1. Several 1-ml syringes containing a 26-ga needle for avertin.
2. 2X Avertin solution.
3. Enough tracers for a final volume of 30 μl per mouse. Bring approximately 10–15% more volume than calculated to account for volume loss during delivery.
4. A 200-μl tip box and 20–200-μl pipette.
5. Blunt-curved forceps.

2.5. Single-Cell Suspension of BAL, Lung, and Lung-Draining Lymph Node

1. Need: Scissors, blunt-curve and fine-point straight-tip forceps, 18-ga needle, 3-ml syringe, 30-ml syringe, 35 mm × 10 mm round culture dishes, 26-ga needles, 1-ml syringe, case of glass Pasteur pipettes, and a rubber bulb.
2. Buffers: 1X × PBS without calcium and magnesium, 1X Hank's Buffered Salt Solution (HBSS) without calcium and magnesium. Buffers must be ordered without calcium and magnesium.
3. A stock solution of 0.5 M EDTA, pH 8.0.
4. 100 mM EDTA, pH 8.0, stored at room temperature.
5. EDTA-pretreated fetal calf serum (FCS): In 10 ml FCS add EDTA to a final concentration of 0.02 mM EDTA. Then make 1 ml aliquots and store at −20°C.

6. Hank's buffered salt solution complete buffer (HBSS complete): To a 500-ml bottle of HBSS without calcium and magnesium add 1 ml 30% BSA (e.g., from Sigma) and 300 μl 0.5 M EDTA.
7. FACS blocking and staining solution: To a 15-ml conical tube add 5 ml PBS, 5 ml HBSS complete, 100 μl normal mouse serum, 100 μl normal rabbit serum, 100 μl normal human serum, 333 μl 30% BSA (*see* **Note 8**).
8. For tissue digestion: Make a 10X stock solution of collagenase D in RPMI. Aliquot 1–2 ml stock solutions and store at –20°C. When needed make fresh 1X collagenase D in HBSS without calcium and magnesium and add 1% pretreated FCS with 0.02 mM EDTA.

2.6. FACS Staining for Single-Cell Suspension of BAL, Lung, and Lung-Draining Lymph Node

1. The following antibody clones that are demonstrated to be specific are purchased from eBioscience and BD Pharmingen: PE-conjugated mAbs to CD103, CD8, MHC-II-IA/IE (clone M5/114.15.2), or B220; PerCP-conjugated mAb to Gr-1 (recognizes Ly-6C and Ly-6G) or CD11b; and APC-conjugated mAb to CD11c and MHC-II-IA/IE (clone M5/114.15.2). Conjugated isotype-matched control mAbs were also obtained from eBioscience or BD Pharmingen (*see* **Note 9**).
2. HBSS complete and FACS blocking and staining solution (as in **Section 2.5**).
3. Centrifuge and FACS machine.

3. Method[2]

3.1. Anesthetization of Mouse

1. Average-sized 7–8-week-old mouse should be completely anesthetized by injecting i.p. 300 μl of 2X avertin per mouse. Do not anesthetize more than five mice at any given time until the tracer delivery has been performed (*see* **Note 1**).
2. Once the mouse is no longer moving in its cage, make sure to remove the mouse away from the bedding and onto a pad or paper towel.

[2] A portion of the methods was reprinted from *J Immunol Methods*. Sep 15;337(2):121–31, Jakubzick C, Helft J, Kaplan TJ, and Randolph GJ, Optimization of methods to study pulmonary DC migration reveals distinct capacities of DC subsets to acquire soluble versus particulate antigen. 2008 with permission from Elsevier.

3. After 5 min check that the mouse is fully anesthetized by pinching its feet (*see* **Note 2**). If the mouse shakes avidly more avertin will be required. Only add 50–100 μl of 2X avertin and wait for 5 min for the avertin to take effect (*see* **Notes 3 and 4**).

3.2. Labeling Pulmonary Dendritic Cells

1. Shortly after full anesthetization, hold the mouse in a vertical position perpendicular to the bench.
2. Pull the tongue out with blunted forceps and hold it with the thumb and the middle finger without obstructing the nasal cavity.
3. While holding the tongue, place 30 μl of labeling solution on top of the nasal opening, which should be facing up toward the ceiling. Make sure to observe full inhalation of the tracer.
4. Continue to hold the mouse's tongue in the vertical position for an additional 30 s post-delivery to ensure that the tracer is inhaled by the lungs and not swallowed (*see* **Note 5**).
5. Lay the mouse to rest on top of the cage frame in a prone position. This ensures that the resting mouse has a clear breathing space between the cage bars.
6. After 20 min of observation during recovery from anesthesia, the mouse can be returned to its cage and placed on a paper towel to prevent bedding inhalation and suffocation.

3.3. Single-Cell Suspension of Airway Cells – Bronchoalveolar Lavage (BAL)

1. Sacrifice the mouse by putting it in a CO_2 chamber and following a standard protocol prescribed by your institution.
2. Immediately after sacrifice, obtain single-cell suspensions of the bronchoalveolar space by exposing the mouse's trachea. To expose the trachea, first cut-off the tracheal skin area. Under the skin, there are two large masses of tissue. Gently separate the tissue with forceps (do not cut). Then the trachea will be easily seen and exposed. Grab the outer thick membrane covering the trachea with forceps and cut away the membrane and ligaments to expose the cartilage rings of the trachea.
3. Position the mouse upright and insert one side of a blunt forceps behind the trachea.
4. Through the largest upper-cartilage ring, insert an 18-ga needle, beveled-side opening facing you, attached to a 3-ml syringe containing 1 ml of 0.5 mM EDTA/HBSS. Do not insert the needle deep into the trachea. Only insert it deep enough to sufficiently cover the needle opening. After needle insertion, clamp down on the needle with the other side of the blunt forceps to hold the needle in place (*see* **Note 6**).

5. Flush the airway four times with 1 ml of 0.5 mM EDTA/HBSS. Do not add more volume to the syringe (*see* **Note 7**).
6. Collect flushed cells in a 15-ml conical tube and spin at 1,200 rpm for 5 min at 4°C.
7. Aspirate buffer, place on ice, and add FACS blocking and staining solution for 10 min or until ready to stain with specific antibodies for FACS analysis.

3.4. Single-Cell Suspension of Lung and Lymph Nodes

1. To remove the lungs, place the mouse in a supine position and cut open the thoracic cavity of the lung along the lower part of the rib cage, then cut up along the rib cage toward the axillary region of the mouse. The mouse should not be bleeding.
2. Pull back the sternum with blunt forceps and locate the largest mediastinal lymph node (MLN) under the right side of the heart below the thymus. A small blood vessel perpendicular to the trachea and superior vena cava indicates the location of the large MLN, which is slightly below this small blood vessel. There are also two very small lymph nodes slightly above the blood vessel, which are easily seen in an inflamed mouse but not in a steady-state mouse. Since the left-side MLNs are very difficult to find, even during inflammation, only extract the right side of the MLNs for consistency and proper data analysis. Place the MLN in a 35-mm culture dish containing 1 ml collagenase D (alternatively use 1.75 mg/ml Liberase Blendzyme 3 in RPMI for 15 min at 37 C) for digestion. Keep on ice till all samples have been collected.
3. Expose and perfuse the heart with 1X PBS. Lungs should be perfused with PBS via the heart to remove blood cells. During perfusion, lungs will turn white. Remove lobes individually and place them in a 35-mm culture dish containing 1 ml collagenase D for digestion. Keep on ice till all samples have been collected.
4. Digestion: For the lungs, cut lungs into very tiny pieces with scissors. For MLN, tease each sample excessively with two 26-ga needles attached to 1-ml syringes. To tease, hold down the lymph node with one needle while breaking open the lymph node with the other needle. When teasing is done correctly, concentrated cells bursting from the lymph node are easily observed in the media.
5. Place minced and teased cells in the incubator for 30 min at 37°C.
6. Following digestion, add 100 μl of 100 mM EDTA for 5 min at 37°C.

7. Place culture dishes on ice and filter cells through a 70-μm nylon filter (*see* **Note 10**) by gently mashing the tissue through with the plunger portion of a 3-ml syringe. Collect cells with a glass Pasteur pipette and a rubber bulb into a 15-ml conical tube. Wash over mashed cells 2X with 2 ml HBSS and collect into the same 15-ml conical tube.
8. Spin cells at 1,200 rpm for 5 min at 4°C.
9. Aspirate supernatant up to 200 μl volume from the cells. Make sure not to aspirate up to the cells because cells could be aspirated away (*see* **Note 11**).

3.5. Stain Single-Cell Suspension of Airway, Lung, and Lymph Nodes for FACS

1. After aspiration, add 50 μl of FACS blocking and staining solution to all samples, mix, and leave for 10 min on ice.
2. Make an antibody master mix for FACS staining. Use approximately 1 μl of antibody per sample in a final volume of 100 μl (exception: the MHC-II (IA/IE) antibody is very strong, use 0.2 μl). Thus, if there are five samples that require the same stain, add 5 μl per antibody of interest in 500 μl HBSS complete. Then add 100 μl of the antibody mix onto the sample. The best two combinations used – CD11c-APC with CD11b-PERCP and CD11c-PECy7 with CD11b-PERCP – easily separate macrophages from DCs. Both combination stains allow for the separation

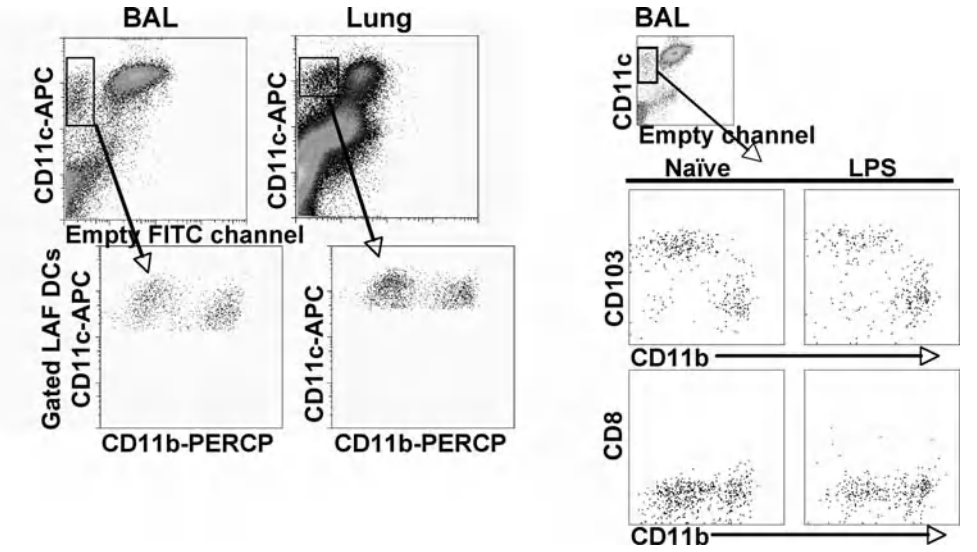

Fig. 24.1. BAL and lung DCs identified using autofluorescence. $CD11c^+$ BAL and lung DCs were gated by low autofluorescence (LAF) (macrophages are the $CD11c^+$ highly autofluorescent cells) by plotting CD11c versus an empty FITC fluorescent channel, and then the DCs were stained for CD11b to illustrate the two DC subsets (*right panel*). BAL DCs were stained for CD103 or CD8 versus CD11b in naïve and mice treated with 1 μg LPS to (*left panel*) illustrate the two DC subsets: $CD11c^{hi}CD11b^{lo}CD103^+$and $CD11c^{hi}CD11b^{hi}CD103^-$ DCs. The left panels of this figure were reprinted from Jakubzick et al. (10). Optimization of methods to study pulmonary DC migration reveals distinct capacities of DC subsets to acquire soluble versus particulate antigen; 2008, with permission from Elsevier.

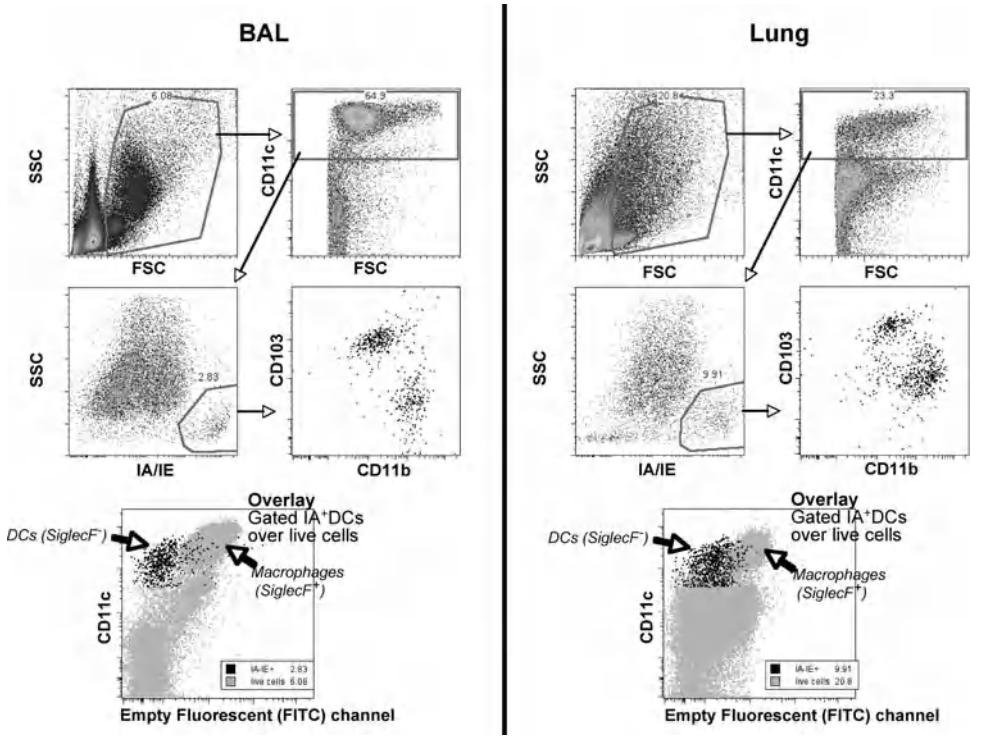

Fig. 24.2. BAL and lung DCs identified by MHC-II. CD11c$^+$ BAL and lung DCs were gated by using MHC-II expression on low SSC cells. To gate DCs with an MHC-II stain, gated live cells were plotted as CD11c versus FSC. The CD11c+ cells were then plotted as SSC versus MHC-II. DCs are low SSC, MHC-II$^+$ cells. To make sure both DC subsets were present, the low SSC MHC-II$^+$CD11c$^+$ cells were plotted as CD103 versus CD11b. Bottom plots are an overlay of gated low SSC MHC-II$^+$CD11c$^+$ DCs (*black*) on top of the gated live cells (*gray*). The overlay was performed to show that the gated low SSC MHC-II$^+$CD11c$^+$ DCs (*black*) have low autofluorescence compared to macrophages found in the total live cell gate.

of autofluorescent macrophages from DCs. In addition, it is necessary to leave open a "free" channel, in this case the FITC channel (or PE channel, use the CD11c-APC with CD11b-PERCP stain), as doing so allows one to clearly gate on low autofluorescent DCs or high autofluorescent macrophages (*see* **Note 9**) (**Fig. 24.1**). Both combination stains work well when gating on lung and BAL DCs by low side-scatter and by the expression of CD11c and MHC-II (*see* below **Section 3.5**, Item 3). In addition, both stains identify migratory DCs in the MLN, since there are no highly autofluorescent cells present in the MLN (**Fig. 24.3**). It is best to use already conjugated antibodies since airway and lung cells are "sticky." Attempts should be made to avoid secondary antibodies, particularly those using biotin–streptavidin, in the lung. Make sure to run both positive and negative single-stain antibody controls for all the samples.

3. There are two ways to find DCs in the airways and the lungs. One is by autofluorescence, leaving the FITC channel empty (**Fig. 24.1**), and the other is with MHC-II and low

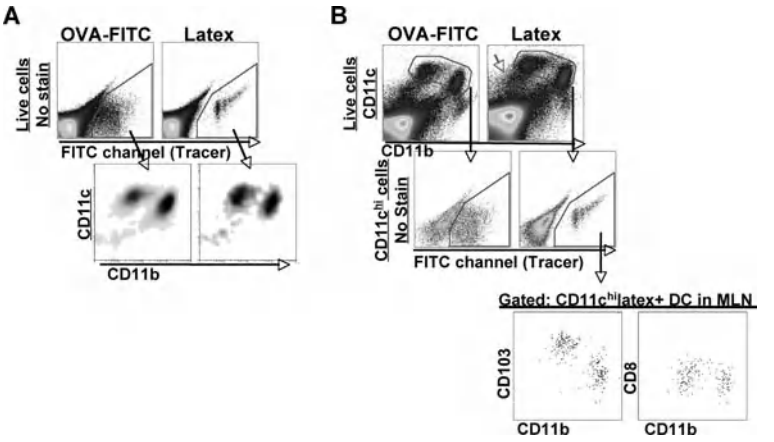

Fig. 24.3. Two ways to gate on MLN migratory tracer$^+$ DCs. (**A**) Gated live cells from MLN were plotted as an empty channel versus FITC (channel that detects the tracer) and all tracer$^+$ cells were gated. Then, gated MLN tracer$^+$ cells were plotted as CD11c versus CD11b (same DC subsets found in the upstream tissue). (**B**) All live cells are plotted as CD11c versus CD11b and then all CD11c^{hi} cells in the MLN were gated, two main subsets are observed: CD11c^{hi}CD11b^{lo}and CD11c^{hi}CD11b^{hi} DCs. Within these subsets, tracer$^+$ migratory DCs were extracted. All gated CD11c^{hi} cells were then plotted as an empty channel versus tracer and all tracer$^+$ cells were gated on (these are tracer$^+$ migrating pulmonary DCs). Small gray arrow in the latex method top panel represents the CD11c^{int}CD11b$^-$cells, where the majority of these cells are B220$^+$ plasmacytoid DCs. To show upstream specificity, the gated latex particle$^+$ DCs were stained for CD103 or CD8 versus CD11b. The latex$^+$ DCs had the same phenotype as the DCs found in the upstream tissue. These mice were treated with 1 μg LPS to enhance DC migration. This figure was reprinted from Jakubzick et al. (10). Optimization of methods to study pulmonary DC migration reveals distinct capacities of DC subsets to acquire soluble versus particulate antigen; 2008, with permission from Elsevier.

side-scatter (SSC) (**Fig. 24.2**). To gate DCs by the MHC-II stain, perform the following steps in order: (1) gate on live cells, (2) gate on CD11c$^+$ cells, (3) take the CD11c$^+$ cells and plot as SSC versus MHC-II, and (4) gate on MHC-II$^+$, low SSC cells (adding SiglecF antibody to this mix definitively removes the SiglecF+ macrophages from the SigelF- DCs) (12). Check this gating strategy by plotting the DCs as CD103 versus CD11b (**Fig. 24.2**). There should be two DC populations, as seen in **Figs 24.1** and **24.2**. In the two gating strategies, DC gating by autofluorescence works well under steady-state or inflammatory conditions. When gating DCs using MHC-II, under steady-state conditions mainly DCs are MHC-II positive. However, during inflammation, in addition to DCs SSC^{hi} macrophages also express MHC-II and CD11b (pulmonary macrophages normally do not express these markers under steady-state conditions) (17) so be aware of this and make sure to gate MHC-II$^+$ low SSC cells and then plot these cells as CD103 versus

CD11b, any cell population in between these two subsets is most likely macrophages.

4. In the MLN, there are two clusters of DCs: migratory DCs that come from the upstream tissue and resident DCs that are not found in the upstream tissue (10, 18). Tracer$^+$ DCs in the MLN could be gated on if the FITC and PE channels are not used (**Fig. 24.3**). It is imperative not to use the PE channel so that the migratory tracer$^+$ DCs do not scatter throughout the gates.

