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Lymphocyte Trafficking in Health and Disease

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Birkhäuser Verlag
Basel · Boston · Berlin

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Library of Congress Cataloging-in-Publication Data

Lymphocyte trafficking in health and disease / Raffaele Badolato, Silvano Sozzani, editors.
p. ; cm. -- (Progress in inflammation research)
Includes bibliographical references and index.
ISBN-13: 978-3-7643-7308-5 (alk. paper)
ISBN-10: 3-7643-7308-3 (alk. paper)
1. Lymphocytes. 2. Biological transports. I. Badolato, Raffaele, 1965- II. Sozzani, Silvano. III. PIR (Series)

QR185.8.L9L884.2006
616.07'9--dc22

2006043595

Bibliographic information published by Die Deutsche Bibliothek
Die Deutsche Bibliothek lists this publication in the Deutsche Nationalbibliografie;
detailed bibliographic data is available in the internet at http://dnb.ddb.de

ISBN-10: 3-7643-7308-3 Birkhäuser Verlag, Basel – Boston – Berlin
ISBN-13: 978-3-7643-7308-5 Birkhäuser Verlag, Basel – Boston – Berlin

Part of Springer Science+Business Media
Printed on acid-free paper produced from chlorine-free pulp. TCF ∞
Cover design: Markus Etterich, Basel
Cover illustration: Monocyte-derived dendritic cell obtained *in vitro*. With the friendly permission of Fabio Facchetti.
Printed in Germany
ISBN-10: 3-7643-7308-3 e-ISBN-10: 3-7643-7442-X
ISBN-13: 978-3-7643-7308-5 e-ISBN-13: 978-3-7643-7442-6

9 8 7 6 5 4 3 2 1 www.birkhauser.ch

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Lymphocyte trafficking: from immunology paradigms to disease mechanisms

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Intensive investigation of lymphocyte trafficking has revealed the key role of chemokines and adhesion molecules in the regulation of lymphoid organ development and lymphocyte homeostasis [1]. Lymphocyte development takes place in primary lymphoid districts within bone marrow and thymus, where lymphoid progenitors differentiate before emigrating to the peripheral tissues. After complete maturation, naïve lymphocytes continuously recirculate from the blood to lymph nodes and back to the blood. In lymph nodes, naïve T cells scan local dendritic cells (DC) that present processed antigens in the context of appropriate costimulatory molecules. The engagement of the T cell receptor induces clonal expansion and maturation into effector T cells, which are then able to exit lymph nodes and preferentially home to inflamed tissues, where they are needed to eliminate antigens in the effector phase of adaptive immune responses. The selective ability of naïve T cells to enter lymph nodes through high endothelial venules relies on the expression of an appropriate combination of adhesion molecules and chemokine receptors, namely CD62L and CCR7. Similarly, the ability of T effector lymphocytes to home to the proper tissues is dependent on the expression of appropriate combinations of adhesion molecules and chemokine receptors: the addressins. Tissue origin of DC determines the homing of elicited T cells [2]. For instance, only DC present in Peyer's patches induce T cells to home to the small intestine. Similarly, DC present in skin draining lymph nodes permit the subsequent localization of effector T cells to the skin [3]. Similar rules apply for the migration of central and effector memory cells to lymph nodes and peripheral tissues, respectively [4].

The study of genetically determined defects of immune system, which are commonly known as primary immunodeficiencies, have provided substantial support for these findings [5]. In this perspective, the investigation of the pathogenesis of primary immunodeficiencies constitutes a powerful tool to reveal or define the normal mechanisms that regulate generation of hematopoietic cells from bone marrow or from thymus, and their trafficking between blood, secondary lymphoid organs and peripheral tissues. Some immunodeficiencies are characterized by

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impaired maturation of lymphocytes that prevents their emigration into the blood from the primary lymphoid organ. This is observed in diseases characterized by lymphopenia, such as severe combined immunodeficiencies or X-linked agammaglobulinemia [6]. In other diseases, lymphocytes are able to differentiate into mature cells, but cannot migrate in response to chemokines, which are required for their proper trafficking to peripheral blood or secondary lymphoid organs. This condition is observed in Wiskott-Aldrich syndrome (WAS) patients, where a defect in cell migration is associated with an impaired delayed-type hypersensitivity response, with bacterial and viral infections, and with abnormalities in both cellular and humoral immune responses [7].

Chemokines and adhesion molecules such as integrins are implicated in the process of mobilization of mature hematopoietic cells from bone marrow to blood and in the subsequent migration to inflammatory tissues. Patients affected by type-I leukocyte adhesion deficiency (LAD-I) have a selective defect in the expression and/or functional activation of beta2-integrins, one of the major subfamily of integrins expressed by neutrophils, monocytes and DC. This defect results not only in a defect in innate immune responses, but also in a reduced ability of myeloid DC in the activation of T and B cells. Other rare defects of leukocyte migration are observed in type-II LAD (LAD-II), which is characterized by defective rolling of leukocytes on endothelial cells, due to lack of fucosylated glycoconjugates, such as CD15A, and type-III LAD, which is a genetically undefined defect in the activation of beta1 and beta2 integrins avidity following chemokine receptor activation [8].

All the immunodeficiences mentioned so far are characterized by general defects in cell migration due to abnormal activation of the cytoskeleton machinery or impaired functioning of adhesion molecules. However, also the exaggerated chemotactic response of leukocytes results in compromised functioning of the immune system. This is the case in the primary immunodeficiency characterized by recurrent infections, hypogammaglobulinemia, myelokathexis and leukopenia known as WHIM syndrome [9]. In WHIM patients, lymphocytes display an exaggerated response to CXCL12/SDF-1 that prevents the emigration of mature cells to the blood circulation, and interferes with the correct positioning and recirculation of neutrophils and lymphocytes in and out lymphoid organs [10].

This book offers a series of contributions which deal with the most challenging aspects of lymphocyte migration in homeostasis and pathological conditions. The reviews presented here range from the current knowledge on the molecules involved in lymphocyte extravasation to the recent advances in the understanding of the molecular basis of immunodeficiencies. In some of these areas exciting progress has been made very recently, and the content of this book reflects these new concepts and presents new perspectives of future immune intervention.

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Physiology of lymphocyte trafficking

Biology of chemokines

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Chemokines and their receptors

The chemokine superfamily consists of cytokine-like, low-molecular-mass proteins (8–12 kDa) that are critical for cell migration, and also promote other activities such as inflammation, angiogenesis, spread of tumor metastasis, anti-microbial effects, T cell activation, development and organogenesis. Members of this superfamily share structural similarities, including four conserved cysteine residues that are crucial to the tertiary structures. Based on the location of the first two cysteines in their protein sequence, the chemokine family can be divided into four subfamilies, the CC, CXC, C and CX_3C chemokines. To date, the official nomenclature accounts for 43 human chemokines, including 24 CC (CCL1-CCL28), 16 CXC (CXCL1-16), 2 C, and 1 CX_3C subfamily member. Chemokines interact with specific receptors that belong to the superfamily of seven-transmembrane-domain G protein-coupled receptors (GPCRs). So far, ten CC-, six CXC-, and one each of CX_3C- and C-chemokine receptors have been described. Since the search for new chemokines and receptors has almost been completed, these numbers may remain largely unchanged for the years to come. Table 1 lists the human chemokines and receptors identified so far. We have distinguished the homeostatic from the proinflammatory chemokines. Although this does not represent an absolute distinction, the homeostatic chemokines are largely involved in lymphoid organogenesis, vasculogenesis and brain development, while proinflammatory chemokines regulate the traffic of leukocytes, endothelial cells (ECs), and other somatic cells involved in host defense and repair processes. Of note, Table 1 does not include information concerning the isoforms, polymorphism, splice variants, enzymatically processed forms of human chemokines, nor the chemokines encoded in viral genomes. For more detailed information, we refer our readers to the comprehensive reviews on this subject [1, 2].

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Table 1 - Human chemokines and their receptors

Systemic names	Original names	Receptors
CXC chemokines		
CXCL1	GROα/MGSAα	CXCR2, CXCR1
CXCL2	GROβ/MGSAβ	CXCR2
CXCL3	GROγ/MGSAγ	CXCR2
CXCL4*	PF4	CXCR3b
CXCL5	ENA-78	CXCR2
CXCL6	GCP-2	CXCR1, CXCR2
CXCL7	NAP-2	CXCR2
CXCL8	IL-8	CXCR1, CXCR2
CXCL9**	MIG	CXCR3
CXCL10**	IP-10	CXCR3
CXCL11**	I-TAC	CXCR3
CXCL12*	SDF-1α/β	CXCR4
CXCL13*	BCA-1	CXCR5
CXCL14*	BRAK/bolekine	Unknown
CXCL15		Unknown
CXCR16**		CXCR6
C chemokines		
XCL1**	Lymphotactin/SCM-1α	XCR1
XCL2**	SCM-1β	XCR2
CX3C chemokine		
CX3CL1**	Fractalkine	CX3CR1
CC chemokines		
CCL1	I-309	CCR8
CCL2	MCP-1/MCAF/TDCF	CCR2
CCL3	MIP-1α/LD78α	CCR1, CCR5
CCL4	MIP-1β	CCR5
CCL5	RANTES	CCR1, CCR3, CCR5
CCL7	MCP-3	CCR1,CCR2,CCR3
CCL8	MCP-2	CCR3, CCR5
CCL11	Eotaxin	CCR3
CCL13	MCP-4	CCR2, CCR3
CCL14	HCC-1	CCR1, CCR5
CCL15	HCC-2/Lkn-1/MIP-1γ	CCR1, CCR3

Table 1 (continued)

Systemic names	Original names	Receptors
CCL16	HCC-4/LEC/LCC-1	CCR1, CCR2
CCL17**	TARC	CCR4
CCL18*	DC-CK1/PARK/AMAC-1	Unknown
CCL19*	MIP-3β/ELC/exodus-3	CCR7
CCL20**	MIP-3α/LARC/exodus-1	CCR6
CCL21*	6Chine/SLC/exodus-2	CCR7
CCL22**	MDC/STCP-1	CCR4
CCL23	MPIF-1/CKβ8/CKβ8-1	CCR1
CCL24	Eotaxin-2/MPIF-2	CCR3
CCL25**	TECK	CCR9
CCL26	Eotaxin-3	CCR3
CCL27	CTACK/ILC	CCR10
CCL28	MEC	CCR3/CCR10