4. Notes

1. It is best not to anesthetize more than five mice at any given time because precise tracer delivery and respiratory uptake works best in mice that are recently anesthetized. If the tracer is not delivered in all five mice within 20 min post-anesthesia then reduce the number of mice per working session.
2. A fully anesthetized mouse should not be moving. It is important that the feet do not shake when the footpad is pinched. There is a fine line between fully anesthetized and over-anesthetized. Make sure not to over-anesthetize the mouse. However, it is important to avoid under-anesthetizing the mice to avoid having mice that swallow tracer, which in turn will lead to uneven delivery of tracers among the cohort of mice.
3. It is not recommended to inject avertin more than two times (although it is best to inject only once) because over-anesthetization will lead to mouse death due to drowning after tracer inhalation.
4. If 300–400 μl of 2X avertin does not fully anesthetize a 7–8-week-old ~20 g mouse, then re-make the 2X avertin solution. In addition, older and larger mice will require more avertin to reach a fully anesthetized state; therefore, it is best to use 400–500 μl instead of 300–400 μl during the first injection.
5. While holding the mouse for 30 more seconds post-intranasal delivery of tracer, make sure that the chest cavity is rapidly expanding and contracting (faster than normal). This movement signifies that the solution entered the lungs appropriately. If the lungs are not moving rapidly, then the mouse is over-anesthetized. If the mouse can pull back its tongue from your fingers, then the mouse is not anesthetized enough.

6. If the needle appears to be suctioned, rotate the tip a bit to release suction or slightly expose needle opening to the air to release suction.
7. Never place more than 1 ml of 0.5 mM EDTA/HBSS (or PBS) into the airways. Excess fluid forced in the airways will cause lung injury and bleeding will occur. Fluid extracted from airways should not contain red blood cells. If this happens, there are mainly two reasons: (1) the bronchoalveolar lavage was not extracted immediately after mouse sacrifice or (2) fluid is being injected into the mouse too aggressively. It is absolutely necessary to include EDTA in the buffers because it helps to detach macrophages and DCs along the surface of the airways.
8. If during the staining procedure the secondary antibody is against one of the non-specific serums added in FACS staining solution, then remove that serum from the FACS staining solution. Otherwise, this would lead to false-positive staining and extensive background signal.
9. Other color combinations of these antibody clones could be used in the lung and BAL. Do not use CD11c-FITC or CD11c-PE to stain in the lung and BAL if DCs will be gated on by autofluorescence.
10. Do not use a 40-μm nylon filter or any filter smaller than 70 μm, because DCs may not easily pass through a filter that is too fine. Hence, a 40-μm nylon filter may result in reduced recovery of DCs for FACS analysis.
11. Lung cells and sometimes lymph nodes should be re-filtered through a 70-μm or 100-μm nylon filter to remove clumped cells that could clog FACS machine.

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Chapter 25

Measuring pH, ROS Production, Maturation, and Degradation in Dendritic Cell Phagosomes Using Cytofluorometry-Based Assays

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Abstract

Phagosomes are complex organelles that form after ingestion by phagocytic cells of pathogens, dying cells, or cell debris. Highly dynamic interactions of phagosomes first with endosomes and then with lysosomes lead to the maturation of phagosomes into phagolysosomes. Contrary to other phagocytes, which degrade ingested particles to amino acids, dendritic cells only partially degrade ingested proteins, preserving short peptides for the onset of adaptive immune responses. We have modified a series of latex bead-based techniques, previously reported, in order to analyze phagosome maturation using flow cytometry. The analysis of the phagosomal pH, degradation, or oxidation relies on techniques based on the fate of specific probes bound to particles to be phagocytosed. These techniques are very sensitive and quantitative.

Key words: Dendritic cell phagosome, phagosome maturation, phagosomal pH, phagosomal degradation, phagosomal ROS generation.

1. Introduction

Phagocytosis represents both one of the earliest responses of the innate immune system against pathogens and a critical process for the initiation of adaptive immune responses. While neutrophils and macrophages use phagocytosis to eliminate invading microorganisms, dendritic cells (DCs) use it to generate antigenic determinants to be loaded onto MHC molecules and presented to T lymphocytes for activation. According to these different cellu-

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lar functions, phagosomal functions are differentially regulated in the different types of phagocytes, leading to diverse fates for the ingested particles. While neutrophils and macrophages display a variety of strategies aimed to destroy the capture particles, DCs seem to protect the phagosome contents from complete destruction, optimizing the generation of peptides that can be loaded onto MHC molecules. NOX2 activation (1, 2), inefficient assembly of the V-ATPase (3), the presence of phagosome protease inhibitors (4, 5), all concur to maintain an alkaline pH and a low level of protease activity in DC phagosomes (1, 2, 6) .

A lot of what we know about the regulation of macrophage and DC phagosomes was found using latex beads as model particles for phagocytosis. Latex beads have been widely used both to purify phagosomes (using their unique very low density) and to monitor phagosomal functions (after modifying the particles with different probes). It is also possible to bind certain ligands to the beads, in order to analyze phagocytosis through different receptors; we will not describe these approaches herein. In this review, we will focus on a series of techniques that we have developed or adapted from previous publications. These techniques are based on the use of fluorescence-activated cell sorting (FACS) to measure phagosomal functions. The FACS analysis is performed either on cells that have phagocytosed beads bound to different phagocytosis probes or on isolated phagosomes, after disruption of the cells (**Fig. 25.1**). In the former case, it is possible to analyze phagosomal functions on subpopulations of cells, after labeling the cells with specific markers. In the latter case, antibodies specific to proteins exposed on the cytosolic side of phagosomes are detected using specific antibodies. It is also possible to use antibodies directed to a protein bound to the beads, after permeabilization of the phagosomal membrane. These FACS-based techniques proved to be extremely sensitive and reliable: thousands of events (cells or phagosomes) can be monitored independently and quantitatively. We could thus analyze the pH and the production of ROS in DC phagosomes. The use of FACS allows the labeling of specific cell subpopulations and the measurement of the pH or oxidation selectively in those cells (**Fig. 25.1**). These FACS-based techniques also allow the analysis of the maturation of phagosomes and protein degradation. In this case, the FACS analysis is performed on phagosomes and not on cells, which allows the simultaneous analysis of different parameters on individual organelles (**Fig. 25.1**).

2. Materials

2.1. Phagosomal pH

1. Polybead amino 3.0 μm microspheres (Polysciences, 9003-53-6).

2. Fluorescein isothiocyanate (FITC) is dissolved at 50 mg/ml in dimethyl sulfoxide (DMSO) and stored in single-use aliquots at −20°C.
3. FluoProbes 647, SE (Fluo Probes, FP-AK7740) is dissolved at 1 mg/ml in DMSO and stored in single-use aliquots at −20°C.
4. CO_2-independent medium.
5. Conditioned-complete medium: Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin–100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol and granulocyte-macrophage colony stimulating factor-containing medium as previously described (7).
6. Triton X-100: Prepare 10% in PBS and store at room temperature.

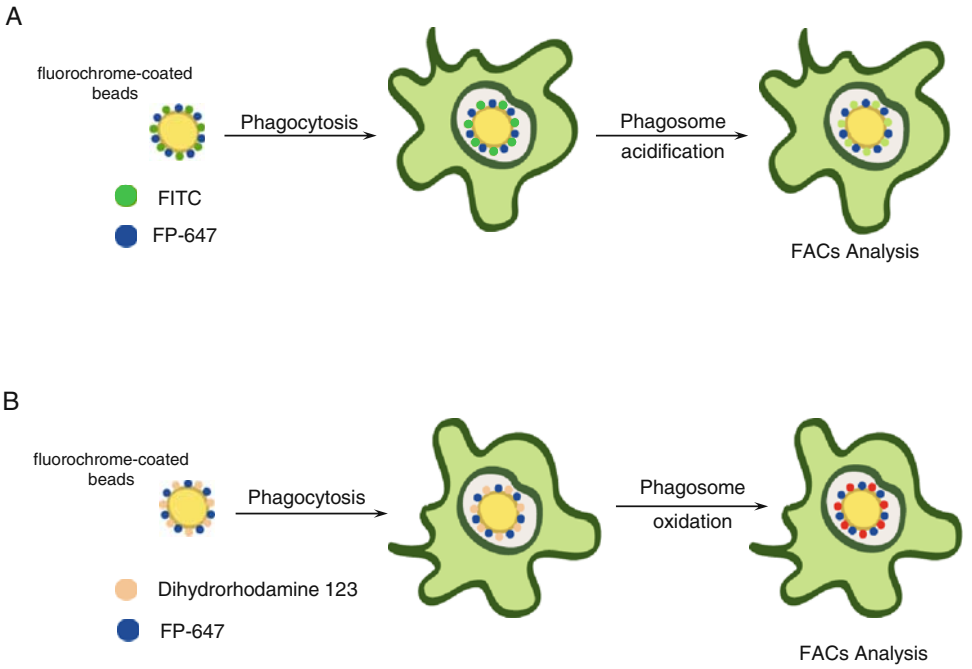

Fig. 25.1. FACS-based determination of phagosomal functions. Fluorochrome-coated beads for pH (**A**) or oxidation (**B**) measurements are internalized by DCs. After different time of phagocytosis, cells are analyzed by cytofluorometry to detect any change in the fluorescence of FITC in **A** or of DHR in **B**. DCs are allowed to phagocytose OVA-coated beads. Lysis with detergents (**C**) causes the rupture of both plasma and phagosomal membranes allowing the isolation of beads. Remaining non-degraded OVA is detected on beads with a specific antibody and analyzed by FACS. Contrary to lysis with detergents, mechanic disruption of cells after phagocytosis (**D**) permits to conserve the membranes of phagosomes. After isolation several membrane markers can be detected by FACS using specific antibodies.

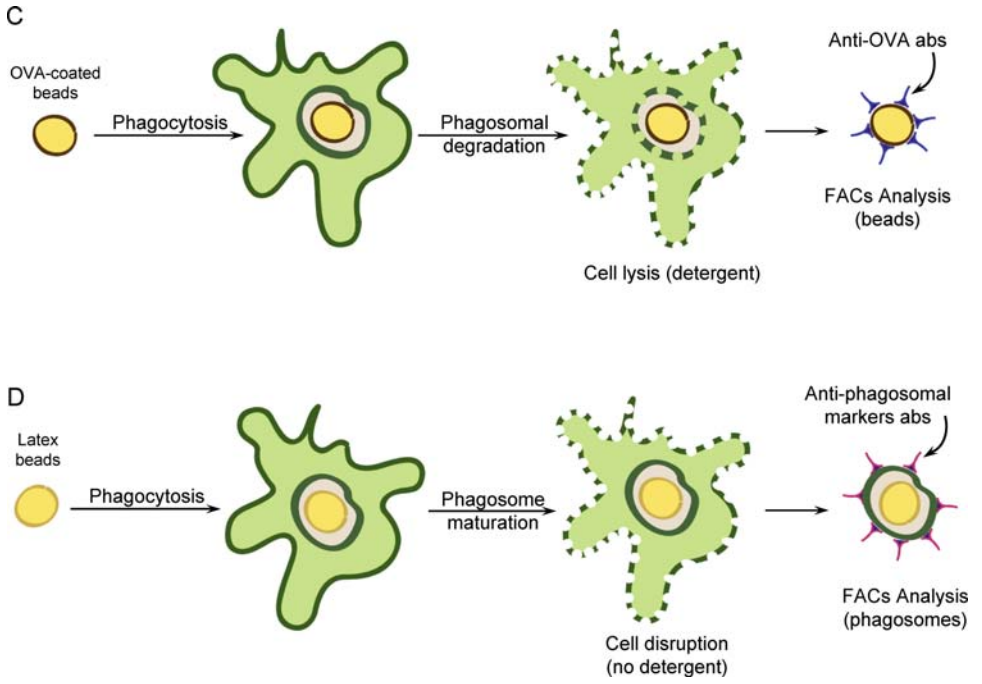

Fig. 25.1. (continued)

2.2. Oxidative Capacity of Phagosomes

1. Polybead amino 3.0 μM microspheres (Polysciences).
2. Dihydrorhodamine 123 (Fluo Probes) is dissolved at 20 mg/ml in DMSO and stored in single-use aliquots at −80°C.
3. FluoProbes 647, SE is dissolved at 1 mg/ml in DMSO and stored in single-use aliquots at −20°C.
4. CO_2-independent medium.
5. Conditioned-complete medium: Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine (GIBCO), 100 U/ml penicillin–100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol and granulocyte-macrophage colony stimulating factor-containing medium as previously described (7).
6. Diphenyleneiodonium chloride: Prepared at 10 mM in DMSO and stored in single-use aliquots at −20°C.

2.3. Proteolytic Activity of Phagosomes

1. Polybead amino 3.0 μm microspheres.
2. Glutaraldehyde 8% in PBS, stored in the dark at room temperature.
3. Ovalbumin (OVA, Worthington, LS003049) at 10 mg/ml in PBS, stored in single-use aliquots at −20°C.
4. Polyclonal anti-OVA antibody (Sigma, C 6534).

5. Anti-rabbit antibody labeled with a fluorochrome adapted to flow cytometry.
6. Conditioned-complete medium: Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin–100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol and granulocyte-macrophage colony stimulating factor-containing medium as previously described (7).
7. Cell lysis buffer: 50 mM Trizma base (buffered at pH 7.4 by addition of HCl), 150 mM NaCl, 0.5% NP40, 1 mM dithiothreitol (DTT), 10 μg/ml DNase I, and a cocktail of protease inhibitors (Roche).
8. Cell washing buffer: 50 mM Trizma base (buffered at pH 7.4 by addition of HCl), 150 mM NaCl, 0.5% NP40.
9. Staining buffer: 1% BSA in PBS.

2.4. Membrane Protein Content of Phagosomes

1. 3.0 μm Polybead amino microspheres.
2. Glutaraldehyde 8% in PBS, stored in the dark at room temperature.
3. Paraformaldehyde (PFA) 2% in PBS, stored in single-use aliquots at −20°C.
4. Glycine 0.5 M in PBS.
5. Ovalbumin at 10 mg/ml in PBS, stored in single-use aliquots at −20°C.
6. Conditioned-complete medium: Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin–100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol and granulocyte-macrophage colony stimulating factor-containing medium as previously described (7).
7. 2-ml Syringes and 25-ga needles.
8. Homogenization buffer: 8% sucrose in PBS, 3 mM imidazole, 1 mM dithiothreitol (DTT).
9. Staining buffer: 1% BSA in PBS.
10. Polyclonal anti-OVA antibody.
11. Anti-LAMP-2 antibody (BD Biosciences).

3. Methods

Flow cytofluorometry is a powerful quantitative approach commonly used to quantify fluorescent signals from single cells contained in mixed populations. It was recently extended to explore

the properties and functions of DC phagosomes, including the regulation of the luminal pH, oxidative and proteolytic activities, and the recruitment of membrane proteins. The ionization equilibrium of certain fluorochromes leads to changes in the pH-dependent absorption and emission over determined ranges of proton concentrations. Such fluorochromes can be used as pH indicators. This is the case of fluorescein isothiocyanate, a fluorescein derivative that displays high photo-stability and low temperature-dependent fluorescence variation. In addition, technological manipulation of some fluorophores led to the generation of non-fluorescent molecules such as dihydrorhodamine 123, which becomes fluorescent only upon oxidation and can thus be used to measure the generation of ROS in living cells. Both types of probes can be coated on latex beads to provide specific information about the pH and oxidative state of the phagosomal lumen. Coupling of the probes to the beads must be efficient and stable, since leakage of the probe outside phagosomes would generate erroneous measurements. The molecular structure of the fluorescent dyes is generally reactive toward nucleophiles, including amine groups, making it possible to couple the probes to proteins or to latex beads bearing primary amines (called here as amino beads). Additionally, a succinimidyl-ester functional group present in the main core of the dyes creates another common amine reactive derivative that has much greater specificity toward primary amines in the presence of other nucleophiles.

3.1. Phagosomal pH

3.1.1. Preparation of Fluorochrome-Coated Beads for pH Measurement

1. Place 50 μl of a 2.59% aqueous suspension of 3 μm amino beads in an eppendorf centrifuge tube (1.5 ml capacity).
2. Add 0.5 ml of sterile PBS and cap tightly.
3. Centrifuge for 2 min in a microcentrifuge at 15,000 rpm. Remove supernatant carefully using a Pasteur pipette or a vacuum pump. Discard the supernatant.
4. Resuspend the pellet in 400 μl of sodium hydrogen carbonate 0.1 M buffered at pH 8.5. Vortex until the pellet is completely dispersed.
5. Add 50 μl of a 50 mg/ml fluorescein isothiocyanate (FITC) solution plus 50 μl of a 1 mg/ml of FluoProbes 647 (FP647) solution to the bead solution. Cap tightly and cover the tube with aluminum to protect its content from light.
6. Leave on a rotator for at least 2 h at room temperature with continuous rotation.
7. Centrifuge for 2 min in a microcentrifuge at 15,000 rpm. Remove and discard the supernatant.
8. Resuspend the pellet in 0.5 ml of 0.1 M glycine in PBS. Vortex until the pellet is dispersed. Add 0.5 ml

of 0.1 M glycine in PBS to obtain a total volume of 1 ml.

9. Centrifuge for 2 min in a microcentrifuge at 15,000 rpm. Remove supernatant carefully using a Pasteur pipette or a vacuum pump. Discard the supernatant.
10. Repeat **Steps 8** and **9** once.
11. Resuspend the pellet in PBS. Vortex until the pellet is dispersed. Add 0.5 ml of PBS to obtain a total volume of 1 ml.
12. Centrifuge for 2 min in a microcentrifuge at 15,000 rpm. Remove supernatant carefully using a Pasteur pipette or a vacuum pump. Discard the supernatant.
13. Resuspend the pellet in 40 μl of sterile PBS to obtain a bead dilution equivalent to the initial one (1.7×10^6 beads per μl for 3 μm amino beads).
14. Cap tightly and cover the tube with aluminum paper to protect its content from light. Beads are ready to use and can be stored at 4°C for a few days. Do not freeze.

3.1.2. Measurement of Phagosomal pH by Cytofluorometry (FACS)

1. A total of 1×10^6 of immature bone marrow-derived DCs (BMDC) are required for a pH measurement performed over 120 min. In addition, 1×10^6 cells per point are also required to obtain the pH standard curve. To collect the cells from the dishes, take first the cells in suspension. Then wash the dishes once with cold PBS, collect the cells, and pool them with the first-cell suspension. Add 10 ml of cold PBS and incubate the plate for 20–30 min at 4°C. Collect the cells again and pool them with the previous cell suspension (*see* **Note 1**).
2. Set a water bath at 37°C.
3. Cells are disposed in two 15-ml tubes, one for phagosome pH measurement and one for the standard curve, labeled as "kinetics" and "standard curve," respectively. Cells are washed twice in PBS.
4. Resuspend the cell pellet in 95 μl of CO_2-independent cell culture medium by pipetting gently.
5. Add 5 μl of fluorochrome-coupled beads solution and mix gently by pipetting (vortex carefully the bead solution before adding to cells).
6. Place the tubes containing the cells and beads at 37°C in the water bath for 15 min to allow phagocytosis. This step is called "pulse."
7. Add 10 ml of ice-cold PBS to stop the internalization and cap the tube tightly. Shake the tube vigorously for 10 s to

remove the non-internalized beads that remain attached to the cell surface. From this step any manipulation must be done at 4°C.

8. Centrifuge for 4 min at 1,000 rpm and 4°C (*see* **Note 2**). Remove the supernatant carefully using a Pasteur pipette or a vacuum pump.
9. Repeat **Steps 7** and **8** twice.
10. Resuspend the cell pellets in 1 ml of conditioned-complete medium by pipetting gently.
11. For phagosome pH measurement, place the cell suspension of the tube "kinetics" in the incubator at 37°C and 5% CO_2. This is the time 0 of the kinetics. Take 250 μl of the cell suspension 10, 20, 30, 60, and 120 min later. Analyze the sample in the cytofluorometer immediately (*see* **Note 3**).
12. Select the population that has internalized only one bead (population *a*, **Fig. 25.1**) (*see* **Note 4**). The cytometer settings for the analysis are 488 nm (excitation laser) and FL1 (emission) for FITC and 633 nm (excitation laser) and FL4 (emission) for FP647. Calculate the mean fluorescence intensity (MFI) for FL1 and FL4. Determine the MFI ratios for the one-bead DC population (*see* **Note 5**). To obtain the real pH value corresponding to each time-point ratio, an extrapolation to the standard curve is required (*see* below) (*see* **Note 6**).