Abbreviations: AMAC, alternative macrophage activation-associated CC chemokine; ATAC, activation-induced, chemokine-related molecule; BCA, B cell-attracting chemokine; BRAK, breast and kidney-expressed chemokine; CK, chemokine; CTACK, cutaneous T cell-activating chemokine; DC-CK1, dendritic cell-derived CC chemokine; ELC(Ebl-1), EBL-1-ligand chemokine; ENA, epithelial neutrophil activating; GCP, granulocyte chemotactic protein; GRO, Growth-related oncogene; HCC, human CC chemokine; I-309, a nameless human chemokine; ILC, IL-11 receptor alpha-locus chemokine; IP, IFN-γ-inducible protein; I-TAC, IFN-γ-inducible T cell chemoattractant; LARK, liver- and activation-regulated chemokine; LD78, macrophage inflammatory protein-11; LEC, liver-expressed chemokine; Lkn, leukotactin; MCAF, monocyte chemotactic and activating factor; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MEC, mucosae-associated epithelia chemokine.; MGSA, melanoma growth stimulatory activity; MIG, monokine-induced by IFN-γ; MIP, macrophage inflammatory protein; MPIF, myeloid progenitor inhibitory factor; NAP, neutrophil-activating peptide; PARC, pulmonary and activation-regulated chemokine; PF, platelet factor; SCM, single C motif; SDF-1, stromal cell-derived factor 1; SLC, secondary lymphoid tissue chemokine; STCP, stimulated T cell chemoattractant protein; TARC, thymus- and activation-regulated chemokine; TDCF, tumor-derived chemotactic factor; TECK, thymus-expressed chemokine.

The unstarred chemokines are all proinflammatory.

**Homeostatic chemokines characterized by being constitutively produced and involved in leukocyte trafficking under physiological conditions.*

***Chemokines that have both homeostatic and proinflammatory inducible properties.*

Chemokine signaling

Although substantial progress has been made in our understanding of chemokine signal transduction, the pathways involved are yet too complicated for us to provide a detailed and comprehensive model. The challenge is that we know too many of players, but not the complex relationships between them. Moreover, it is obvious that multiple interdependent networks, rather than a linear pathway, must be involved in signaling cascades following chemokine stimulation. Thus, in this section, instead of attempting to provide a comprehensive model, we will highlight several points of interest in the field that we believe are critical for chemokine signaling (Fig. 1). For more details on this subject, we refer our readers to excellent recent reviews [3–7].

Activation of chemokine receptor-associated G proteins

Chemokines initiate intracellular signaling through seven-transmembrane domain GPCRs. Binding of a chemokine to its receptor results in changes in the tertiary structure of the receptor, allowing the intracellular part to bind and activate heterotrimeric G proteins [8]. The activated G proteins exchange guanosine diphosphate (GDP) for guanosine triphosphate (GTP) and dissociate into α- and βγ-subunits. Chemokine receptors can couple to several different Gα isotypes; however, for most chemokines, only the functional coupling of the receptor to $G\alpha_i$ is required, because migration is completely inhibited by treatment of the cells with pertussis toxin, a specific $G\alpha_i$ inhibitor [9, 10]. The βγ-subunits released from $G\alpha_i$ mediate chemokine-induced signaling pathways by activating multiple downstream effectors. Although precisely how this is achieved is currently unclear, the recent evidence points to a critical role for lipid signaling and PH domain-containing proteins in molecular relays leading to chemotaxis.

Relevance of phosphotidylinositide-3 kinase pathways

It is documented that the βγ-subunits released from the heterotrimeric G proteins mediate chemokine-induced signals, at least partially, by activating the phosphotidylinositide-3 kinases (PI3Ks). Numerous reports implicate PI3Ks as central mediators linking early chemoattractant signals with downstream components of the chemotaxis response [6, 11]. Pharmacological inhibition of PI3Ks in *D. discoideum* amoebae and in a variety of mammalian cell types causes inhibition of cell migration to varying degrees. Myeloid leukocytes derived from mice lacking PI3Kγ have defects in polarity and show impaired chemotaxis. However, lymphocyte chemotaxis is not so severely compromised in PI3Kγ-deficient mice [12–14]. This implies the existence

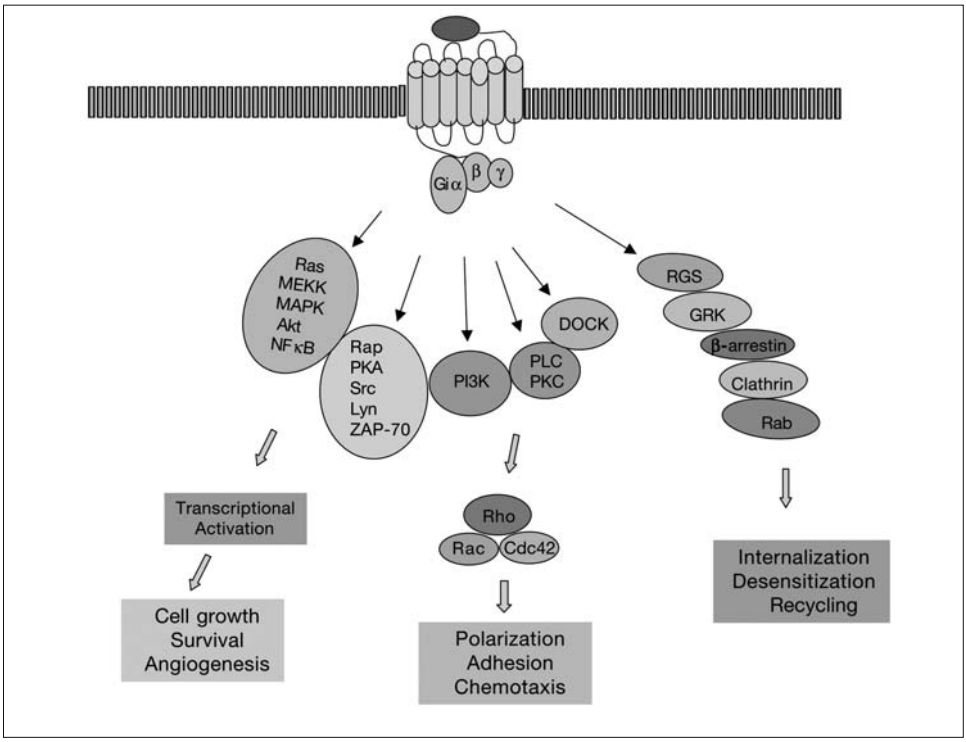

Figure 1
Graphic view of the chemokine signal cascade
G-protein α-, β-, γ-heterotrimeric G-proteins are composed of α-, β- and γ-subunits; RGS, regulators of G-protein signal; GRK, G-protein coupled receptor kinase; β-arrestin, scaffold protein associated with G-protein coupled receptors; Rab, small GTPases associated with transportation and fusion; DOCK, 2-downstream of Crk-180 homolog-2; PLC, phospholipase C; PKC, protein kinase C; Rho, Rac and cdc42, small GTPases associated with cell adhesion; PI3K, phosphoinositide 3-kinase; Rap, small GTPase associated with integrin-mediated cell adhesion; PKA, cAMP-dependent protein kinase; Src and Lyn, tyrosine kinases; ZAP-70, Zeta-associated protein of 70 kDa a tyrosine kinase; Ras, small GTPase associated with cell adhesion; MAPK, mitogen-activated protein kinase; MEKK, MAPK/ERK kinase (MEK)-extracellular regulated kinase (ERK) kinase; Akt, phosphatidylinositol-3-OH kinase is also known as protein kinase B (PKB); NF-κB, nuclear factor kappa B

of compensatory mechanisms that prevail in chemokine-induced lymphocyte migration, or that other PI3K isotypes could play a more important role. The roles of PI3K are multifold, but principally, it functions as a lipid kinase by generating phosphotidylinositol-3,4,5-trisphosphate (PIP3), which recruits and activates multiple PH

domain-containing substrates to the leading edge of the cells, generating the intracellular molecular gradient that is essential for cell movement.

Small GTPase connection

Leukocytes stimulated by chemoattractants assume a polarized morphology. They become elongated and develop a wide lammellipod (pseudopod) at the leading edge and a tail-like projection at the trailing end (uropod). The generation of filamentous actin at the pseudopod leads to its extension, which together with uropod retraction drives cell locomotion. Small GTPases of the Rho family, Rac, Cdc42, and Rho, are pivotal regulators in these processes [15]. It is generally assumed that the major way to activate Rho GTPases is via activation of specific guanine nucleotide exchange factors (GEFs), most notably Tiam1, Sos and Vav, that have been known to bind via their PH domain to PIP3 [16–20]. Different GTPases may play distinct roles, collaborating in cellular locomotion. Rac was reported as indispensable for cellular polarization prior to migration [21]. Its downstream effectors include p21-activated kinase (PAK) and the Wiskott-Aldrich syndrome protein (WASp) homologue, WAVE, which stimulate actin-related protein 2/3 (ARP2/3). This induces focal actin polymerization, responsible for the development and forward extension of the pseudopod. Cdc42, on the other hand, is required for the establishment of orientation machinery at the pseudopod [22, 23]. Without Cdc42, leukocytes exhibit a random walk, rather than directed migration, when placed in a chemoattractant gradient. The events that lead to Cdc42-mediated orientation and Rac-mediated actin polymerization explain what happens in the pseudopod, i.e., the front part of cells, although the specific mechanism has not been determined. In parallel to the sequence leading to Rac and Cdc42 activation at the pseudopod, another small GTPase Rho was activated by Rho GEFs at the uropod, the trailing end of the cells. The activated Rho, through its effector p160ROCK, a serine/threonine kinase, induces focal activation of myosin II, and formation and contraction of actin-myosin complexes, thereby leading to retraction of the uropod [24–26]. In addition, Rho prevents the formation of lamellipodia at the trailing end. Together, these two alternative signaling pathways involving Rac, Cdc42, or Rho induce and maintain functional and morphological cell polarity and drive locomotion.

Activation of the mitogen-activated protein kinase cascade

It is well known that the mitogen-activated protein kinase (MAPK) cascade is activated following chemokine stimulation, although the exact consequence is currently unclear. As MAPK can phosphorylate and activate various transcription factors [27], it is assumed that chemokines may be involved in regulation of gene expres-

sion through this pathway. Indeed, the activation of NF-κB, a multifunctional transcription factor, was also observed concomitantly with MAPK activation by chemokine stimulation [28, 29]. Richmond et al. [30] demonstrated that GROα activates NF-κB through a pathway involving Ras, MAP/ERK kinase kinase-1 (MEKK1), and p38. MAPK was also reported to phosphorylate and activate cytoplasmic phospholipase A2 (cPLA2), leading to release of arachidonic acid and phospholipids [31]. Arachidonic acid-induced leukotriene production is essential for actin polymerization. These data also indicate a chemokine-induced pathway involving MAPK and cPLA2 that may regulate cytoskeletal changes necessary for cell migration. However, this function may not be critical, as the p38-MAPK inhibitor SK&F 86002 and MEK inhibitor PD98059 have no significant effect on chemokine-mediated chemotaxis [32].