3.1.3. Standard Curve for pH Measurements

1. Place the "standard curve" tube in the incubator at 37°C, 5% CO_2 for 30 min.
2. Centrifuge for 4 min at 1,200 rpm and 4°C. Remove the supernatant carefully using a Pasteur pipette or a vacuum pump. Discard the supernatant. Add a large volume of PBS. Resuspend the cell pellet by pipetting gently and leave the tube on ice.
3. Prepare defined pH solutions using CO_2-independent culture medium. pH is adjusted using citric acid or NaOH and a basic pH meter. *See* **Note 7**.
4. Split the cell suspension into various 15-ml tubes according to the number of pH solutions prepared.
5. Centrifuge for 4 min at 1,000 rpm and 4°C. Remove the supernatant carefully using a Pasteur pipette or a vacuum pump. Discard the supernatant.
6. Add 200 μl of each pH solution to the cells. Mix carefully by pipetting. From this moment working on ice is not required anymore.
7. Add Triton X-100 to each tube at a final concentration of 0.1%. Mix gently. Leave for 2 min at room temperature and

immediately analyze the bead-associated fluorescence in the cytometer. It is recommended to perform this experimental step tube-by-tube (*see* **Note 8**).

8. The analysis described in **Note 2** is performed.
9. Build the curve by plotting the MFI ratio values obtained from each cell suspension against the corresponding pH (**Fig. 25.2**) (*see* **Note 9**).

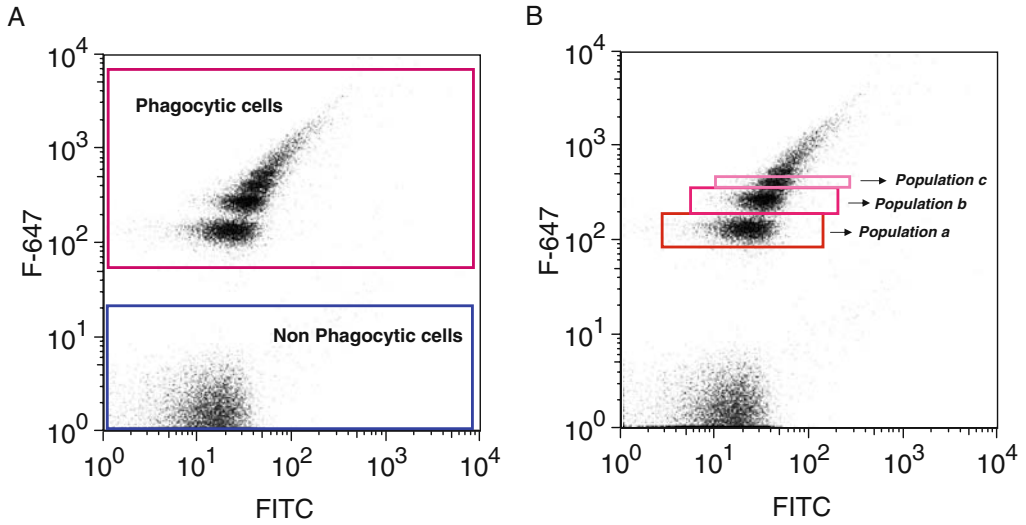

Fig. 25.2. Measurement of phagosomal pH by cytometry. (**A**) An example of an FL4/FL1 plot after beads internalization. Two different cell populations (phagocytic and non-phagocytic) can be distinguished due to the presence on the beads of pH-insensitive fluorochrome (F-647). (**B**) A detailed analysis of the phagocytic population in the same plot makes evident the presence of different cell populations according to the number of beads internalized by an individual cell (*population a*: cells with one bead; *population b*: cells with two beads; *population c*: cells with three beads; etc.).

3.2. Oxidative Capacity of Phagosomes

3.2.1. Preparation of Fluorochrome-Coated Beads for ROS Production Measurement

1. Place 50 μl of a 2.59% aqueous suspension of 3 μm amino beads in an eppendorf tube (1.5 ml capacity).
2. Add 0.5 ml of sterile PBS and cap tightly.
3. Centrifuge for 2 min in a microcentrifuge at 15,000 rpm. Remove supernatant carefully using a Pasteur pipette or a vacuum pump.
4. Resuspend the pellet in 400 μl of sodium hydrogen carbonate 0.1 M buffered at pH 8.5. Vortex until dissolving the pellet.
5. Add 50 μl of the dihydrorhodamine 123 (DHR) (20 mg/ml) and 50 μl of the FP647 (1 mg/ml) solutions. Cover the tube with aluminum to protect its content from light.

6. Leave on a rotator for at least 2 h at room temperature with a continuous rotation.
7. Centrifuge for 2 min in a microcentrifuge at 15,000 rpm. Remove the supernatant carefully using a Pasteur pipette or a vacuum pump.
8. Resuspend the pellet in 0.5 ml of 0.1 M glycine in PBS. Vortex until the pellet is dissolved. Add 0.5 ml of 0.1 M glycine in PBS to obtain a 1 ml final volume.
9. Centrifuge for 2 min in a microcentrifuge at 15,000 rpm. Remove the supernatant carefully using a Pasteur pipette or a vacuum pump.
10. Repeat **Steps 8** and **9** once with 0.1 M glycine in PBS and once with PBS alone.
11. Resuspend the pellet in 40 μl of sterile PBS to obtain a bead dilution equivalent to the initial one (1.7×10^6 beads per μl for 3 μm amino beads).
12. Cap tightly and cover the tube with aluminum. Coupled beads must be used on the same day of preparation to avoid DHR auto-oxidation. Keep them at 4°C until use.

3.2.2. Measurement of NOX2-Dependent Phagosomal ROS Production by FACS

1. A total of 1×10^6 of immature bone marrow-derived DCs are required for a kinetic over 60 min. To collect the cells from the dishes, take first the cells in suspension. Then wash the dishes once with cold PBS, collect the cells, and pool them with the first-cell suspension. Add 10 ml of cold PBS and incubate the plate for 20–30 min at 4°C. Collect the cells again and pool them with the previous cell suspension.
2. Set a water bath at 37°C.
3. Split the cells into two 15-ml tubes: one for kinetics measurement of phagosome oxidation and one for an NOX2 inhibitor (DPI) control.
4. Wash the cells twice with PBS.
5. Resuspend the cell pellet in 95 μl of CO_2-independent cell culture medium by pipetting gently.
6. Add DPI to the "NOX2 inhibitor" condition to a final concentration of 5 μM.
7. Incubate both tubes for 5 min in the water bath at 37°C.
8. Add 5 μl of the DHR-coated bead solution and mix gently by pipetting. Vortex carefully the bead solution before adding to the cells.
9. Put back the tubes at 37°C for 15 min to allow phagocytosis. This step is called "pulse."

10. Add 10 ml of ice-cold PBS to stop bead uptake and cap tubes tightly. Shake the tubes vigorously for 10 s to remove the non-internalized beads that remain attached to the cell surface. From this step any manipulation must be done on ice.
11. Centrifuge for 4 min at 1,000 rpm and 4°C (*see* **Note 2**). Remove supernatant carefully using a Pasteur pipette or a vacuum pump.
12. Repeat **Steps 7** and **8** twice.
13. Resuspend cell pellets in 750 μl of conditioned-complete medium by pipetting gently. Place cell suspensions in the incubator at 37°C and 5% CO_2. This corresponds to the time 0 of the kinetics measurements. Take 250 μl of cell suspension 10, 30, and 60 min later. Analyze the samples in the cytofluorometer immediately (*see* **Note 3**).
14. Select the population that has internalized only one bead (population *a*, **Fig. 25.1**) (*see* **Note 5**). The cytometer settings are 488 nm (excitation laser) and FL2 (emission) for DHR and 633 nm (excitation laser) and FL4 (emission) for FP647. Calculate the MFI for FL2. Determine the MFI ratio for the DC population containing one bead (*see* **Note 10**). The increase over time in the fluorescence intensity represents the oxidation capacity of the phagosomal compartment.

3.3. Phagosome Maturation: Proteolytic Activity of Phagosomes and Recruitment of Proteins to Phagosomes

The techniques described here are aimed to quantitatively assess the degradation of proteins in phagosomes. Degradation of the proteins coupled to the latex beads is monitored at different time-points after phagocytosis, by lysing the cells (including the phagosome membrane) and analyzing the amount of protein that remains on the beads using flow cytometry. We describe the procedure for ovalbumin (OVA). However, these methods have been applied successfully to other polypeptides such as horse radish peroxidase, bovine serum albumin, and hen egg lysozyme. Two unique requirements need to be fulfilled: (1) the protein needs to include lysine residues (at least one) in the primary structure and (2) a specific polyclonal antibody directed against the protein for analysis by flow cytometry needs to be available. This technique has also been modified to analyze in parallel the degradation of a protein bound to the bead together with the acquisition of a lysosomal marker on the phagosome (*see* **Fig. 25.1**). To do so, the cells are disrupted mechanically, in order to preserve the phagosomal membrane around the latex bead. The phagosomes are then eventually permeabilized and analyzed by FACS using antibodies to the protein bound to the bead (to asses degradation) and to the cytosolic region of a lysosomal marker (to measure phagosome maturation).

3.3.1. Coupling OVA to Amino Beads

1. Place 100 μl of a 2.5% aqueous suspension of beads in a 1.5-ml eppendorf tube.
2. Fill the tube with PBS and cap tightly.
3. Centrifuge for 4 min in a microcentrifuge at 15,000 rpm.
4. Remove and discard the supernatant.
5. Resuspend the pellet in 500 μl of PBS and vortex until the pellet is dissolved.
6. Centrifuge for 4 min in a microcentrifuge at 15,000 rpm and discard the supernatant.
7. Repeat **Steps 5** and **6** once.
8. Resuspend the pellet in 0.5 ml of 8% glutaraldehyde.
9. Mix for 4–6 h (or O/N) at room temperature on a rocker table, rotary shaker, or any other kind of shaker which provides end-to-end mixing.
10. Centrifuge for 4 min in a microcentrifuge at 15,000 rpm and discard the supernatant.
11. Repeat **Steps 5** and **6** once.
12. Resuspend in 0.5 ml of PBS and add 25 μl of the protein solution (final concentration 0.5 mg/ml). Leave O/N at 4°C in the cold room with gentle end-to-end mixing (use a wheel).
13. Centrifuge for 4 min (14,000 rpm) and discard the supernatant.
14. Resuspend the pellet in 1 ml of 0.5 M glycine and mix gently for 30 min at room temperature. This step serves to block unreacted sites on the microparticles.
15. Centrifuge 4 min in a microcentrifuge (14,000 rpm) and discard supernatant.
16. Repeat **Steps 5** and **6** twice. OVA is now covalently coupled to amino beads. Resuspend the pellet in the original volume of beads to maintain the starting concentration of particles (1.7×10^6 beads per μl for 3 μm amino beads). Protein-coated beads can be stored for 1 week at 4°C.

3.3.2. Measurement of the Degradation of OVA Bound to Beads in Phagosomes

1. A total of 10×10^6 of immature bone marrow-derived DCs are required for a kinetic experiment over 240 min (four times points). To collect the cells from the dishes, take first the cells in suspension. Then wash the dishes once with cold PBS, collect the cells, and pool them with the first-cell suspension. Add 10 ml of cold PBS and incubate the plate for 20–30 min at 4°C. Collect the cells

again and pool them with the previous cell suspension (*see* **Note 11**).

2. Cells are placed in 15-ml tubes and centrifuged for 5 min at 1,200 rpm. Discard the supernatant.
3. Set a water bath at 37°C.
4. Wash the cells twice with PBS.
5. Resuspend the cell pellet in 200 μl of CO_2-independent cell culture medium by pipetting gently. Add the protease inhibitors to the control tube and place the cell suspensions in a water bath at 37°C for 5 min.
6. Add 30×10^6 beads (18 μl of OVA-coated beads, ratio 3:1 beads to cells) and mix gently by pipetting (vortex carefully the bead solution before adding to cells).
7. Place the tubes containing the cells and the beads at 37°C in the water bath for 10 min to allow phagocytosis. This step is called "pulse."
8. Add 1 ml of ice-cold PBS to stop the internalization and cap the tube tightly. From this step any manipulation must be done at 4°C.
9. To wash out non-internalized beads an FCS flotation cushion is recommended. Place 2 ml of cold FCS into a 15-ml tube. Add very carefully the cell suspension (1.2 ml) over the serum (two different phases should be observed). Before adding to the serum, it is important a good cell resuspension by pipetting to remove the sticky beads.
10. Centrifuge for 5 min at $150 \times g$ and discard carefully the supernatant with a Pasteur pipette. Resuspend the cells in 1 ml of cold PBS.
11. Repeat **Steps 9** and **10** twice.
12. Resuspend the cells in 2 ml of conditioned-complete medium by pipetting gently. Add the protease inhibitors to the control tube.
13. Place the tube in a cell incubator and take 500 μl aliquots after 0, 30, 120, and 240 min of chase. Place immediately each aliquot in 1 ml of cold PBS to stop phagosome maturation.
14. Centrifuge for 5 min at 1,200 rpm and 4°C. Discard the supernatant.
15. Lyse the cells by adding 200 μl of a lysis buffer. It is recommended to perform the lysis O/N at 4°C in a conic 96-well plate (avoid using round or flat plates). However, 1 h of lysis at 4°C should be enough to recover the beads without membranes.

16. Centrifuge the plate at 3,000 rpm for 5 min at 4°C. Discard the supernatant by inversing the plate.
17. Wash twice with 150 µl of the washing. Non-degraded OVA will be detected on the beads (phagosomes) placed at the bottom of the wells (*see* **Note 12**).
18. Add 100 µl of a 1/750 dilution in PBS/1% BSA of a rabbit anti-OVA antibody (SIGMA product number C6534). Incubate for 30 min at 4°C.
19. Wash twice in cold PBS/1% BSA and incubate with a dilution of an anti-rabbit antibody labeled with a fluorochrome adapted to cytometry. Analyze the fluorescent signal coming from the beads in the cytofluorometer (*see* **Notes 13** and **14**).

3.3.3. Measurement of the Phagosomal Acquisition of a Lysosomal Marker During Phagosome Maturation

We here describe a protocol to assess phagosome maturation by determining acquisition by phagosomes of lysosomal markers such as LAMP-2. This assay could be extended to other phagosomal membrane proteins.

1. **Steps 1–14** are performed as described in the previous part (**Section 3.3.2**), except that the amount of cells needed for each pulse is 50×10^7 and the ratio of beads to cells is 5:1. All of this is directed to obtain a greater rate of bead internalization. As the number of cells is higher, the pulse is also performed in a bigger volume of medium (1 ml) to keep the concentration of cells at 50×10^6 per ml (*see* **Note 15**).
2. Resuspend the cell pellets in 500 µl of the homogenization buffer and transfer the suspension to a 2-ml eppendorf tube. From this step it is essential to work on ice.
3. Count the cells by using Trypan Blue exclusion to assess viability.
4. Take the total cell volume in a 2-ml syringe harboring a 22-ga needle. Push strongly the suspension of cells through the needle maintaining the tube on ice. Repeat this action ten times avoiding making a high amount of bubbles.
5. Count the cells again with Trypan Blue to obtain new information of cell mortality. **Step 17** must be repeated until reaching about 70% of cell mortality.
6. Once cell mortality desired is obtained in each tube, centrifuge for 4 min at $150 \times g$ and 4°C.
7. Recover carefully the post-nuclear supernatant (PNS), which contains the phagosomes, and transfer to new eppendorf tubes.

8. Centrifuge for 4 min at 3,000 rpm and 4°C. Discard the supernatant with a Pasteur pipette. Resuspend the pellet in 1 ml of cold PBS.
9. From this step two options can be considered: (a) to perform directly a staining with antibodies that recognize epitopes located at the cytosolic face of phagosomal membranes or (b) to fix/permeabilize the phagosomes to stain for intra-phagosomal epitopes.
10. For fixation–permeabilization, add 1 ml of 2% PFA (prepared in PBS) to the 1 ml of PBS containing the phagosomes. Incubate for 10 min at 4°C in the refrigerator (avoid leaving on ice). Stop fixation by adding 2 ml of 0.4 M glycine (prepared in PBS).
11. Centrifuge for 4 min at 3,000 rpm and 4°C.
12. Resuspend in PBS/2% BSA and distribute in 96-well conical plates.
13. The OVA staining is performed as described in Section **3.3.2** (**Step 18**). For the membrane protein use the anti-LAMP-2 antibody (final concentration 10 μg/ml) and a secondary antibody labeled with a fluorochrome adapted to flow cytometry.
14. Analyze the fluorescent signal coming from the beads in the cytofluorometer (*see* **Note 16**).

4. Notes

1. In order to preserve the protein composition of the external cell surface it is recommended not to trypsinize the cells, but rather wash the plates once with cold PBS.
2. Centrifugation conditions (rpm and time) were set up to pull down the cells but not the non-internalized beads, which remain in suspension when contained in a large volume.
3. For the flow cytometer analysis, gate first on the cells plotted in the Forward Scatter (FCS) and Side Scatter (SSC) to perform further analysis on living DCs. A second gate FL1/FL4 has to be defined from living DCs, to select for cells that have or have not internalized beads. The presence of a pH-insensitive dye on beads permits to distinguish between different phagocytic DC populations based on the amount of beads internalized by each cell (**Fig. 25.2**, populations *a*, *b*, *c*, ...). It is worth mentioning that this approach also allows measuring the phagocytic

capacity of a cell population when calculating the percentage of the populations shown in **Fig. 25.2A**.

4. It is assumed that the amount of each fluorochrome per bead is equivalent. Because FP647 is not sensitive to pH, its fluorescence intensity depends only on the amount of the dye coated on the bead and does not change over time. In contrast, the fluorescence intensity of FITC depends not only on the amount coated on the bead but also on the pH of the bead microenvironment. Considering that the variability of FITC binding among beads is almost negligible, it is assumed that the changes in FITC MFI result from pH modification.
5. Gating on the population that has internalized one bead only increments the analysis accuracy. In contrast to other strategies used to measure phagosomal pH or ROS production measurements, the cytometer-based approach described here is statistically powerful since it permits to measure the pH of over a thousand phagosomes per condition and per experiment.
6. Considering that the MFI of FP647 does not change over time for a determined population, it is possible to extrapolate directly the data obtained with FITC to the standard curve (without calculating the MFI ratio between both dyes). In that case, the standard curve should be built using the FITC MFI values instead of the FL1/FL4 ratios.
7. It is convenient to prepare buffers that differ by 0.5 units of pH ranging from 5 to 8.
8. Triton X-100 is added to the samples in order to slightly permeabilize the plasma and phagosomal membranes, allowing the external fixed pH solution getting access of the phagosome lumen. This treatment may affect the FCS/SSC distribution of the DC population. In this case, the gate established on non-permeabilized cells should be maintained even if the amount of cells is markedly diminished. To avoid excessive cell damage it is important to respect the triton concentrations and times of incubation.
9. Because the fluorescence intensity of FITC diminishes as the pH decreases, it is important to control that the decrease in the ratio between the two fluorochromes is not due to the loss of FITC from the bead under acidic conditions. To do so, an aliquot of an acidic cell suspension should be neutralized by adding 0.1 M NaOH (change in the color of the medium confirms neutralization) and immediately analyzed in the cytometer. The level of fluorescence intensity in FL1 must be recovered under such con-

ditions. A typical histogram of a standard curve is shown in **Fig. 25.3A**.