Receptor dimerization and JAK/STAT pathway activation?

The involvement of receptor dimerization and JAK/STAT pathway activation in chemokine signaling has been a controversial issue [33, 34]. A number of reports suggested that chemokines, like cytokines, induce receptor dimerization, which results in the activation of the JAK-STAT pathway [35, 36]. However, these observations, although interesting, have not been supported by other groups. O'Shea's group [37] recently reported that lack of JAK2 or JAK3 had no effect on CXCL12 signaling or its receptor mediated chemotaxis, nor did overexpression of wild-type versions of the kinases, strongly arguing against CXCL12 signaling being dependent on JAK2 and JAK3, as previously reported.

Receptor desensitization and internalization

It is intriguing that the mechanism of chemokine receptor activation contains a built-in shut-off sequence, thus providing additional opportunities for antagonizing chemokine signaling pathways. To date, little is known of the down-regulatory mechanisms in the cellular response to chemokines. Molecules called regulators of G protein signaling (RGS) can modify the GTPase activity of the Gα subunits and thereby initiate inhibitory mechanisms of chemokine signaling [38, 39]. Additional mechanisms inhibiting chemokine receptor signaling include G protein-coupled receptor kinase (GRK) family members, which cause rapid receptor desensitization by inducing receptor phosphorylation upon chemokine stimulation, and β-arrestin or adaptin-2, which result in down-regulation of receptors by sequestration and internalization through clathrin-coated pits or caveolae [40–42]. Rab, a small GTPase family member, has been shown to be critical for determining the fate of chemokine receptors, degradation or recycling, following their internalization [43, 44].

GPCR undergo two types of ligand-mediated inactivation; phosphorylation and internalization. Endocytosis can proceed through clathrin-dependent and -independent pathways [45], but other factors regulating receptor internalization remain poorly understood [46]. It appears that a higher concentration of ligand is needed to trigger internalization than is required to induce signal transduction [47]. Subsequent to ligand binding to the receptor, beta-arrestin relocates to the cell membrane and interacts with C-terminal domains of activated chemokine receptors, followed by recruitment of chaperone molecules that leads to receptor localization in endosomes [41, 48, 49]. Several researchers have proposed that internalization is likely to be a means to fine tune the local immune response since it is the first step toward determining if receptors are recycled or proteolyzed [44, 47]. In addition to the ultimate fate of a receptor, clearance of a ligand-receptor complex from the cell surface has the effect of removing ligand from the local environment [50], resulting in a diminished chemokine gradient and inhibition of cellular migration. Thus, receptor internalization, like phosphorylation, is a stop signal for migration.

Chemokines in development and organogenesis

One of the homeostatic chemokine receptors, CXCR4, participates in morphogenetic processes beginning with the earliest stages of development. Perhaps the most convincing data supporting a role for CXCR4/CXCL12 in mammalian embryogenesis are that mice deficient in either CXCL12 or CXCR4 die *in utero* or shortly afterwards due to extensive abnormalities in cerebellar and vascular development, including failed cardiogenesis, and limited hematopoietic cell development [51–53]. Further, transfer of CXCR4-deficient murine hematopoietic progenitor cells from fetal liver to adult bone marrow does not support long-term reconstitution of T or B cells in adult hematopoietic organs. There are fewer B cell progenitors in the liver of CXCR4-deficient embryos, which could contribute to the failed reconstitution [54]. However, CXCR4 also contributes to the retention of progenitor cells in the bone marrow and progenitor survival [55, 56], suggesting that these activities may also contribute to B cell development in primary lymphoid tissues. While the population of T cell progenitors found in the thymus of CXCR4- and CXCL12-deficient mice is comparable to that found in wild-type mice, the number of double-positive thymocytes was reduced fivefold, suggesting that the CXCR4 signal is required for T cell expansion in the thymus [57, 58]. Thus, CXCR4 and its select agonist, CXCL12, participate in many stages of lymphocyte development. In addition, *plt* mice, which have mutated hypo-functional variants of both CCL19 and CCL21 chemokines, exhibit defective lymphoid organogenesis and have an immunosuppressed phenotype [59–62]. Deletion of other chemokines or their receptors does not interfere with gross development, but often results in impaired host defense against infectious challenges as will be discussed.

Chemokines and lymphocyte development

While the initial stages of T and B precursor cell development occur in the bone marrow, T cell precursors are recruited to the thymus for selection and differentiation into a diverse T cell population exhibiting self tolerance. These CXCR4$^+$ and CCR9$^+$ precursors are attracted to the thymic cortex by CXCL12 and CCL25 (TECK), respectively [63–65]. Following entry into the thymus, CCR9 expression is decreased, while CCR4 expression is increased, which enables the T cell precursors to migrate into the thymic medulla [66]. This step is likely to be redundant with other chemokine receptors since CCR4-deficient mice do not show a defect in thymocyte development [67]. Following selection to the single-positive stage, either CD4 or CD8, the mature but naïve lymphocytes express CCR7 [66, 68], and are ready to move to secondary lymphoid tissue expressing CCL19 and CCL21. In the case of extrathymic differentiation of T cells that reside in the intestine, either CCR6 or CCR9 expression is required for T cell homing to the gut [69]. However, unlike other naïve lymphocytes, these extrathymic-derived cells are unlikely to recirculate from peripheral blood to other tissues in search of antigens.

The importance of CCR7 in the trafficking of lymphocytes into and out of secondary lymphoid tissue has been demonstrated using CCR7-deficient [70, 71] and *plt* mice, which are deficient in CCL19 and CCL21 [62]. Following their activation, dendritic cells (DCs) are also induced to express CCR7, and can then migrate to the draining lymph nodes, in response to CCR7 ligands [72]. Activated DCs present antigen and costimulatory molecules to naïve T cells in the T cell zone, which leads to persistent interaction of DC and antigen-specific T cells for at least 15 h prior to their proliferation and differentiation into effector T cells [73, 74]. Th1 and Th2 effector T cells express distinct patterns of chemokine receptors, e.g., Th1-polarized cells express CXCR3 and CCR5, while Th2-polarized cells express CCR4 and CCR8 both *in vivo* and *in vitro*. Thus, chemokines and their receptors coordinate lymphoid development from the initial embryonic hematopoietic cell stage to the mature polarized lymphocytes.

CXCR5 and its ligand, CXCL13, are essential for the organogenesis of peripheral lymph nodes and Peyer's patches along with splenic architecture [75, 76]. CXCR5, in combination with CCR7 is essential for efficient development and maintenance of secondary lymphoid tissue and lymphocyte migration through these tissues [76, 77]. B cells move into secondary lymphoid tissue through high endothelial venules (HEVs), where they mature into long-lived follicular B cells [78] and, with T cell help, will generate antibody responses. T cells also enter secondary lymphoid tissue through HEVs, but they are segregated into T cell zones where they may encounter antigen-presenting DCs. Activated T cells rapidly up-regulate CXCR5, which allows a small fraction of them to move to the B cell follicle border [79]. Activated B cells rapidly up-regulate CCR7, which allows them

to also move to the border of the B cell follicle near T cells [80]. Thus, through a poorly understood but closely choreographed process, both B and T germinal center cells are produced.

Surprisingly, several tumor necrosis factor (TNF) family members also contribute to the generation of secondary lymphoid tissue. Lymphotoxin (LT)-α, LT-β, and LT-β receptor-deficient mice lack CXCL13 expression and are deficient in peripheral lymph nodes and Peyer's patches [81]. TNF and TNFRI-deficient mice lack Peyer's patches. Further, splenic architecture is disrupted in all these animals. Thus, several cytokines, by inducing chemokines and their receptors, participate in secondary lymphoid tissue development and recruitment of naive lymphocytes.

Chemokines and angiogenesis

Chemokines also regulate the vascularization of lymphoid organs and inflammatory tissues because ECs express many proinflammatory chemokine receptors. Robert Strieter and colleagues [82] first reported that the ELR^+ CXC chemokines induced migration and proliferation of ECs and were angiogenic, while those ELR^- CXC chemokines that interact with CXCR3 inhibited the proliferation and chemotaxis of ECs and were anti-angiogenic. More recently, a variant CXCR3B receptor was reported to be responsible for the anti-angiogenic effects of CXCR 9, 10, 11 and platelet factor 4 (PF4/CXCL4) [83]. Furthermore, ELR^- CXCL12 interactions with CXCR4 were proangiogenic, as were some CC chemokines (MCP-1/CCR2 and eotaxin/CCR11) and fractalkine CX3CL1 [84]. Thus, the processes of chemokine-induced lymphoid organogenesis and leukocyte infiltration of inflammatory sites is enabled by the capacity of many of the proinflammatory chemokines to promote the development of supportive tissue vascularization. Presumably, the anti-angiogenic effects of platelet-derived CXCL4 inhibits capillary formation within the vascular compartment, while the interferon-induced CXCR3 ligands down-regulate inflammatory vasculogenesis.

Virally encoded modifiers of chemokines

Several classes of large DNA viruses circumvent the host immune system by targeting the chemokine system. Viruses utilize three approaches to co-opting the host by chemokine mimicry, chemokine receptor mimicry and by encoding chemokine-binding proteins as reviewed [85–88]. The broadly reactive nature of these virally encoded biological modifiers suggests that they are likely to influence all aspects of the host immune response, and can therefore be utilized to elucidate the role of chemokines in immune function and develop more targeted therapies.

Chemokine mimics

One of the most extensively studied viral chemokines is vMIPII (viral-monocyte inflammatory protein-2). It is encoded by human herpes virus 8 (HHV8) and possesses both agonist and antagonist activity due to its interaction with at least six chemokine receptors [85]. The migration of monocytes induced through activation of CCR1 or CCR5 is inhibited by vMIPII, while migration of Th2 lymphocytes and eosinophils is enhanced by vMIPII binding to CCR3 and CCR8. The in vivo effect of vMIPII during the initial stages of infection is to inhibit the recruitment of Th1-polarized cells to sites of infection [89]. Additional studies indicate that vMIPII actually enhanced IL-10 expression and reduced costimulatory molecule expression [90], suggesting that vMIPII can suppress host Th1 responses and enhance Th2 responses. Limited studies of HHV8-encoded vMIPI and vMIPII indicate they bind to CCR8 and CCR4, respectively [91]. The $CD4^+CD25^+$ regulatory cell population uniquely expresses both CCR4 and CCR8 [92], leading to the speculation that viral chemokines may guide regulatory cells to sites of infection and inflammation to suppress lymphocyte activation. In contrast, the CCR2 agonist encoded by human herpesvirus 6 (HHV6) [93], U83, and the CXCR2 agonists encoded by cytomegaloviruses (CMV), human vCXCL1, murine MCK-1 and MCK-2, appear to promote host granulocyte and lymphocyte migration to sites of infection [94] potentially providing more infectable cells. Thus, viruses have successfully purloined chemokines to shape their environment.