10. FP647 is not sensitive to oxidation. Its fluorescence intensity depends only on the amount of dye bound to the bead and should therefore not change over time. Because DHR is not sensitive to pH, it is assumed that the changes in DHR MFI strictly result from its oxidation. Treatment with DPI increases cell auto-fluorescence. A correction must therefore be performed to compare DPI-treated and untreated cells. This is achieved by subtracting the FL2 MFI value displayed by the non-phagocytosing cell population from the FL2 MFI value displayed by the cells that have internalized one bead.
11. It is suggested to perform a control with a mix of protease inhibitors to inhibit the majority of the proteases present in phagosomes. In that case an equal number of cells should be considered for this condition. The protease inhibitors have to be present during the pulse and the chase.
12. Depending on the rates of phagocytosis, the number of beads recovered is or is not sufficient to see a pellet at the bottom of the wells. Consequently, the absence of a pellet does not mean that there are no phagosomes at the bottom of the wells.
13. A first FCS/SSC analysis on the cell lysate must be done to distinguish the beads population (phagosomes) from the rest of the cellular debris as shown in **Fig. 25.4A** (red circle). The fluorescence intensity from this population is obtained and plotted. Typical histograms of a kinetic experiment are shown in **Fig. 25.4B**.

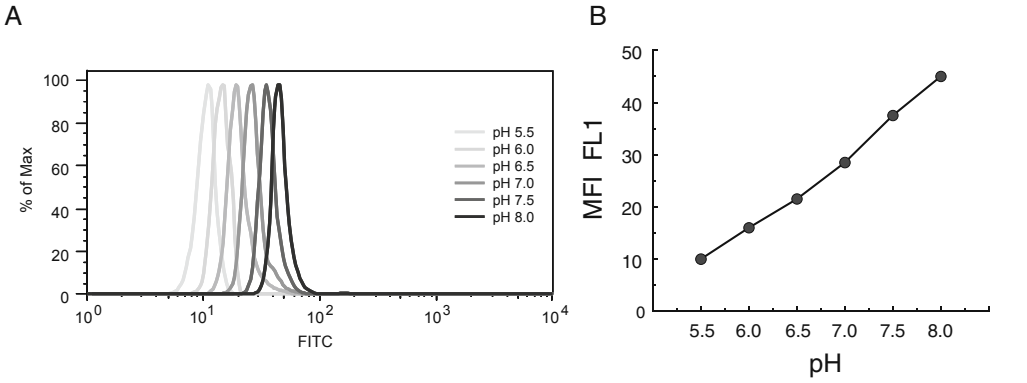

Fig. 25.3. Building the standard curve for cytometer-based phagosomal pH measurement. (**A**) After internalization cells are suspended into fixed pH buffers and immediately analyzed by cytometer. A typical histogram showing the MFI in FL1 obtained for each pH value. (**B**) A curve is built from these values. Extrapolation of MFI values from a given cell population allows to determine its phagosomal pH.

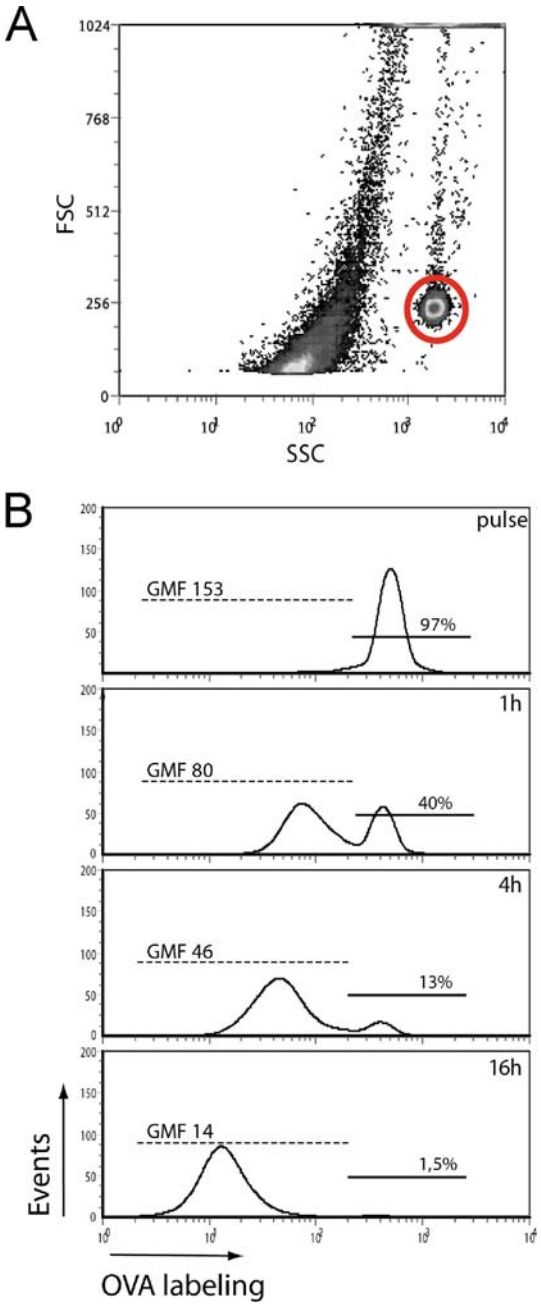

Fig. 25.4. Proteolytic activity of phagosomes. (**A**) An example of an FSC/SSC plot obtained when acquiring beads from cells that were lysed in the presence of detergent. The population of beads is highlighted as circle and can be clearly distinguished from the cell debris. (**B**) The amount of remaining OVA (non-degraded) at the surface of the beads is therefore detected with a specific antibody and analyzed by gating on this population at the different time-points.

14. There are different ways to express protein degradation in this experiment. The simplest one is by analyzing the decrease in the mean fluorescence intensity over time (comparing with protease inhibitor-treated cells), as shown in the histograms of **Fig. 25.4B**. The geometric mean of fluorescence intensities was chosen in this case, because statistically, this parameter gives equivalent weight to each plotted point. An alternative way of analysis is calculating the rate of degradation at each time-point considering the protease inhibitor-treated cells as 100% non-degraded OVA. In case that the protease inhibitors control is not performed, it is also possible to consider 100% of non-degraded OVA to the MFI value obtained in the condition "pulse" of the kinetics (time 0).
15. Because the aim of this experiment is to detect proteins at the phagosomal membrane during phagosome maturation, it is essential to preserve it as much as possible. Instead of using detergents to lyse the cells; a mechanic disruption of plasma membrane will be performed to maintain the integrity of phagosomal membranes.
16. The double staining (OVA and LAMP-2) allows monitoring in an individual phagosome as to how protein degradation and phagosome maturation are synchronized over time as shown in **Fig. 25.5**.

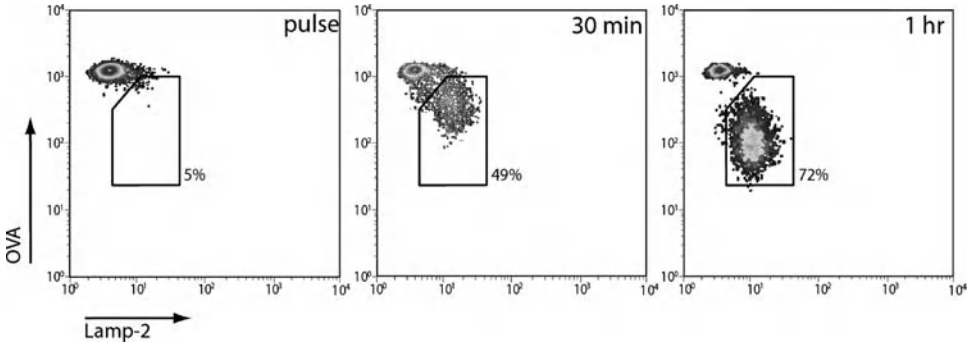

Fig. 25.5. Antigen degradation and protein content of phagosomes. Typical dot plot showing OVA degradation and LAMP-2 staining in individual phagosomes are depicted. After different chase times the cells are mechanically cracked and the phagosomes containing beads are stained with different combinations of antibodies. OVA degradation is simultaneously followed with the acquisition of LAMP-2 allowing the characterization of the stage of phagosome maturation at which they acquire their proteolytic capacity.

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Chapter 26

Adaptive Treg Generation by DCs and Their Functional Analysis

Li Wang

Abstract

Naturally occurring thymus-derived $CD4^+CD25^+Foxp3^+$ regulatory T cells (nTregs) are critical regulators of immune tolerance. $Foxp3^+$ Tregs can also be induced from $CD25^-$ naïve CD4 T cells in vivo and ex vivo. This conversion process requires cytokines such as IL2 and TGFβ as well as suboptimal TCR stimulation, thus is regulated by the co-stimulatory status of antigen-presenting cells (i.e., dendritic cells). Although mature dendritic cells (DCs) are potent initiators of adaptive immune response, immature steady-state DCs contribute to immune tolerance. In this chapter, we summarize methods that use ex vivo splenic DCs to induce the conversion of naïve $CD4^+$ T cells to adaptive $Foxp3^+CD4^+$ regulatory T cells (aTreg) in the presence of TGFβ.

Key words: Dendritic cells, $CD8\alpha^+$ DC subset, adaptive Tregs, conversion, Foxp3, immune suppression.

1. Introduction

Naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory T cells (nTregs) represent 5–10% of peripheral CD4 T cells and are critical regulators of immune tolerance (1). The transcription factor Foxp3 is a specific lineage marker for nTregs and is both necessary and sufficient for Treg function (2). It is established that naïve $CD4^+CD25^-Foxp3^-$ T cells can convert into $Foxp3^+$ regulatory T cells (aTregs). In vitro conversion occurs in the presence of TGFβ (3), typically under conditions of low co-stimulation (4, 5). This process requires CTLA-4-mediated negative co-stimulation (6). The aTregs resemble nTregs both phenotypically and

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functionally (7–9). In vivo, the extra-thymic induction of Foxp3$^+$ aTregs from naïve CD4$^+$ T cells occurs upon subimmunogenic antigen stimulation (10–13). Consistent with in vitro studies, TGFβ signaling and B7 co-stimulation are required for peripheral conversion (13, 14).

Since resting DCs are constantly presenting tissue under subimmunogenic conditions, it is imperative to understand their potential roles in the peripheral tolerance. One of the key questions is whether and how steady-state DCs regulate the de novo induction of Foxp3$^+$ aTregs. In this chapter we describe procedures that examine the capacity of ex vivo splenic DC subsets to induce Foxp3 expression in the presence of TGFβ. We show that among the splenic DC subsets, the CD8α$^+$ DCs exhibit a superior capacity to drive conversion. Multiple co-stimulatory and co-inhibitory molecules have been identified to non-redundantly regulate this process. In particular, PD-L1 expression on DCs is required for conversion. The suppressive function of DC-induced aTregs is also examined using the in vitro suppression assay.

In this chapter we describe the isolation of splenic DCs, the ex vivo induction of Foxp3$^+$ aTregs as well as the in vitro suppression assay of aTregs.

2. Materials

2.1. Isolation of Splenic Dendritic Cells and CD4$^+$ T Cells

1. Flow staining buffer: 1X HBSS with calcium and magnesium, supplemented with 5% BCS (bovine calf serum) and 10% heat-inactivated rat serum (*see* **Note 1**).
2. Complete culture medium: RPMI-1640, 10 mM HEPES (pH 7.5), 1X sodium pyruvate, 1X non-essential amino acids, 2 μM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol (*see* **Note 2**) and 10% fetal calf serum.
3. Serological sterile plastic pipets: 5, 10, and 25 ml.
4. Sterile conical tubes: 15 ml and 50 ml.
5. Trypan Blue: 0.4% in saline.
6. Neubauer hemocytometer
7. Liberase CI:
   To make 40X stock at 16.7 mg/ml in LPS-free H_2O, reconstitute 250 mg (1,000 Wunch U) in 15 ml sterile LPS-free H_2O, leave on ice for 30 min, and filter sterilize through a low protein-binding 0.22 μm polysulfone membrane (Millipore Millex-GP syringe filter). Dispense into 125-μl aliquots and store at –80°C
8. DNase I: Make 20 mg/ml (100X) stock solution in plain RPMI-1640, aliquot, and store at –80°C.

9. LPS-free PBS/5 mM EDTA.
10. CD11c (N418) microbeads (Miltenyi).
11. CD4 T-cell isolation kit (Miltenyi).
12. MACS buffer: PBS, 2 mM EDTA, 1% FCS.
13. FACS buffer: PBS, 0.5 mM EDTA, 0.02% azide, 1% FCS.
14. LS MACS column (Miltenyi).
15. 40-μm Cell strainer.

2.2. In Vitro Conversion Assay

1. Complete RPMI medium (*see* above).
2. Recombinant human IL2 (PeproTech): To make a stock solution, we first dissolve and activate huIL2 in a small volume of acid buffer (0.1 M acetic acid), then add more RPMI medium to make a stock of 400 U/μl.
3. Recombinant human TGFβ1 (PeproTech): To make a stock solution, we first dissolve and activate huTGFβ1 in a small volume of acid buffer (0.1 M HCl + 1 mg/ml BSA), then add more RPMI medium to make a stock of 2 μg/ml.
4. Synthetic $OVA_{323–339}$ peptide: To make a stock solution, we first dissolve lyophilized peptide in acid buffer (0.1 M HCl), then add more PBS to make 1 μg/μl stock.
5. Round-bottomed 96-well plates.

2.3. In Vitro Suppression Assay of DC-Induced Adaptive Tregs

1. 10 mM CFSE stock in DMSO, aliquoted, and store desiccated at –20°C.
2. Round-bottomed 96-well plate.
3. Rabbit complement (Dynal Biotech).
4. Anti-thy1.2 mAb (HO13.4, ascites).
5. ACT blood lysis buffer.

3. Methods

3.1. Isolation of Splenic Dendritic Cell Subsets

3.1.1. Making Spleen Single-Cell Suspensions

1. Collect spleens in 5–10 ml plain RPMI, keep on ice.
2. Make enzyme solution, add 125 μl liberase and 50 μl DNAase in 5 ml plain RPMI. Use 2 ml per spleen.
3. Inject 2 ml enzyme solution (25-ga needle; choose —three to five sites along the spleen) while holding spleen with a pair of curved forceps above a 15-ml tube. Some solution will be retained in the spleen (swelling) but most of it will drain through releasing cells.

4. Let spleen slide to the bottom of the tube, completely immersing in the enzyme solution; incubate for 30 min at 37°C
5. Pass cell suspension and digested spleen through 40-μm strainer into a 50-ml Falcon tube. Use 3-ml syringe plunger to grind spleen in the strainer, followed by several washes with PBS/EDTA. Repeat until all spleen tissue is eluted.
6. Spin down cells at 450 × *g* for 10 min, and resuspend them in MACS buffer (200 μl per spleen) for CD11c purification (*see* **Note 3**).

3.1.2. Enrichment of Splenic DCs

Splenic DCs are a small percentage of total splenic cells. The most efficient strategy to isolate DCs is to positively select using CD11c magnetic beads (*see* **Note 4**).

1. Dilute CD11c beads (50–100 μl per spleen) in cold MACS buffer (200 μl per spleen) and F_CR block 2.4G2 (1:5) (*see* **Notes 5** and **6**).
2. Combine 1:1 volume of cells and beads (400 μl per spleen). Mix gently and leave in the fridge (~4–8°C) for 10–15 min.
3. Wash cells by adding 20X labeling volume of MACS buffer and spin at 450 × *g* for 10 min.
4. Resuspend in MACS buffer (500 μl per spleen) and filter through a 40-μm cell strainer to remove any potential cell clumps (*see* **Note 7**).
5. Pre-wet an LS column with 3 ml MACS buffer.
6. Load cell suspensions onto the column. We use 1X LS column for a maximum of four spleens.
7. Wash three times with 3 ml MACS buffer.
8. Remove column from the magnetic separator and place it on a 15-ml collection tube.
9. Pipet 5 ml MACS buffer onto the column and immediately flush out magnetically labeled cells using the plunger. A typical yield is ~2–4 million cells per spleen.

3.1.3. FACS Sorting of Splenic DC Subsets

Enriched DCs were resuspended at 10 million per ml and stained using surface markers CD11c-PE and CD8-APC (eBioscience, 0.5 μg/ml final concentration) in 1X HBSS with 10% heat-inactivated rat serum, 20 min on ice. Cells are then washed and resuspended in complete RPMI medium, and FACS sorted into $CD11c^{high}CD8\alpha^{+}$ and $CD11c^{high}CD8\alpha^{-}$ populations on BD FACSAria.

3.2. Isolation of Naïve CD4 T Cells

OVA-specific TCR-transgenic mice OTII have been bred onto the Foxp3-GFP reporter background. To obtain $Foxp3^{-}$ naïve OTII CD4 T cells, total CD4 T cells are isolated using the

MACS® CD4 T-cell isolation kit prior to FACS sorting into Vbeta5^{+}Foxp3GFP^{-}CD25^{-} populations.

1. Spleens and lymph nodes are dissected out and kept in sterile complete RPMI-1640 medium. Single-cell suspensions are prepared by mechanical disruption using sterile-frosted microscopic slides. Cells are then passed through 40-μm cell strainers, spun, and resuspended in cold MACS buffer at 400 μl per spleen (~1 × 10^8 cells).
2. Add 100 μl of biotin-antibody cocktail per 1 × 10^8 total cells. Mix well and incubate for 10 min in the fridge (~4–8°C).
3. Add 300 μl of MACS buffer and 200 μl of anti-biotin microbeads per 1 × 10^8 cells.
4. Mix well and incubate for 15 min at ~4–8°C.
5. Wash cells with 20X labeling volume of MACS buffer, spin at 450 × *g* for 10 min.
6. Resuspend cells in 500 μl of MACS buffer per 1 × 10^8 total cells.
7. Pre-wet LS column with 3 ml MACS buffer. We use 1X LS column for a maximum of four spleens.
8. Apply cell suspension onto the column.
9. Allow cells to pass through column and collect the unlabeled fractions as enriched CD4 T cells.
10. Wash column with 4 × 3 ml MACS buffer and collect all the flow-through as well.
11. Enriched CD4 T cells are then spun at 450 × *g* for 10 min and resuspended in flow-staining buffer containing the following antibodies: PE-conjugated Vβ5 (BD Biosciences, 0.5 μg/ml) , APC-conjugated CD25 (eBioscience, 0.5 μg/ml) and PerCP-conjugated CD4 (BD Biosciences, 0.5 μg/ml). After 20 min incubation on ice, cells are washed twice with HBSS/5% BCS and resuspended in complete RPMI medium.
12. Naïve CD4^{+} T cells are obtained by FACS sorting gated on Vβ5^{high}CD25^{-}FoxP3^{-} (BD FACSAria). Purity typically exceeded 95%.

3.3. In Vitro Conversion Assay

1. FACS-sorted naïve OTII CD4 T cells and splenic DC subsets are resuspended in complete RPMI medium at 1 million per ml, and 50 μl of each (50,000 cells) is added to duplicate wells in a round-bottomed 96-well plate (*see* **Note 8**).
2. Cytokines are prepared at 2X concentration in complete RPMI medium (i.e., recombinant human IL2 (100 U/ml),

synthetic $OVA_{323–339}$ peptide (500 ng/ml), and indicated concentration of human recombinant TGFβ1) (*see* **Note 9**) and are added to the wells in 100 μl volume.

3. Cells are analyzed by FACS on day 5 or according to a time course. Cells are first transferred to a non-sterile V-bottomed 96-well plate, spun, and resuspended in flow-staining buffer containing APC-conjugated CD4 (eBioscience). After 20 min incubation on ice, cells are washed twice with HBSS/5% BCS and resuspended in FACS buffer (PBS with 1% BCS and 0.05% azide) and analyzed by flow cytometry. Percentage of $Foxp3GFP^+$ cells among gated $CD4^+$ cells indicates the induction of adaptive Tregs. An example is shown in **Fig. 26.1**, reproduced from Wang et al. (15) (Copyright 2008 PNAS.)

3.4. In Vitro Suppression Assay Using DC-Induced OVA-Specific Adaptive Tregs

1. Thaw rabbit complement and anti-thy1.2 mAb (HO13.4, ascites) on ice (*see* **Note 10**).
2. Make splenocyte single-cell suspensions by homogenizing the spleen between two sterile-frosted microscopic slides, then pass cells through a 40-μm cell strainer.