Chemokine receptor mimics

A second approach to subvert the chemokine system by viruses is to encode chemokine receptors. For example, the human β herpesvirus CMV encodes four chemokine receptor-like genes, of which only US28 has been extensively studied [86]. US28 is a constitutively active seven transmembrane receptor that signals through three heterotrimeric G-proteins; $G_{\alpha q}$, $G_{\alpha i}$ and $G_{\alpha 16}$ [95]. US28 binds CC and CX3C chemokines with the highest affinity being for CX3CL1 [96]. US28 has been implicated as being causative in atherosclerosis and restenosis [97]. While several components of the US28 signal cascade are constitutively actively, especially NF-κB and phospholipase C (PLC) [95], others, including calcium flux [98], and chemotaxis [97], can be induced by host-produced CC chemokines. The rapid internalization of US28 suggests it also acts as a chemokine scavenger [50]. Thus, CMV-encoded US28 has several signaling and trafficking approaches by which it can condition the local immune environment to the viruses' advantage.

In addition to vMIPI-III, HHV8 also encodes ORF74, a constitutively active seven transmembrane receptor, that has been implicated as causative in Kaposi's sarcoma, primary effusion lymphoma and multicenter Castleman's disease [99]. The

clearest indication that ORF74 can condition the host response to HHV8 comes from transgenic mouse studies, where the ORF74-expressing mouse develops Kaposi's sarcoma-like lesions [100].

An additional approach used by viruses to exploit the chemokine system is regulation of host chemokine receptors. Human γ herpesviruses, Epstein-Barr virus (EBV) and HHV6a, HHV6b, HHV7, up-regulate CCR7. Although it remains to be proven, this appears to be a mechanism used by these viruses to ensure that infected cells and uninfected cells localize to lymphoid tissues, thus providing more cells to be infected [101]. In conclusion, several large DNA viruses both encode constitutively active seven transmembrane receptors and/or regulate host chemokine receptor expression to condition the host.

Chemokine-binding proteins-vCKBP

Virally encoded chemokine binding proteins (vCKBPs) can be subdivided based on their mechanisms of action. The so-called T7 chemokine-binding proteins compose the first class. They are expressed by some but not all poxviruses and structurally resemble soluble interferon gamma receptor. vCKBP-1 is thought to incapacitate CXC, CC and XC chemokines by binding to the glycosaminoglycan (GAG) binding site found in the C-terminal domain of most chemokines, as reviewed in [102]. A member of the vCKBP-1 class has been shown to block inflammation *in vivo* [103].

The second class of chemokine-binding proteins are 35-kDa proteins expressed by many poxviruses, including some vaccinia virus strains [87]. This class of proteins appears to bind to the same molecular surface of CC chemokines that binds to CC receptors blocking chemokine-receptor interactions. Infection of mice with strains of vaccina virus expressing vCKBP-2 resulted in lower chemokine levels in bronchoalveolar lavage fluids (BAL) compared to BAL from mice infected with vaccina virus not expressing vCKBP-2.

The third class of chemokine-binding proteins has only one member. M3 is expressed by murine γ-herpes virus (MHV-68), and has been shown to bind human and murine CXC, CC and CX3C chemokines [87]. The mechanism employed by M3 to inhibit chemokines is very unique. An asymmetric dimer of M3 becomes symmetric when it has bound two chemokine monomers blocking their N-terminal amino and 40s loop structures [104]. Thus, M3 blocks receptor and GAG binding by chemokines. M3 has been shown to block CCL19 (ELC) and CCL21 (SLC), which are essential for lymphocyte trafficking through secondary lymphoid tissue. Selective expression of M3 in the pancreas of transgenic mice blocked lymphocyte recruitment [105].

The fourth class of chemokine-binding proteins are expressed by ungulate α-herpes-virus, but not by human herpes simplex viruses or varicella zoster virus

[106]. These glycoproteins are anchored in the host cell membrane through their C terminus, potentially acting as chemokine receptor decoys. Additionally, soluble forms are found in the serum of infected animals. These proteins bind to CXC, CC and CX3C chemokines potentially blocking heparin and receptor binding [106].

Overall, regardless of the mechanism by which chemokine-binding proteins block chemokine function, they appear to broadly disrupt chemokine-mediated innate and adaptive lymphocyte-dependent immune functions. Thus, these proteins provide tools to dissect the role of entire classes of chemokines in the immune systems of intact adult animals and fetal development.