3.4.1. Preparation of T cell-Depleted Splenocytes

3. Wash with 1X HBSS and resuspend in HBSS + 5% BCS (5 ml per spleen).
4. Add 150 μl thy1 antibody, incubate at 4°C for 30 min, and mix occasionally.
5. Wash with 1X HBSS and resuspend in HBSS + 5% BCS (5 ml per spleen).
6. Add rabbit complement (200 μl), incubate at 37°C for 40 min.
7. Wash with 40 ml 1X HBSS and resuspend cell in cold ACT buffer (2 ml per spleen), incubate for 2 min at 37°C, and then wash immediately with cold HBSS.
8. Resuspend cells in 5 ml HBSS per spleen and irradiate at 20 Gy.
9. Resuspend cells in complete RPMI medium at 3×10^6/ml and set on ice until plating.

3.4.2. Preparation of Effector T cells and aTregs

1. Naïve OTII CD4 T cells are isolated from non-reporter naïve mice by first using the CD4 isolation kit (Miltenyi), then FACS sorted into $V\beta5^{high}CD25^{-}CD62L^{high}$ populations, and are then resuspended in plain RPMI (without FBS supplement) at 40 million per ml.
2. 10 μM CFSE stock is prepared in plain RPMI by diluting the 10 mM stock (*see* **Note 11**) and added at 1:1 volume ratio to OTII cells (the final labeling concentration of CFSE is 5 μM). Cells are incubated at 37°C for 10 min, washed

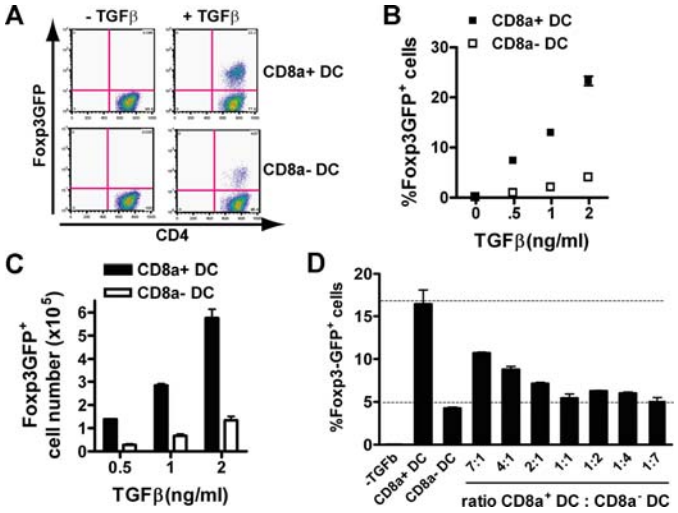

Fig. 26.1. Ex vivo splenic CD8α$^+$ DCs induce Foxp3 expression more efficiently than CD8α$^-$ DCs in the presence of TGFβ. Freshly isolated splenic DC subsets (CD8α$^+$ CD11c^{high} and CD8α$^-$ CD11c^{high}) were co-cultured with naïve OTII CD4$^+$ T cells (CD25$^-$Foxp3GFP$^-$) in the presence of $OVA_{323–339}$ peptide (500 ng/ml) and increasing amounts of TGFβ1. Foxp3GFP expression was analyzed on day 5 by flow cytometry. Total cell number was counted, and the number of Foxp3$^+$ cells was calculated and plotted. (**A**) Representative FACS plots showing Foxp3GFP induction among OTII CD4$^+$ T cells. (**B** and **–C**) The percentage and absolute number of Foxp3GFP$^+$ cells per well after 5 day culture. (**D**) CD8α$^-$ DCs were mixed with CD8α$^+$ DCs at indicated ratios to stimulate OTII CD4 T cells in the presence of TGFβ (2 ng/ml). Total number of DCs per well was kept constant. Foxp3GFP expression was analyzed as above. All conditions were performed in duplicate wells and reported as mean ± SEM. Shown were representative results of three independent experiments.

twice with complete RPMI medium (supplemented with 10% FCS), and resuspended at 1 million per ml.

3. Converted Foxp3GFP$^+$ aTregs from conversion cultures are FACS sorted on day 5 based on GFP expression. Cells are then resuspended in complete RPMI medium at various concentrations so that when 50 μl cells is added to the wells, the ratio of aTreg to Teff will be at 1:1, 1:2, 1:4, and 1:8.

3.4.3. Set Up the Suppression Assay Culture

To wells of a round-bottomed 96-well plate

1. Add 50 μl (150,000 cells) of T cell-depleted splenocytes (3×10^6 cells/ml).
2. Add 50 μl (50,000 cells) of CFSE-labeled effector CD4 T cells (1×10^6 cells/ml).
3. Add 50 μl of Foxp3$^+$ aTregs at various concentrations (*see* **Note 12**).
4. Add 50 μl complete RPMI medium supplemented with antigenic peptide ISQ (4 μg/ml) (*see* **Note 13**).

5. Culture for 72 h at 37°C/5% CO_2.
6. Analyze proliferation of effector CD4 T cells by monitoring CFSE dilution profiles. Cells are collected into a V-bottomed 96-well plate and T cells are stained using CD4-APC prior to being analyzed by flow cytometry. An example of the suppressive function of aTregs is shown in **Fig. 26.2**, reproduced from Wang et al. (15) (Copyright 2008 PNAS.)

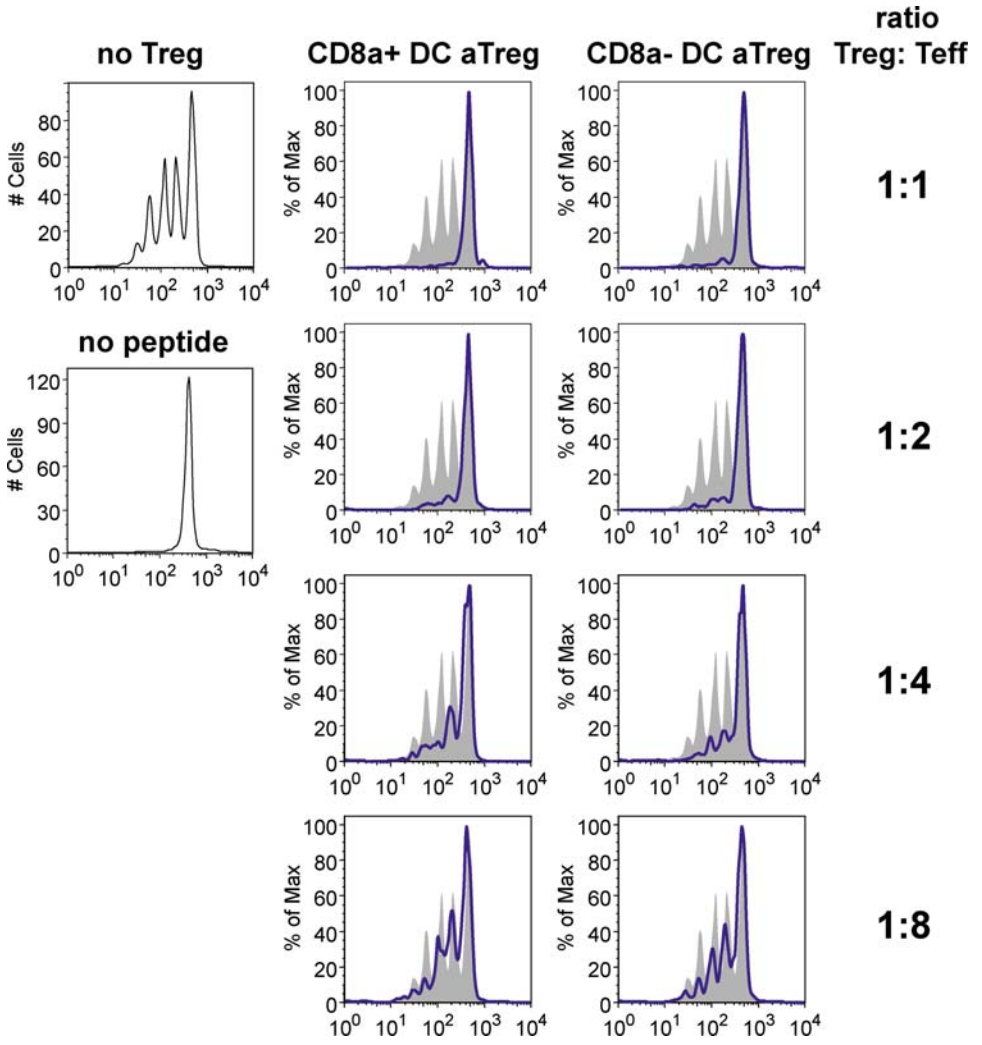

Fig. 26.2. Induced Foxp3$^+$ OTII cells from DC subset co-culture are suppressive in vitro. Foxp3GFP$^-$ OTII CD4 T cells were cultured for 5 days with CD8α^+ and CD8α^- splenic DC subsets in the presence of TGFβ (10 ng/ml). Induced Foxp3$^+$ OTII cells, designated as CD8α^+ DC aTreg and CD8α^- DC aTreg, were then sorted based on GFP expression. Congenically mismatched naïve CD25$^-$ CD4 OTII responder T cells (50,000) were CFSE-labeled and stimulated with spleen APCs (150,000) and antigenic peptide ISQ (1 μg/ml), in the presence of graded numbers of Foxp3GFP$^+$ aTregs. CFSE-dilution profile was analyzed after 3 days. Representative flow plots from duplicate wells were shown. Similar results were obtained from two independent experiments.

4. Notes

1. For staining with rat monoclonal antibodies, we recommend the addition of 10% heat-inactivated rat serum, which helps to reduce non-specific staining.
2. β-Mercaptoethanol is a critical component for murine T-cell cultures. Make 1 M stock in sterile H_2O, store at RT, and use it within 2 weeks.
3. Do not lyse red blood cells as it might affect cell viability as well as cause column clogging.
4. Alternatively, a negative enrichment strategy can be applied by depleting B cells and T cells using CD19 microbeads and anti-biotin microbeads (Miltenyi) bound with biotin-conjugated TCR antibody, prior to FACS sorting of the DC subsets. The advantage of this strategy is to generate "untouched" DCs.
5. The Miltenyi kit instruction suggests using 100 μl CD11c-microbeads per spleen. However, we have found that similar purity and yield could be achieved with much less amount of beads.
6. Keep buffer cold during the procedure to improve cell viability.
7. It is important to add this extra filtration step to remove any potential cell clumps that could clog the column, especially when more than 1X spleen worth of cells are loaded onto the column.
8. Dendritic cells are excellent antigen-presenting cells, albeit at limited numbers, especially for $CD8\alpha^+$ DCs. We typically obtain ~1 million of $CD8\alpha^+$ DCs from six to seven spleens and use at 1:1 ratio to CD4 T cells. But we have found that the ratio of DCs/CD4 T cells could go as low as 1:3 and still maintain good proliferative stimuli for the T cells.
9. The preparation procedure for cytokine stock solution affects their specific activity. Both human IL2 and recombinant TGFβ1 need to be dissolved in small amount of acidic buffer (i.e., 0.1 N HCl with 1 mg/ml BSA) prior to being diluted further to the working concentration, to achieve their maximum activity.
10. We titrate the amount of rabbit complement and anti-thy1.2 (HO13.4 ascites) for every new batch to achieve optimal depletion results with minimum toxic effects.
11. Carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) is dissolved in DMSO to make 10 mM

stock solution, aliquoted in small volume, and stored desiccated at –20°C. When needed, take one tube, thaw at 37°C, and use immediately. Avoid repeated freezing and thawing.

12. Replace with complete medium for the "no-suppression control well." Cells in this well will proliferate at maximum capacity.
13. Replace with complete medium without the peptide for the "no-proliferation control well." Cells in this well will not divide and their CFSE profile will represent the parental peak.

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Chapter 27

Generation of Parabiotic Mice for the Study of DC and DC Precursor Circulation

Claudia Waskow

Abstract

Dendritic cells (DCs) are the main regulators of adaptive immune responses and considerable interest currently focuses on the mechanisms of DC homeostasis. Understanding the mechanisms and regulation of DC generation may provide cues on how to modulate DC numbers and their longevity, an issue of wide interest in translational research. Surgical joining of the blood circulation of two mice (parabiosis) results in the equal distribution of lymphocytes between the parabiotic partners after a short period of time. In contrast, DCs fail to equalize even after prolonged periods of parabiosis suggesting that self-renewing precursors replenish mature DCs in situ. We have shown that mature DCs are constantly replenished by blood-borne precursors. Furthermore, the low but sustained exchange rate of DCs during parabiosis depends on *fms*-like tyrosine kinase 3 (Flt3)-mediated signals, which are important regulators for maintenance of normal DC numbers in the spleen by controlling their division in situ.

Key words: Parabiosis, flow cytometry, joined blood circulation, hematopoiesis, conventional dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs), Red-pulp macrophages (RP-Mp), polymorphonuclear neutrophils (PMN), monocytes, hematopoietic stem cells (HSCs).

1. Introduction

Dendritic cells (DCs) are crucial for innate and adaptive immune responses against pathogens and also maintain tolerance against self-tissues (1). Their pivotal role in controlling immune responses suggests that targeted regulation of DCs will provide therapies for dysfunctional immune responses. Activation and antigen presentation of DCs seem to prolong their life span suggesting a direct link between DC function and homeostasis (2–4).

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Thus, understanding mechanisms responsible for DC generation and maintenance may be a premise for regulating DC function.

DCs are descendants from bone marrow-resident hematopoietic stem cells (HSCs), and lineage-restricted progenitor populations have been identified in the BM (5–8) and spleen (9). DCs are short-lived and the entire compartment is renewed within 2 weeks (4), suggesting that DC homeostasis is a tightly regulated process. However, the regulation of DC development in the BM and in peripheral lymphoid organs is incompletely understood.

The union of two organisms by sharing a common blood circulation, parabiosis, allows the transfer of blood cells to the parabiotic partner, and the use of congenic mice permits tracking the origin of leukocytes. DCs never equilibrate during parabiosis, and the majority of the DC compartment is constituted of endogenous cells, thus, the minority of DCs are of exogenous origin, suggesting that in situ proliferating progenitors replenish the DC compartment (10). We could show that the failure of DCs to distribute equally is based on an incomplete exchange of blood-resident precursors and that the low but sustained DC chimerism in lymphoid organs reflects the DC precursor composition in the blood (4).

Using parabiosis we could also show that the growth factor receptor Flt3 is crucial for DC generation outside the BM (8). The development of HSCs and lineage-restricted macrophage DC progenitors (MDP) is independent from Flt3-mediated signals (8), and, accordingly, both cell types show a similar, very low exchange rate during parabiosis. Low replacement of wild-type DCs by $Flt3^-$ DCs in the spleen, but equal exchange of BM-resident HSCs and MDP during parabiosis of wild-type and Flt3-deficient mice suggested a regulatory role for Flt3 in DC development beyond the BM. We showed that Flt3 is required for normal DC homeostasis by regulating their division in the spleen (8).

In this chapter, we describe joining of the blood circulation and analysis between wild-type and Flt3-deficient mice. The origin of leukocytes is detected by differential expression of the CD45 alloantigen.

2. Materials

2.1. Mice

C57BL/6 (called B6 throughout) and congenic C57BL/6 Pep^{3b} (called B6/SJL throughout) mice were obtained from Jackson Laboratories or were bred at the Rockefeller University. B6 mice are genetically identical to B6.SJL mice except that B6 mice express the CD45.2 alloantigen and that B6/SJL mice express the CD45.1 alloantigen, which is expressed on all leukocytes.

Flt3$^-$ mice expressing the CD45.2 alloantigen were generated and provided by I. Lemischka (11). Mice were maintained in specific pathogen-free conditions, and mouse protocols were approved by the Rockefeller University Animal Care and Use Committee.

2.2. Anesthesia

To make the stock solution (100%) dissolve 10 g of 2,2,2-tribromoethanol (synonym: tribromoethyl alcohol, Fluka) in 10 ml 2-methyl-2-butanol (synonym: *tert*-amyl alcohol, Fluka). Dissolve 2,2,2-tribromoethanol by warming the mixture to 40°C, but prevent over heating. 2,2,2-Tribromoethanol is light sensitive, so the mixture should be covered with aluminum foil and kept at 4°C. The stock is good for ~ 2 years.

To produce the final anesthesia solution (2.5%) mix 0.25 ml of the stock solution by gentle stirring or inversion of the tube with 10 ml room temperature (RT) PBS (Gibco). To monitor the pH, any growth medium containing Phenol Red can be used as an alternative solvent. If the mixture becomes acidic (yellow), it should be discarded. Filter the final solution (0.2 μm) to remove undissolved crystals and warm to 37°C before injection. Wrap the final solution with aluminum foil, and store at 4°C. This solution can be used for ~1 week.

2.3. Surgery

a. Razor (Braun).
b. Prepare 70% ethanol and a piece of cotton wool.
c. Surgery tools: dissecting forceps (Aesculap), microforceps (Aesculap), scissors (straight Supercut iris scissors (Miltex)), straight Castroviejo needle holder with locking mechanism (Robbins Instruments), Autoclip wound clip applier and wound clips (9 mm, BD/Sparks), chromic gut surgical suture (Ethikon). For an overview of the surgery tools *see* **Fig. 27.1**.
d. Dry sterilizer (Germinator, WPI). The dry sterilizer with glass beads sterilizes microdissecting tools in seconds (260°C).
e. Sterile pads (Johnson&Johnson).
f. Prepare saline (0.9% NaCl, Baxter) for subcutaneous injection.
g. Pre- and post-surgery analgesic: Add ibuprofen into the drinking water at a concentration of 50 mg/l for 3 days after the surgery and then switch to 0.16 mg/l for the next 3 weeks. To adapt the mice to altered taste, add ibuprofen to the drinking water 3 days prior to the surgery.
h. Post-surgery anti-inflammatory treatment: Add neomycin to the drinking water at a concentration of 1.17 g/l. Neomycin is light sensitive; therefore, the use of dark brown drinking

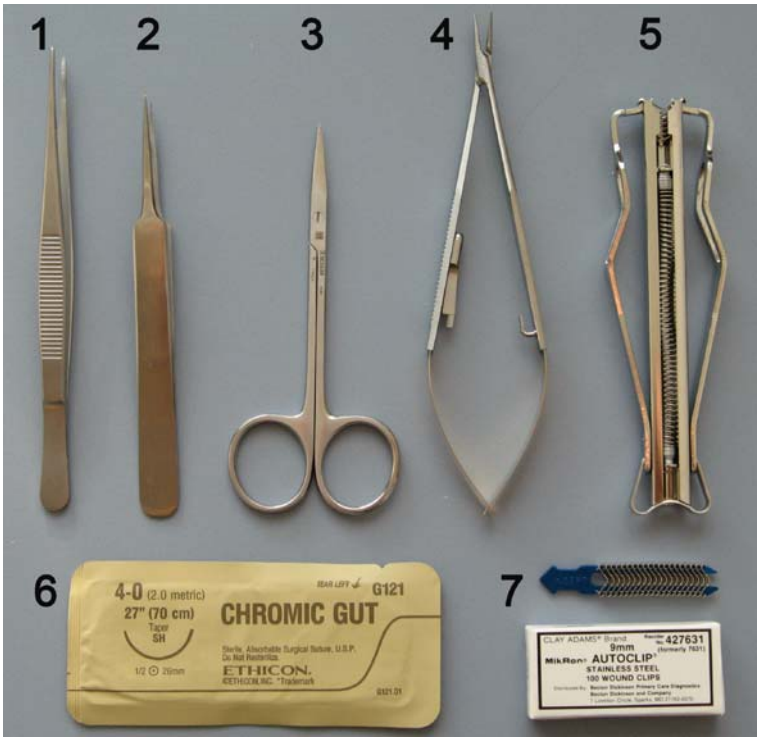

Fig. 27.1. Surgery tools. (1) Dissecting forceps, (2) microforceps, (3) supercut scissors, (4) Castroiejo needle holder, (5) wound clip applier, (6) surgical suture, and (7) wound clips.

bottles is recommended. Replace the drinking water every third day for 3 weeks after surgery.

2.4. Preparation of Cell Suspensions

a. Dissolve heparin (Biochrom) in sterile deionized water at a concentration of 5,000 U/ml to prepare a stock solution. Keep the stock solution at 4°C. Right before use, prepare the working solution by diluting the stock solution in PBS (1:10). Use 0.1 ml of the working solution to collect ~0.7 ml blood per mouse.

b. Use heparinized microhematocrit capillary tubes (Fisherbrand) to collect the blood out of the retro-orbital sinus of the mice.

c. Prepare PBS/5% FCS. To prevent complement-mediated lysis of cells, heat-inactivate the FCS in a water bath at 56°C for 45 min prior to use.

d. Glass slides with frosted ends (MarketLab) to allow gentle disruption of the spleen and lymph nodes.

e. Collagenase D (Roche). The specific activity of each lot of collagenase D is given on the homepage of Roche. Dissolve collagenase D powder in HBSS (w/o CaCl, w/o MgCl, w/o Mg sulfate, w/o Phenol Red, Gibco) to obtain a stock of 3 U/ml. Use collagenase D at a final concentration of 0.4

U/ml. Pour collagenase D on top of the HBSS and allow it to dissolve over night at 4°C in a beaker covered with aluminum foil. Afterward, sterilize collagenase D solution using a 0.2 μm filter, and store frozen at −20°C in 10-ml aliquots. Collagenase D can be thawed and refrozen once without considerable loss of activity.

f. 15 ml Falcon tubes (BD).

g. Pasteur pipettes.

h. Prepare 0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8.0 (Gibco).

i. Nylon filter mesh (*see* **Note 4**) (100 μm pore size, Sefar).

j. 23 gauge Needles.

k. 2 ml Syringes (BD).

l. ACK lysing buffer (Gibco).

2.5. Analysis by Flow Cytometry

a. Perform the staining procedure in PBS/5% FCS.

b. Use a Neubauer counting chamber/hemocytometer.

c. For blocking of unspecific binding sites, use purified rat gamma globulin (Dianova) at a final concentration of 25 μg per million cells in a 50 μl volume. To block the low affinity receptors for the mouse IgG Fc portion, use the monoclonal antibody 93 (eBioscience) or the 2.4G2 antibody (BD/Pharmingen) at a final concentration of 5–8 μg per million cells in a 50 μl volume. The low affinity receptors for the mouse IgG Fc portion are the FcγRII (CD32) and the FcγRIII (CD16) receptors, which are expressed by B cells, monocytes, macrophages, NK cells, and neutrophils.

d. The antibodies used for specific cell surface stainings are listed below. The optimal concentration for each antibody should be determined by titration, since there can be differences between each batch and each supplier. As a guideline, use the antibodies at 0.3–3 μg per million cells in 50 μl total staining volume. The clones of the monoclonal antibodies that recognize (*see* **Note 3**) the indicated antigen are in brackets.

BM: CD3 PE-Cy7 (2C11), CD11c PE-Cy7 (HL3) (BD/Pharmingen), Ter119 PE-Cy7 (Ter119), CD19 PE-Cy7 (1D3), B220 PE-Cy7 (RA3-6B2), NK1.1 PE-Cy7 (PK136), CD11b PE-Cy7 (M1/70), Gr-1 PE-Cy7 (RB6-8C5), Sca-1 PE-Cy5 (D7), Kit APC (2B8), CD115 PE (AFS98), CD45.1 FITC (A20), CD45.2 Alexa 700 (104) (eBioscience).

Spleen: CD3 APC (2C11), CD19 APC (MB19-1), NK1.1 APC (PK136), Ter119 APC (Ter119), F4/80 PE-Cy5 (BM8), CD11c PE-Cy7 (HL3), CD11b Alexa

750 (M1/70), Gr-1 pacific blue (RB6-8C5), I-Ab biotin (M5/114.15.2), CD45.2 Alexa 700 (104) (eBioscience), CD45.1 FITC (A20) (Pharmingen), PDCA-1 PE (JFOS-1C2.4.1) (Miltenyi).

Blood: CD3 PE-Cy7 (2C11), Ter119 PE-Cy7 (Ter119), NK1.1 PE-Cy7 (PK136), Gr-1 PE-Cy5.5 (RB6-8C5), CD11b Alexa 750 (M1/70), CD45.1 APC (A20), CD45.2 FITC (104) (eBioscience), CD19 PE-Cy7 (1D3) (BD/Pharmingen).

e. Use secondary reagents to detect biotinylated antibodies. Streptavidin (SA)-conjugated Pacific Orange (Molecular Probes) can be used as a secondary reagent at the final concentration of 0.001 μg per million cells in 50 μl total staining volume.

f. For analysis, use the LSRII FACS Scan (BD) with the following filter configuration:

Laser	Optical filter	Fluorochrome
Blue	530/30	FITC
Blue	582/42	PE
Blue	670/14	PE-Cy5
Blue	710/50	PE-Cy5.5
Blue	780/60	PE-Cy7
Violet	525/50	Pacific Orange
Violet	450/50	Pacific Blue
Red	660/20	APC
Red	710/50	Alexa 700
Red	780/30	Alexa 750

g. Analyze the data with DiVa (BD) or FlowJo (TreeStar) software according to the manufacturer's instructions.

3. Methods

To establish parabiosis, use weight-matched mice as parabiotic partners. Suturing the skin of two mice together allows the formation of microvasculature at the site of inflammation. This permits the exchange of blood between both parabionts. Perform the surgery under general anesthesia to prevent exposing the animals to pain. To ensure rapid performance and a successful surgery, sterilize all materials before starting the anesthesia and

make them readily available for the procedure. Great care is to be taken to be certain that the anesthesia is effective at the start of the surgery.

3.1. Anesthesia

1. Before anesthesia is started, all materials should be readily available. Sterilize surgery tools in the dry sterilizer for ~2 min or in 70% ethanol for ~30 min. Keep the tools sterile by placing them on a sterile pad.
2. Weigh the animals and inject the calculated dosage of anesthesia. Inject 0.015–0.017 ml of final anesthesia solution per gram body weight into the peritoneal cavity (a 25 g mouse gets 0.4 ml). Warm the final anesthesia solution to ~37°C before injection.
3. Anesthesia works within 5–10 min and is sufficient when the animals do not respond after squeezing the paw. If necessary, inject more anesthesia solution (about 1/10th of initial dosage).

3.2. Surgery and Post-surgery Care

1. After the mice are anesthetized, shave the hair from the left side of the right parabiotic partner and from the right side of the left parabiotic partner.
2. Wipe the shaved skin with 70% ethanol.
3. Place the mice on sterile pads.
4. Lift the side skin with a pair of blunt forceps and cut the skin from the elbow joint of the forelimb to the knee joint of the hindlimb on each animal. For the left parabiotic partner, the incision is on the right side and for the right parabiotic partner, the incision is on the left side. Care should be taken not to injure the body wall.
5. Suture the ligaments of the two elbows together and then the ligaments of the two knees using chromic gut held by stitching an instrument tie. Stitching an instrument tie: Allow the short end of the thread to lie freely while holding the long end with the left hand. Form a loop by placing the needle holder on side of the thread away from the operator and wind the thread around the needle holder once. With the needle holder in the right hand, grasp the short end of the thread and pull it through the loop. Tighten the knot by gently pulling on both ends. Release the needle holder from the short end of the thread. Pull the long end of the thread toward the operator and form a loop around the needle holder by placing needle holder on side of thread toward the operator. Finish the knot by pulling the short end of the thread through the loop and tighten by gently pulling on both ends.

6. Lay the mice side-by-side so that there is contact between the body walls. The dermis of the parabiotic partners is pressed together excluding epidermal layers in the junction of the dermis.
7. Close the skin incisions with wound clips. A demonstration of how to close a wound with wound clips is given online on the homepage of the National Institute of Health: http://mammary.nih.gov/tools/mousework/cunha001/index.html.
8. Suture skin incisions at sites that are difficult to reach with the wound clip applier with chromic gut suture.
9. Directly after surgery, water consumption of the mice is reduced to approximately half of the normal amount. To prevent dehydration, inject 0.5 ml warmed (~37°C) saline subcutaneously into the neck. To inject, lift the skin with a pair of blunt forceps and then hold between two fingers. Saline is injected under the skin shortly below fingers.
10. To prevent pain after anesthesia, add an analgesic (ibuprofen) to the drinking water of the mice.
11. To prevent infection, add an antibiotic to the drinking water.

3.3. Preparation of Cell Suspensions

To determine the origin of distinct hematopoietic cell lineages in both parabiotic partners, prepare cell suspensions from collected blood, spleen, and bone marrow.

1. Retro-orbital blood collection. The peri-orbital sinus of the mouse is used as a source of venous blood, because the mouse has a large peri-orbital venous sinus that fills the bony orbit of the eye.
   a. Anesthetize both parabiotic partners with avertin (*see* above).
   b. Gently open the eyelid of the mouse with two fingers of the same hand.
   c. Collect blood by inserting a microhematocrit tube through the conjunctiva and into the orbital sinus by quickly rotating the tube. The eye is not damaged because the tube passes under the eye.
   d. Blood flows through the microhematocrit tube into an eppendorf tube containing 0.1 ml diluted heparin. Collected blood is transferred to 15 ml Falcon tubes.
2. Preparation of spleen cell suspension. Dendritic cells are tightly associated with connective tissue and other cells of the mononuclear phagocyte system and are only released upon digestion with collagenase D.

a. To collect the spleen, lay the mouse on its right side and make a small incision below the chest. Make a small incision through the body wall where the spleen can be seen.
b. Remove the spleen and place it in 3.5 ml PBS/FCS containing 0.5 ml collagenase D.
c. Cut the spleen into three pieces using glass slides.
d. Gently rub the spleen pieces between the frosted ends of two glass slides and then incubate the spleen for 40 minat 37°C to allow tissue to digest.
e. To disintegrate the spleen further, pipette the spleen pieces rigorously up and down using Pasteur pipettes.
f. Add 0.1 ml EDTA (final concentration: 12.5 mM) and incubate the spleen suspension for 5 more min at 37°C.
g. Pipette the disintegrated spleen up and down again and transfer it into a 15 ml Falcon tube by filtering through a mesh. The filter step removes remaining non-disintegrated connective tissue.

3. Preparation of Bone Marrow (BM) Cell Suspension.
   a. To collect the thighbone, place the mouse on its belly and make a small incision into the skin within the lower third of the back.
   b. Pull the skin apart (fingers) thereby removing the skin from the legs.
   c. Expose the femuras by removing the muscle tissue around it. For collection, cut the leg below the knee joint and gently pull the femura out of the hip joint and cut at its base.
   d. Remove the remaining muscle tissue and place the bone in PBS/FCS.
   e. To collect the marrow, cut both ends of the bone (epiphysis) and flush the marrow out with a syringe.
   f. To prepare a cell suspension, pipette the flushed marrow up and down once using a 23 gauge needle.
   g. Transfer the BM cell suspension into a 15 ml Falcon tube by filtering it through a mesh to get rid of small bone pieces.

3.4. Red Blood Cell Lysis

To facilitate analysis, remove the red blood cells (RBC) by lysis.

1. Spin down blood and BM and spleen cell suspensions (500 $\times$ *g* for 10 min) and discard the supernatant by inversion of the tube.
2. Resuspend BM and spleen cells in remaining buffer.

3. Add 0.5 ml ACK lysis buffer to BM and spleen cell suspensions and incubate at room temperature for 20 and 40 s, respectively.
4. Add 1 ml of ACK lysis buffer to the blood and incubate for 5 min at 37°C.
5. Fill the 15 ml Falcon tube with PBS/FCS and spin the cell suspensions down (500 × *g* for 10 min).
6. If RBC lysis worked poorly in the case of BM and spleen, repeat Steps 3 and 5.
7. In case of blood, repeat Steps 4 and 5 three to five times until the pellet appears light red.
8. The cells are now ready for further treatment.

3.5. Staining for Fluorescence-Associated Cell Scan (FACS)

In order to determine the origin of multiple cell lineages at the same time, perform multi-color flow cytometry.

1. To determine the appropriate number of cells for the staining procedure, count the cells using a Neubauer counting chamber.
2. Stain 1×10^6 cells for single color stainings (compensation controls), and stain the following cell numbers for each organ:

Organ	Cell number	Volume of staining solution
BM	1×10^7	0.15 ml
Spleen	2×10^7	0.15 ml
Blood	1×10^6	0.05 ml

   To facilitate the handling of numerous samples simultaneously, stain cells in a 96-well V-bottom plate.
3. To prevent unspecific binding, use total rat immunoglobulin (Ig) and a monoclonal antibody specific for CD16 and CD32 to block unspecific binding sites. Add rat Ig at a final concentration of 0.5 mg/ml (about threefold excess compared to staining antibodies), and dilute anti-CD16/32 50-fold. Incubate the cells for 30 min on ice.
4. Use the incubation time to prepare the antibody dilutions.
5. Antibody mixture for spleen staining: Because the analysis of myeloid cells is hampered by low frequencies, the sample will be electronically depleted of lymphocytes and remaining erythrocytes. Therefore, mix antibodies recognizing T cells (CD3), B cells (CD19), NK cells (NK1.1), and erythrocytes (Ter119) with antibodies specific for polymorphonuclear neutrophils (PMN, Gr-1), red-pulp macrophages (F4/80), plasmacytoid dendritic cells

(PDCA-1), conventional DC (CD11c, I-A^b), and monocytes (CD11b) in a total volume of 0.15 ml PBS/FCS. To discriminate between cells of each parabiotic partner, add antibodies specific for CD45.1 (wild-type) and CD45.2 ($Flt3^-$) alloantigens.

6. Antibody mixture for BM staining: To analyze the compartment of hematopoietic stem cells (HSCs), BM cells will be electronically depleted of mature blood cells. To this end, mix the antibodies specific for mature blood cells with the same fluorochrome together. This mixture constitutes the so-called lineage mix (Lin) and comprises antibodies against erythrocytes (Ter119), B cells (CD19, B220), T cells (CD3), NK cells (NK1.1), monocytes (CD11b), granulocytes (Gr-1), and dendritic cells (CD11c). Use antibodies recognizing the stem cell antigen (Sca-1) and the receptor kinase Kit to identify the so-called KSL (Kit^+ $Sca1^+$ Lin^-) population, which is strongly enriched in HSC activity (12). Add antibodies specific for CD115 to identify cells strongly enriched for dendritic cell progenitor potential (8). To discriminate between cells of each parabiotic partner, add antibodies specific for CD45.1 (wild-type) and CD45.2 ($Flt3^-$) alloantigens.
7. Antibody mixture for blood staining: To obtain clear FACS pictures from blood monocytes, mature blood cells will be electronically depleted using antibodies specific for mature cells in the same fluorochrome (T cells [CD3], B cells [CD19], NK cells [NK1.1], erythrocytes [Ter119]). To discriminate between PMN, inflammatory, and stationary monocytes, label the cells with antibodies specific for Gr-1 and CD62L. To discriminate between cells of each parabiotic partner, add antibodies specific for CD45.1 (wild-type) and CD45.2 (Flt3) alloantigens. Stationary or inflammatory monocytes are identified as $CD3^-$ $CD19^-$ $NK1.1^-$ $Ter119^-$ SSC^{lo} $CD11b^{hi}$ $CD62L^-$ $Gr\text{-}1^-$ or $CD3^-$ $CD19^-$ $NK1.1^-$ $Ter119^-$ SSC^{lo} $CD11b^{hi}$ $CD62L^+$ $Gr\text{-}1^-$ cells, respectively (4, 13, 14). Identify PMN as $CD3^-$ $CD19^-$ $NK1.1^-$ $Ter119^-$ $CD11b^{hi}$ $Gr\text{-}1^{hi}$ cells (8).
8. Spin the cells down (800 × *g*, 3 min, 4°C) and resuspend in the antibody mixture.
9. Incubate the cells for 40 min at 4°C in the dark.
10. Spin the cells down (800 × *g*, 3 min, 4°C). In case no secondary reagent is added to the cells, wash twice in 0.2 ml PBS/FCS. In case a secondary reagent is used (staining of spleen cells), resuspend the cells in the appropriate volume of staining buffer with SA-Pacific Orange.

11. Pass the cells again through a nylon mesh before analysis (pore size: 100 μm).

3.6. Flow Cytometry

1. Because a detailed description of data acquisition on the LSRII is beyond the scope of this manual, please refer to the manufacturer's instructions.
2. The gating strategy used to identify the nature and origin of myeloid cells in the spleen is shown in **Fig. 27.2**. The size gate (**A**) is placed after initial gating on mature cell types (colored) assuring the inclusion of all cells of interest (back-gating). Cells gated through the live gate are analyzed using side scatter width versus side scatter height, which allows to define single cells (exclusion of doublets, doublet discrimination gate) (**B**). Size and single-gated cells are analyzed for the expression of cell surface markers identifying T cells ($CD3^+$), B cells ($CD19^+$), NK cells ($NK1.1^+$), and erythrocytes ($Ter119^+$), and the fraction of

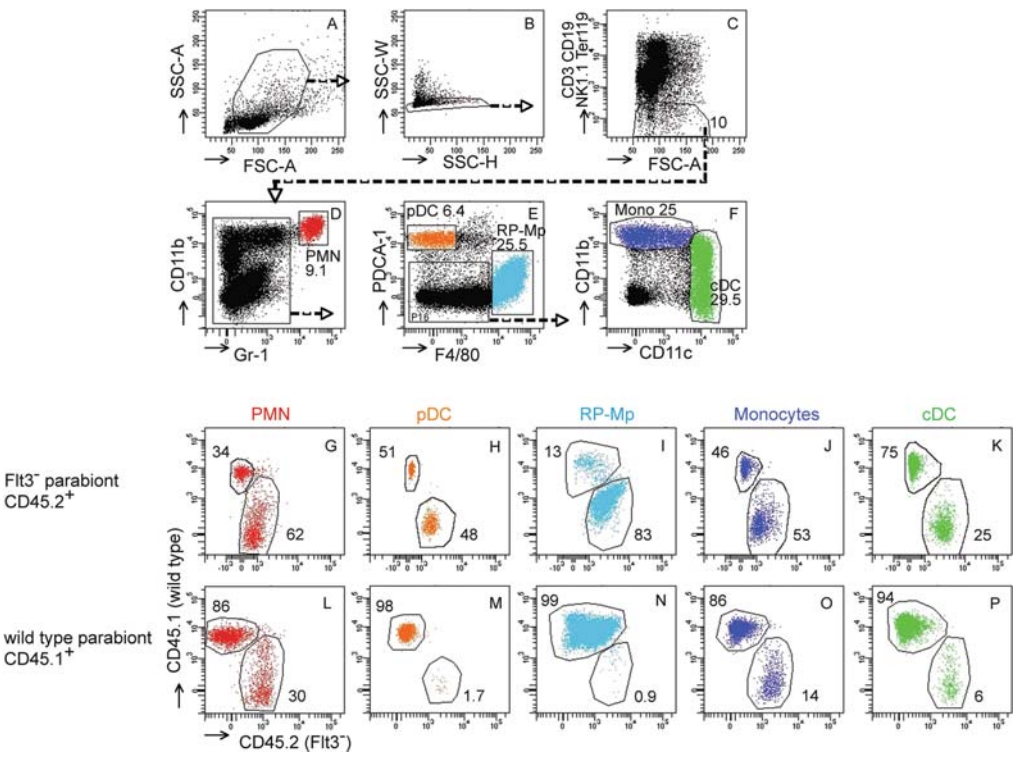

Fig. 27.2. Flow cytometry of spleen cells after 5 weeks of parabiosis between $Flt3^-$ and wild-type mice. Dot plots show spleen cells from $Flt3^-$ (**A**–**K**) and B6.SJL (**L**–**P**) mice stained with a cocktail of antibodies (*see* text). The origin of PMNs (**D**, **G**, **L**), pDCs (**E**, **H**, **M**), RP-Mps (**E**, **I**, **N**), monocytes (**F**, **J**, **O**), and cDCs (**F**, **K**, **P**) is determined in each parabiotic partner ($Flt3^-$ parabiotic partner, $CD45.2^+$ **G**–**K**; wild-type parabiotic partner, $CD45.1^+$ **L**–**P**). Hierarchical gating strategy is applied as indicated by the dotted arrows and the color code.

Table 27.1
Contribution of either parabiotic partner to the indicated hematopoietic cell type in the spleen (PMN, pDCs, RP-Mp, cDC) or BM (KSL, MDP). The analysis was performed after 5 weeks of parabiosis between wild-type B6 mice (*see* Note 1) and congenic wild-type B6/SJL mice. The origin of each cell type was determined by flow cytometry using distinct CD45 alloantigens (CD45.1 origin, upper rows; CD45.2 origin, lower rows). PMN, polymorphonucleated neutrophils; pDCs, plasmacytoid dendritic cells; RP-Mp, red pulp macrophages; Mono, monocytes; KSL, Kit$^+$Sca1$^+$Lin$^-$ cells; MDP, macrophage DC progenitors

Parabiotic partner	CD45 alloantigen	Hematopoietic cell type						
		Spleen					BM	
		PMN	pDC	RP-Mp	Mono	cDC	KSL	MDP
B6 (CD45.2)	CD45.1	33	9	4	38	15	0.2	0.5
	CD45.2	64	88	96	62	85	99.8	99.5
B6/SJL (CD45.1)	CD45.1	62	86	99	74	72	97	97
	CD45.2	36	12	1	25	27	2.8	3

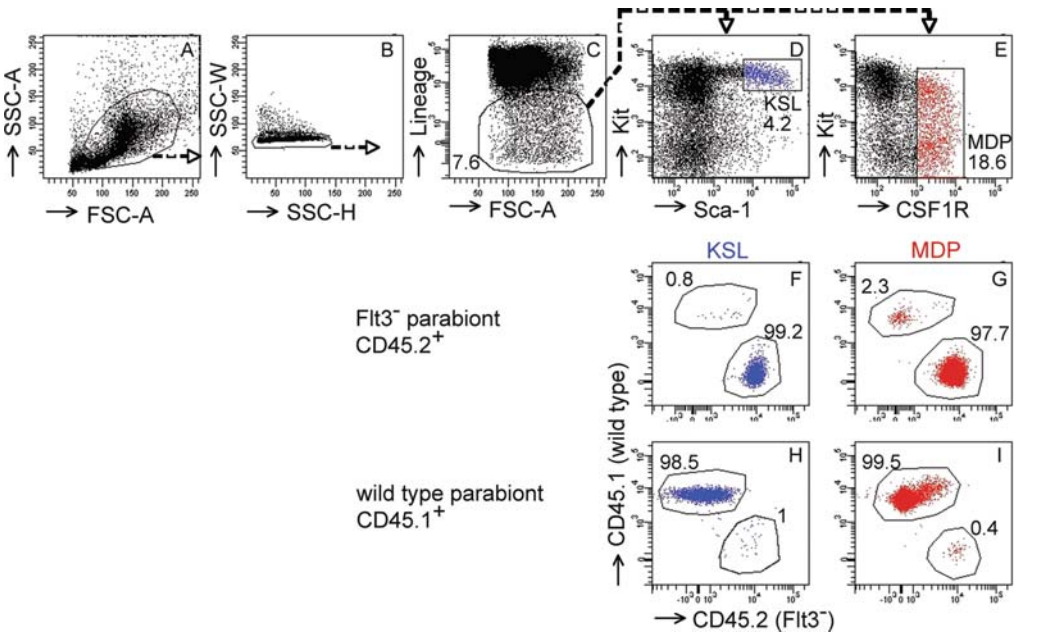

Fig. 27.3. Flow cytometry of BM cells 5 weeks after parabiosis between Flt3$^-$ (**A–G**) and wild-type (**H**, **I**) mice. Dot plots show BM cells stained with a cocktail of antibodies (*see* text) and the origin of the hematopoietic stem cells-containing fraction (KSL, Kit$^+$Sca1$^+$Lin$^-$, **D**, **F**, **H**) and macrophage dendritic cell precursors (MDP, Lin$^-$CSF1R$^+$, **E**, **G**, **I**) is determined in each parabiotic partner (Flt3$^-$ parabiotic partner, **F**, **G**; wild-type parabiotic partner, **H**, **I**). Cells derived from the Flt3$^-$ parabiotic partner express the CD45.2 alloantigen and cells derived from the wild-type parabiotic partner express the CD45.1 alloantigen. Hierarchical gating strategy is applied as indicated by the dotted arrows and the color code.

non-T, non-B, non-NK, and non-erythrocytes is gated for further analysis (gate in **C**). CD11b^{+}Gr-1^{hi} cells are PMN (**D**), and all cells minus PMN are subjected to further analysis (**D**). Plasmacytoid DCs are defined as PDCA-1^{+}F4/80^{-} (**E**) and RP-Mp as PDCA-1$^{-/lo}$F4/80^{+} cells (E). Non-pDC and non-RP-Mp are further analyzed for the expression of CD11b and CD11c (**F**). Conventional DCs are defined as CD11c^{hi}CD11b$^{\pm}$ cells (**F**) and monocytes are (*see* **Note 2**) defined as CD11b^{+}CD11c$^{lo/-}$ cells (**F**). The origin of each cell type is analyzed by the expression of the CD45.1 and CD45.2 alloantigens (**G–P**). For comparison, a quantification of cell exchange during parabiosis of wild-type mice is given in **Table 27.1**. The replacement of pDCs, cDCs, and monocytes in wild-type versus Flt3^{-} mice is different compared to wild-type parabionts (**Table 27.1**), suggesting an effect of Flt3 in the development and/or maintenance of those cell types.

3. The gating strategy used to identify the nature and the origin of HSCs (12) and MDP (8) is shown in **Fig. 27.3**. The placement of the size and doublet discrimination gate is performed as described for spleen cells (**Section 3.6**, Item 2). Size (**A**) and singlet-gated cells (**B**) are analyzed for the expression surface markers identifying mature hematopoietic cells (T cells (CD3), B cells (CD19, B220), NK cells (NK1.1), erythrocytes (Ter119), cDC (CD11c), PMN (Gr-1), and monocytes (CD11b)) (**C**). Immature cells (Lin^{-}) are gated and analyzed for the expression of Kit and Sca–1 (D) or Kit and the CSF1R (**E**). The origin of KSL (**D**, **F**, **H**) and MDP (**E**, **G**, **I**) is determined by analyzing the expression of the CD45.1 and CD45.2 alloantigens (**F–I**). For comparison, the exchange rate of KSL and MDP after parabiosis of congenic wild-type mice is given in **Table 27.1**. There is no difference between the exchange rate of KSL and MDP after parabiosis of congenic wild-type mice compared to exchange rate after the parabiosis of wild-type and Flt3^{-} mice.

4. Notes

1. In the example shown, the blood circulation of the mice was joined for 5 weeks. After approximately 30 days the maximal equilibration of all cell types between parabionts is reached in blood, spleen, and lymph nodes (4). Thus, parabiosis of 5 weeks provides sufficient time to warrant complete exchange of all cell types.

2. To allow discrimination between live and dead cells the nucleic acid-binding dye 4′,6-diaminidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes) can be used (preferentially binds dsDNA). However, using electronic depletion of mature blood cells frequently results in the absence of $DAPI^+$ cells, because dead cells take up fluorochrome-coupled antibodies non-specifically and are therefore segregated together with the mature cells. The maximum excitation/emission wavelength for DAPI is 358/461 nm, and $DAPI^+$ cells are read using the ultra violet (UV) laser. DAPI measurement fails to interfere with the excitation/emission spectra of other fluorochromes.
3. We found the mentioned antibodies to be excellent for flow cytometry. Numerous competitive reagents are available from other commercial sources.
4. The mesh used for filtration of the cells has to be cut into small pieces and can be autoclaved. A commercially available sterile mesh attached to a holder is available from other commercial sources.

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Chapter 28

Defining In Vivo Dendritic Cell Functions Using CD11c-DTR Transgenic Mice

Liat Bar-On and Steffen Jung

Abstract

The study of dendritic cell involvement in complex phenomena that rely on multi-cellular interactions, such as immune homeostasis, stimulation, and tolerization, called for the investigation of dendritic cell functions within physiological context. To this end we have developed a conditional cell ablation strategy that is based on dendritic cell-restricted expression of a Diphtheria Toxin receptor (DTR) using the CD11c/*Itgax* promoter. Here, we provide basic protocols that describe the use of this prototypic dendritic cell ablation model and highlight pitfalls and strengths of the approach.

Key words: Dendritic cells, Diphtheria Toxin, cell ablation.

1. Introduction

Much of our knowledge about DC biology is based on observations made with in vitro generated DCs and these systems continue to provide exciting insights into the unique cell biology of DCs (1–3). In parallel intra-vital imaging approaches (4) and genetic strategies (5–7) have been developed to study DC involvement in complex multi-parameter-driven physiological processes, such as immune responses, the establishment of self-tolerance or its failure resulting in autoimmunity, as well as other hitherto unappreciated processes (8, 9). A major challenge, when moving into the intact organism is the fact that DCs have to be identified in the context of other cells. Furthermore, the in vivo DC compartment itself is characterized by considerable heterogeneity. In the past, DC definitions and classification schemes

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were largely based on phenotypic features, like the expression of surface markers, such as CD8α, and anatomic location in lymphoid and non-lymphoid organs. More recently, major advances in our understanding of DC in vivo differentiation pathways have led to DC genealogy tree charts based on distinct origins (10, 11). However, arguably the ultimate evidence to categorize a certain cell type within the DC family and define DC subsets derives from function-based definitions. Accordingly, the classical experiments of Steinmann et al. defined DCs as "potent stimulators of the primary leukocyte reaction" (12) and ever since their superior ability to act as APC in the priming of naïve T cells has remained the best criterion to distinguish DCs from close relatives, such as macrophages. To achieve a similar function-based DC definition in physiological context we generated a transgenic mouse model that allows the conditional DC ablation in the intact organism. These CD11c-DTR mice harbor a human Diphtheria Toxin (DTx) receptor (DTR) transgene under the CD11c (*Itgax*) promoter (5).

Cytotoxicity of the *Corynebacterium diphtheriae*-derived heterodimeric Diphtheria Toxin (DTx) is strictly dependent on receptor-mediated endocytosis by a cellular DTx receptor (DTR), the heparin-binding EGF-like growth factor (hbEGF) (13) (**Fig. 28.1A**). The critical need of this host component for toxicity is highlighted by the fact that murine cells, which harbor an hbEGF polymorphism that impairs DTx binding, are insensitive to killing by the toxin (14). Moreover, mere transgenic expression of a primate hbEGF with high affinity for DTx renders naturally DTx-resistant mouse cells DTx-sensitive (5, 15). The cell surface

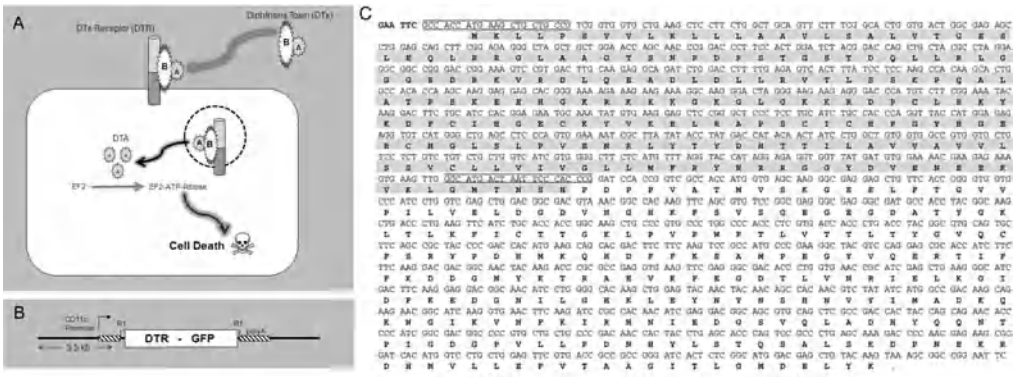

Fig. 28.1. **(A)** Scheme illustrating the obligatory receptor-mediated Diphtheria Toxin (DTx) uptake in cells expressing a high-affinity DTR (heparin-binding EGF-like growth factor (hbEGF). Endosomal release of the DT A subunit blocks protein synthesis. (**B**) Schematic illustration of the CD11c-DTR transgene with the DTR–GFP cassette inserted as an EcoR1 fragment into a rabbit β-globin gene fragment providing the cDNA with an intron and a polyadenylation signal (*see* **Ref.** (17)). (**C**) Sequence of the DTR–GFP cassette, indicating the location of the PCR primers used for the identification of CD11c-DTR transgenic mice (*see* **Note 1**). Bold amino acid sequence: Human hbEGF gene/ DTR (Gene bank accession no. NM_001945 (grey boxed); GFP gene. . Bold nucleotide sequence – EcoR1 restriction sites, framed nucleotide sequence, location of PCR primers.

DTR binds the B subunit of DTx and promotes DTx endocytosis. Upon entry into the cytoplasm, the DTx-A subunit catalyzes an inactivating ADP-ribosylation of elongation factor 2 resulting in termination of protein synthesis and apoptotic death of the target cell within hours. A single molecule of active DTx-A in the cytoplasm is sufficient to kill a eukaryotic cell (16) making DTx-mediated cell ablation very sensitive and efficient.

CD11c-DTR mice harbor a gene that encodes the human DTx receptor as a GFP fusion under the control of the CD11c/*Itgax* promoter (**Fig. 28.1B**, **C**). DTx injection of CD11c-DTR mice results in the rapid ablation of DTR–GFP$^+$ cells (**Fig. 28.2**). The α_x subunit of the β integrin CD11c is expressed on all DCs and the CD11c promoter has been exploited by various researchers to target transgenes to the DC compartment (4, 17). Notably, however, while the 5.5-kbp promoter fragment of the *Itgax* gene had been considered to be DC-specific (17), closer evaluation of the CD11c-DTR transgenic mice revealed additional activity in defined

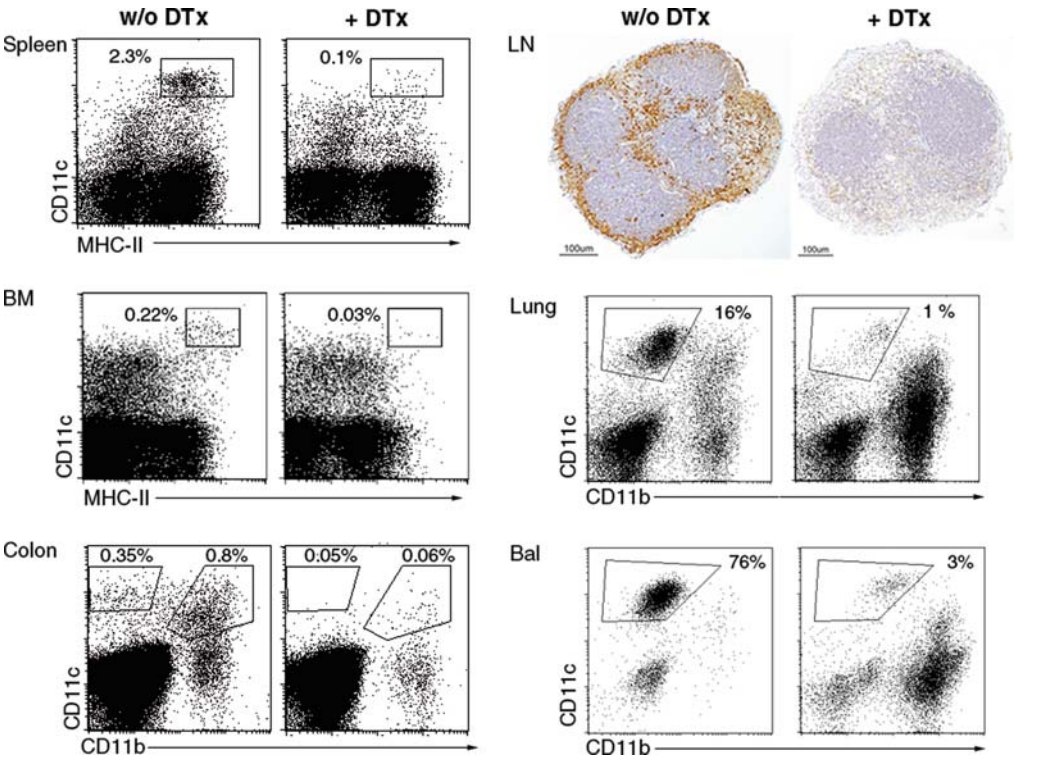

Fig. 28.2. In vivo depletion of CD11c^{high} cells in CD11c-DTR transgenic mice. Flow cytometric analysis of untreated controls and CD11c-DTR transgenic C57BL/6 mice 1 day after injection of DTx (4 ng/body weight). Colon, lung, and BAL cells were analyzed for CD11c and CD11b expression. Spleen and BM cells were analyzed for CD11c and MHC-II expression. Numbers indicate percentage of gated cells from total cells. Immunohistochemistry of popliteal LNs from untreated controls and CD11c-DTR transgenic C57BL/6 mice 1 day after footpad injection of 20 ng DTx. Sections are stained with anti-GFP antibody (brown) (Reproduced with permission from Ref. (23)).

Table 28.1
Toxin sensitivity of mononuclear phagocytes in CD11c-DTR mice*

Organ	DTx-sensitive	DTx-resistant
Spleen	Conventional DC (cDC) (incl. $CD4^+$, $CD8^+$, and DN cDC) Metallophilic MΦ Marginal zone MΦ	Plasmacytoid DC (80%) Monocyte-derived $CD11c^{int}$ DC Red pulp MΦ
BM	$F4/80^+$ bmDC	Perivascular MΦ
Lung	$CD11c^+CD11b^+$ DC $CD11c^+$ alveolar MΦ	
Intestinal lamina propria	$CD11c^{high}CD11b^+$ DC $CD11c^{high}CD11b^-$ DC	$CD11c^-$ $CD11b^+$ MΦ
Skin		Langerhans cells

*Note that in addition to the above-mentioned DC and macrophage populations, the 5.5-kbp CD11c promoter fragment is also transcribed in activated T cell, NK cell subsets, and plasmablasts.

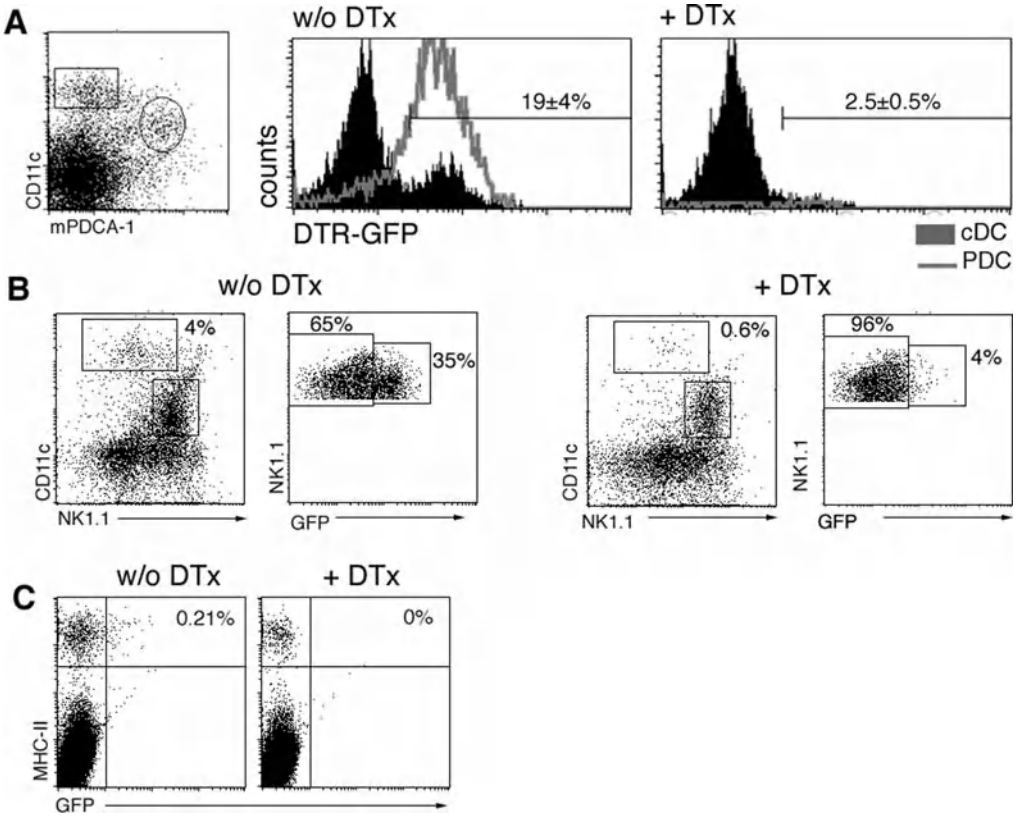

Fig. 28.3. DTx resistance of CD11c-expressing non-cDC in CD11c-DTR transgenic mice. (**A**) GFP expression profiles of splenic $CD11c^{high}$ conventional DC and PDC isolated from CD11c-DTR tg mice that were untreated or treated with DTx. cDCs are gated as $CD11c^{high}$, mPDCA-1^{neg} cells; PDCs are defined as $CD11c^{int}$ mPDCA-1^{pos} cells. Note that the $CD11c^{int}$ PDC population is split into a DTR–GFP^+ DTx-sensitive and DTR–GFP^- DTx-resistant subsets (reproduced with permission from Ref. (23)). (**B**) Flow cytometric analysis of uterine tissue from CD11c-DTR tg mice that were untreated or treated with DTx. Uterine DCs were gated as $CD11c^{high}$ cells, uNK cells were defined by the expression of the pan-NK marker, NK1.1. Note that uNK are homogeneous $CD11c^{int}$, but only a fraction of them expresses the DTR–GFP transgene and is hence DTx-sensitive (9).

macrophage populations, such as alveolar macrophages (18, 19), as well as splenic marginal zone and metallophilic macrophages (20) (*see* **Table 28.1**). Moreover, the *Itgax* promoter is also active in a subset of activated T cells (21), NK cells, and plasmablast (22). On the other hand, the specific *Itgax* promoter fragment used for the generation of the CD11c-DTR transgenic mice (5) seems to lack control elements required for expression in all cells that express CD11c. Thus, the DNA fragment is inactive in some cells, which transcribe their endogenous *Itgax* alleles, including monocyte-derived splenic $CD11c^{int}$ cells (10), $CD11c^{int}$ PDCs (23), and most NK cells (9, 24) (**Fig. 28.3A**, B). Collectively, these observations highlight the fact that when investigating DC functions with the CD11c-DTR mice, one should perform a thorough analysis of the cell types that are targeted in a given tissue. This can be readily achieved through flow cytometric or histological analysis of the expression pattern of the DTR–GFP fusion protein (**Fig. 28.2**). CD11c-DTR mice have become a popular addition to our toolbox to study in vivo DC function (for a recent summary *see* Ref. (25)). Below we provide basic protocols that outline the use of the CD11c-DTR system highlighting the strengths and limitations of the model.

2. Materials

2.1. Animals

Our approaches involve the use of the following mouse strains (*see* **Note 1**).

1. CD11c-DTR transgenic mice (B6.FVB-Tg(Itgax-DTR/GFP)57Lan/J, Jackson laboratory, stock # 004509) that carry a human Diphtheria Toxin receptor (DTR)–GFP transgene under control of the murine CD11c promoter (5).
2. OT-I TCR transgenic mice (C57BL/6) harboring OVA-specific $CD8^+$ T cells that recognize the OVA peptide SIINFEKL in $H2^b$ context (26).
3. OT-II TCR transgenic mice (C57BL/6) harboring OVA-specific $CD4^+$ T cells that recognize the $OVA_{323–339}$ epitope in $I\text{-}A^b$ context (27).

2.2. Media

1. Diphtheria Toxin (DTx; Sigma), 1 mg/ml in PBS, stored in 10 μl single-use aliquots at −80°C. Aliquots are diluted in PBS prior to use.
2. Collagenase D (Roche, Germany), 200 mg/ml in PBS, stored in 20 μl (4 mg) single-use aliquots at −20°C. Aliquots are diluted in PBS with Ca^{+2} and Mg^{+2} ($PBS^{+/+}$) prior to use, to achieve 1 mg/ml working solution.

3. Collagenase type VIII (digestion of intestinal tissue), 1.5 mg/ml in $PBS^{+/+}$ (Sigma Aldrich, Germany).
4. Pre-digestion solution: HBSS, 2 mM EDTA, 1 mM DTT, 5% FBS.
5. Uterine digestion buffer, collagenase type IV 1 mg/ml (Sigma), 0.2 mg/ml DNase (Roche), 1 mg/ml bovine serum albumin (Sigma) in $PBS^{+/+}$.
6. ACK buffer, 0.15 M NH_4C, 0.01 M $KHCO_3$, 8 g of NH_4Cl/1 g of $KHCO_3$ (Merck, Germany)/1 l DDW. Stored in 50-ml aliquots at −20°C.
7. 1 mM DTT (Sigma Aldrich, Germany), 0.1543 mg DTT/ml in PBS without Ca^{+2} and Mg^{+2} ($PBS^{-/-}$)
8. FACS buffer, 1% fetal bovine serum (FBS), 2 mM Na_2EDTA (Merck, Darmstadt, Germany), 0.05% sodium-azide (Merck, Darmstadt, Germany). Fluorochrome-conjugated antibodies used include CD11c-APC or CD11c-PE (eBioscience, clone N418), MHCII-PE (eBioscience, clone M5/114.15.2), mPDCA (eBioscience, clone eBio927), and CD11b-APC (eBioscience, clone M1/70), stored at 4°C. Dilutions are performed in FACS buffer.
9. FACS-blocking reagent: anti-mouse CD16/32-FC (Fc) block (eBioscience, clone 93) or goat IgG (Sigma Aldrich, Israel), stored at 4°C.
10. Magnetic cell sorting (MACS) buffer, 1% fetal bovine serum, 2 mM Na_2EDTA (no azide!) (5 ml of 100 mM EDTA is added to 10 ml of FBS, incubated for 10 min at RT, and then added to 500 ml of PBS without Ca^{++} and Mg^{++} ($PBS^{-/-}$), sterile filtered and degassed.
11. Magnetic cell sorting (MACS) instruments and reagents (Miltenyi Biotec, GmbH, Germany): MiniMACS separator, MS columns, and CD4 microbeads, which should be stored at 4°C and protected from light are used according to the manufacturer's protocols.

3. Methods

Conditional ablation of $CD11c^{high}$ DCs is achieved using a Diphtheria Toxin (DTx)-based cell ablation strategy (5). After entry into the cytoplasm the Diphtheria Toxin A subunit inactivates elongation factor 2 by catalyzing its ADP-ribosylation. The resulting blockade of protein synthesis triggers programmed cell death, with dying cells silently removed by neighboring macrophages.

3.1. Conditional Short-Term Ablation of CD11c^{high} DC

1. For systemic short-term depletion of CD11c^{high} DCs, mice are injected once intra-peritoneally (i.p.) with 4 ng/g body weight DTx (5).
2. Eight hours after i.p. injection, toxin-induced depletion of CD11c^{high} DCs is detected in the spleen, intestine, BM, and LNs (**Fig. 28.1**). cDC depletion persists for 2 days, after which cDC numbers are gradually restored (5).
3. Notably, other cells except DCs, which make use of the *Itgax* promoter are deleted in CD11c-DTR transgenic mice upon administration of DTx (**Table 28.1**). These include alveolar macrophages (18, 19), splenic metallophilic and marginal zone macrophages (20, 22), activated CD8$^+$ T cells (unpublished observation), a subset of PDCs (23), and a subset of uterine NK cells (9). Cells which are sensitive to DTx can be detected by flow cytometric or histological analysis for expression of the DTR–GFP (**Fig. 28.2**).
4. On the other hand, the 5.5-kbp *Itgax* promoter fragment (17) is not active in all CD11c-expressing cells, including monocyte-derived splenic CD11c^{int} cells (10), CD11c^{int} PDCs (23), and most NK cells (9, 24), which transcribe their endogenous *Itgax* alleles, but are resistant to DTx (**Fig. 28.2**; **Table 28.1**).
5. Notably, CD11c-DTR mice as such do not allow for prolonged DC ablation due to the fact that repetitive DTx application results in adverse side effects (28, 29). If the experimental set up allows, this problem can be overcome by use of mixed bone marrow (BM) chimeras generated by reconstitution of lethally irradiated wild-type mice with CD11c-DTR tg BM (28, 29) (*see* **Section 3.2**). An alternative way to circumvent DTx-induced lethality is the development of protocols that aim at local ablation by restricted toxin delivery, as exemplified by the ablation of alveolar macrophages through intra-tracheal installation of DTx (18, 19) and the ablation of DC from skin-draining LNs (23).

3.2. Conditional Long-Term Ablation of CD11c^{high} DC

1. In contrast to CD11c-DTR transgenic mice, syngeneic [DTR > wt] BM chimeras, generated by reconstitution of lethally irradiated wt-recipient mice with CD11c-DTR BM, can be treated with DTx for prolonged periods of time without adverse side effects (28, 29). Moreover, the generation of mixed BM chimeras with wt, mutant, and CD11c-DTR transgenic BM can be a powerful means to investigate molecular contributions of DCs (as exemplified in Ref. (8)).

2. BM chimera generation: C57BL/6 WT mice are exposed to a single-lethal total body irradiation of 950 rad, followed by intra-venous (i.v.) transfer (*see* **Note 2**) of 5×10^6 CD11c-DTR transgenic BM cells one day after. Mice are allowed to rest for 8 weeks before use (*see* **Note 3**).
3. For systemic long-term DC depletion [DTR > wt] BM chimeras are injected every other day i.p. with DTx (8 ng/g body weight).

3.3. Analysis of Depletion Efficiency by FACS

1. Tissues to be examined are isolated, single-cell suspensions are prepared (*see* Sections **3.3.1 and 3.3.2**) and stained with fluorochrome-conjugated monoclonal antibodies (*see* **Notes 4 and 5**).
2. Prior to the staining, cells are incubated with a 1:100 dilution of goat IgG or FC block, for 15 min on ice to block Fc receptors (staining volume ~10 μl/10^6 cells).
3. Cells are then stained for cell-specific markers. We routinely add a 2X concentrated mix of fluorochrome-conjugated CD11c and MHCII or CD11b antibodies and keep the cells for 15 min on ice (final staining volume ~ 20 μl/10^6 cells). All dilutions are performed in FACS buffer.
4. Following staining, cells are washed with FACS buffer (~50X staining volume) and centrifuged at 1,200 rpm for 7 min at 4°C.
5. Cells are resuspended in FACS buffer and analyzed by multicolor flow cytometry on an FACS Calibur cytometer (BD Biosciences) using CellQuest Pro software (BD Biosciences) (**Fig. 28.1**).

3.3.1. Lymphoid Organs (Spleen, LN, BM, Thymus)

1. Mice are sacrificed and the spleen, LNs, BM, and thymus are removed and placed in PBS on ice.
2. The efficient isolation of DCs requires, as opposed to lymphocytes, prior digestion of the tissues with matrix-degrading proteases. To this end the spleen and thymus are injected with 1 ml of collagenase D (1 mg/ml in $PBS^{+/+}$) using a 28-ga 1/2 in needle and incubated for 45 min at 37°C. LNs were mechanically disrupted into 300 μl of the same collagenase D solution for 45 min at 37°C. Collagenase digestion is not required for isolation of BM cells. For BM cells isolation protocol *see* **Note 6**.
3. Following collagenase D digest, spleen, thymus, and LNs are minced and transferred via passage of an 80-μm mesh strainer into a 15-ml tube.
4. The cell suspension is then washed with 10 ml of $PBS^{-/-}$ and centrifuged at 1,200 rpm, 4°C, for 7 min.

5. Erythrocytes in the spleen and BM cell suspensions are lysed by incubation with 1 ml of hypotonic ACK buffer for 2 min at room temperature (RT). Lysis is stopped by dilution of the ACK buffer with $PBS^{-/-}$ (tenfold volume at least). The suspension is then centrifuged and the pellet is washed.

3.3.2. Analysis of Uterine Dendritic Cells

1. Uterine horn are minced into fragments of 1 mm^3 and digested for 35 min at 37°C using uterine digestion buffer. Then, tissues are passed through a mesh and washed in PBS (9).

3.3.3. Analysis of Intestinal Lamina Propria Dendritic Cells

1. Mice are sacrificed and the intestines are isolated. By choosing proximal, central, or distal portions of the intestine the analysis can be focused on the *duodenum, jejunum, ileum,* and *colon*, respectively.
2. Mesenteric tissue (lymph nodes) and fat are removed.
3. The intestine is flushed with $PBS^{-/-}$ to remove all its fecal content.
4. The intestine is opened longitudinally and cut into 0.5-cm pieces.
5. The pieces are incubated in pre-digestion solution for 20 min at 37°C. Incubation is repeated once more, using a fresh solution and a new 50-ml tube. This incubation removes the epithelial cell layer (30).
6. The pieces are incubated for 20 min in 5 ml of digestion solution. This stage is performed at 37°C under rotation (250 rpm) in a thermal incubator.
7. After incubation the cell solution is being vortexed intensively for 30 s and passed through an 80-μm mesh strainer into a 15-ml tube. The dish is washed with $PBS^{-/-}$ in order to collect all released cells.
8. The cells are centrifuged at 1,200 rpm, 10 min, 4°C.

3.3.4. Analysis of Dendritic Cells in the Broncho-Alveolar Space and the Lung Parenchyma

1. Mice are sacrificed, their abdomen is opened and the main artery is cut to allow a maximum blood volume to exit the circulation (*see* **Note 7**).
2. The mouse head is fixed on a Styrofoam board and the neck-skin and salivary glands are removed to expose the trachea.
3. The thin tissue layer covering the trachea is carefully removed using watch-maker forceps and scissors. The trachea rings are now exposed.
4. A 20-cm long sewing thread is placed beneath the trachea and tied around it in proximity to the mouse head.

Holding the string will allow to stretch the trachea for the next stages.

5. Using fine scissors a small hole is cut in the upper side of the trachea, next to, but below, the string.
6. A blunted (cut) 21-ga butterfly needle is inserted into the hole in trachea, and a 3-ml syringe, filled with 1.5 ml of $PBS^{-/-}$, is attached to the needle.
7. A volume of 1 ml $PBS^{-/-}$ is slowly inserted into the mouse trachea through the infusion needle, resulting in the filling of the lungs with liquid.
8. The broncho-alveolar lavage (BAL) containing the cells is slowly withdrawn by pulling the syringe plunger.
9. The syringe is then removed from the infusion needle tube and the BAL is transferred into a 15-ml tube.
10. Steps 7–9 should be repeated three times.
11. The isolation procedure for DCs from the lung parenchyma is similar to the procedure for the splenic DCs, except that the lung tissue is injected with 1 ml of collagenase D solution (4 mg/ml in $PBS^{+/+}$).

3.4. Functional Assays for Absence of Conventional DC

To obtain a functional confirmation for the absence of cDCs upon DTx treatment, cell suspensions of different tissues from DTx-treated CD11c-DTR mice can be tested for their ability to stimulate a mixed leukocyte reaction (MLR), as compared to untreated controls (5).

3.4.1. Mixed Leukocyte Reaction (MLR)

1. Stimulator cells are isolated from spleens, LNs, thymi, and BM of DTx-treated and non-treated CD11c-DTR transgenic C57BL/6 mice ($H2^b$) after collagenase digestion.
2. Responder $CD4^+$ T cells are enriched from spleens of BALB/c mice ($H2^d$) by positive selection with anti-CD4 microbeads according to the manufacturer's protocol (*see* **Notes 8 and 9**).
3. 5×10^5 stimulator cells are irradiated (2,500 rad) and cultured with MHC-mismatched 1×10^5 responder $CD4^+$ T cells (f.i. BALB/c). Cultures are pulsed after 72 h with 1 μCi of [H^3]. Thymidine incorporation is measured 16 h later.

3.4.2. Priming of Antigen-Specific T Cells

1. Ovalbumin (OVA)-specific TCR transgenic $CD8^+$ and $CD4^+$ T cells are isolated from spleens and LNs of OT-I and OT-II mice, respectively (26, 27), enriched by MACS cell sorting with anti-CD8 or anti-CD4 antibodies according to the manufacturer's protocol.

2. Donor mice should carry an allotypic marker, such as CD45.1 that will allow detection of the transferred cells in the recipient mice. Before transfer, the T cells are labeled with the intra-cellular dye CFSE, which allows the monitoring of in vivo proliferation (31). 2×10^6 T cells are co-transferred into WT and CD11c-DTR transgenic C57BL/6 mice. The grafted mice are treated with DTx and, 8 h later, immunized by i.v. injection of soluble OVA (10 μg) or OVA-loaded splenocytes (32).
3. Four days after immunization, the mice are sacrificed, spleens are isolated and analyzed for the proliferation status of the T-cell grafts (23).

4. Notes

1. All mice are maintained under specific pathogen-free (SPF) conditions and handled under institutional protocols according to international guidelines. CD11c-DTR transgenic mice are generally used as heterozygotes. Note that homozygote CD11c-DTR transgenic mice are more sensitive to DTx. Offspring of intercrosses of heterozygote CD11c-DTR mice with WT mice is screened by PCR on tail DNA using the following primers: DTR1, 5′-GCCACCATGAAGCTGCTGCCG-3′; DTR2, 5′-CGGGTGGGAATTAGTCATGCC-3′ (**Fig. 28.1C**). PCR protocol: 94°C 4 min [35 cycles: 30 s 95°C, 1 min 58.5°C, 30 s 72°C], 15 s 72°C, hold at 4°C yielding a product of 625 bp. Tail DNA is prepared as follows: Tails are lysed in eppendorf tube in 500 μl lysis buffer (100 mM Tris–HCL pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 μg/ ml Proteinase K) by agitated overnight incubation at 55°C; samples are spun at 13,000 rpm for 10 min to obtain firm pellet; supernatant is removed and transferred to fresh eppendorf tube and supplemented with 500 μl isopropanol; samples are mixed until viscosity is gone; DNA is recovered as aggregated precipitate (using pipette tips) and transferred to a tube containing 400 μl of TE; samples are incubated at 37 or 55°C, until fully dissolved.
2. Following their isolation, BM cells are resuspended in 200 μl of $PBS^{-/-}$. Recipient mice are moderately heated with an infra-red lamp in order to promote vasodilatation to ease tail vein injection. Cells are injected into recipient mice via the tail vein. Volumes lower than 200 μl can also be injected.
3. Irradiated mice receive transiently (for 1 week) an antibiotic (Ciproxin 0.2%) in their drinking water (20 μg/ml) to

prevent bacterial infections during the period of immunodeficiency (the water bottle should be either dark or covered with aluminum foil to protect the light-sensitive drug).

4. Antibodies are used according to the manufacturer's protocols. All antibodies should be tested and titrated in advance to determine the best staining conditions, before analysis of samples from recipient mice.
5. The suggested total staining volume is 20 µl for up to 2×10^6 cells, 50 µl for 5×10^6 cells. A staining time of 10–15 min on ice is usually sufficient.
6. Femura and tibiae are isolated from donor mice and bone surface is exposed by removing the surrounding soft tissue. Bone is opened at both ends and using a 27-ga fitted to a 1-ml syringe filled with 1 ml of cold $PBS^{-/-}$, the marrow is flushed out of the bone cavity into a 15 ml tube. This step is repeated several times until the bone is empty and becomes white. BM cells are then gently resuspended using 21G1 3-ml syringe until the suspension is homogenous. Cells are washed with 10 ml of $PBS^{-/-}$ by centrifugation at 1,200 rpm at 4°C for 7 min.
7. Blood is absorbed using Kim wipes, in order to prevent blood contamination of BAL fluid at later stages.
8. When working with less than 10^7 cells, use the indicated volumes. When working with higher cell numbers, scale up all of the reagents and total volumes, accordingly.
9. To increase the purity of the magnetically labeled fraction, it can be re-passed over a freshly prepared column.

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