Chemokine decoy receptors

In addition to microbial subversion of the host immune system by chemokine receptor mimicry, the host expresses decoy or scavenger receptors to regulate the immune response. Currently, three of these receptors have been identified, D6, Duffy antigen receptor for chemokines (DARC) and CCX-CKR [107–110]. These chemokine receptors are even more promiscuous in their ligand binding than functional receptors, but ligand binding does not induce chemotaxis nor signal transduction. Many tissues express these receptors, but the highest expression in the case of DARC is found on the surfaces of red blood cells and ECs, including HEVs. DARC binds proinflammatory chemokines CXCL1, CXCL5, CCL2, CCL5 and CCL7, but does not bind the lymphoid chemokines CXCL12, CXCL13, CCL18, CCL19, CCL20 or CX3CL1 [107]. Similarly, D6 binds proinflammatory chemokines, but only CC chemokines. D6 is rapidly internalized and recycled to the cell surface following ligand binding [107]. DARC and D6 are both highly expressed by ECs, suggesting that this serves to regulate inflammatory responses by removing proinflammatory chemokines from the circulation. The rather novel decoy receptor CCX-CKR is also expressed on lymphoid tissue ECs, but unlike DARC and D6, this receptor binds to, and leads to the degradation of, homeostatic lymphoid chemokines, CCL19, CCL21 and CCL25 [108, 109]. The consequences of failing to remove these lymphoid homeostatic chemokines is under evaluation [108, 109].

Chemokines and innate immunity

In mammals and many other species, innate immune responses are initiated by the recognition of molecular patterns commonly associated with invading pathogens, including bacterial lipopolyssacharide (LPS), lipoproteins, peptidoglycan (PGN), and CpG containing un-methylated DNA. These pathogen-derived molecules interact with Toll-like receptors (TLR) and nucleotide-binding site leucine-rich repeat

proteins (NOD), which, upon activation, promote the release of proinflammatory cytokines and chemokines by a variety of host cells. Proinflammatory cytokines such as IL-1 and TNF in turn are also potent inducers of proinflammatory chemokine at sites of inflammation and microbial infection [111]. Proinflammatory chemokines, as listed in Table 1, are largely generated in response to infections and injurious stimuli, and should be distinguished from homeostatic chemokines that are more involved in development and organogenesis. A prominent feature of the innate immune response is the initial recruitment by chemokines of granulocytes followed by monocytes to sites of infection or injury, where these cells phagocytose and degrade the invading pathogens or damaged tissues. Neutrophils infiltrate at the early stage of innate immune responses, through interaction of ELR^+ CXC chemokine ligands such as CXCL8, CXCL5 and CXCL1 with cell surface chemokine receptors, mainly CXCR1 and CXCR2 [5]. This is followed by infiltrating mononuclear phagocytes, including monocytes/macrophages and DCs, which are largely recruited by proinflammatory CC chemokines (i.e., CCL2, CCL3 and others).

Chemokines and natural killer cells

Chemokines and their receptors are also vital in regulating the trafficking and activation of natural killer (NK) cells. NK cells do not undergo genetic recombination during development, and therefore are considered cells of the innate immune system. These cells provide early protection of the host against viral infection. Resting CD56dim NK cells fail to express the chemokine receptor CCR7, which is responsible for lymphocyte homing and DC migration to secondary lymphoid organs [112]. Thus, NK cells instead traffick to non-lymphoid tissues. Reports concerning the pattern of chemokine receptor expression by NK cells are inconsistent. While high level expression of CXCR1, CXCR4 and CX3CR1 and low levels of CXCR2 and CXCR3 have been reported [113], purified resting human NK cells were found to express only CXCR4 [114]. Nevertheless, resting human NK cells are chemoattracted by CXCL9-11 (acting on CXCR3), CXCL12 (acting on CXCR4), XCL1 (acting on XCR1) and CX3CL1 (acting on CX3CR1), suggesting that chemokine receptors for these ligands are expressed by NK cells, but at a relatively low cell surface level [114]. On the other hand, activated NK cells express increased levels of CC chemokine receptors including CCR2, CCR4, CCR7, and CCR8, and are able to migrate *in vitro* in response to a variety of CC chemokines. *In vivo*, CCL3 (MIP-1α) mediates the recruitment of NK cells to the liver in mice infected with CMV, leading to exacerbated inflammation, and increased resistance to infection [115]. Thus, although more studies of the chemokines and receptors of NK cells are needed, the available data indicates that chemokines are clearly involved in NK cell infiltration and activation at sites of inflammation.

Chemokines and adaptive immunity

Although T and B lymphocytes are responsible for adaptive immunity, antigen-presenting cells (APCs) and in particular DCs play a central role in the activation of T lymphocytes [74]. Consequently, chemokines play crucial roles in adaptive immunity based on their effects in directing the migration of APC that deliver antigenic signals as well as of specific antigen-responsive T lymphocytes. Precursors of Langerhans cells are attracted by ligands for CCR6 such as MIP3α/CCL20 to peripheral epidermal sites, while precursors of myeloid and plasmacytoid DCs (mDCs and pDCs, respectively), which express many chemokine receptors, are attracted to inflammatory sites by MCP-1 (CCL2), MIP1α (CCL3) and other chemokines. For example, pDC also respond to the CXCR3 ligand CXCL9 [116, 117]. The proinflammatory cytokines produced at such sites induce immature (iDC) to mature and express comitogenic cell surface markers such as CD80, CD83, CD86 and CD40, and in addition induce the production of proinflammatory cytokines such as IL-12, IL-1 and TNF [118]. These mDCs are also induced to express CCR7, and to decrease their expression of CCR6 and other chemokine receptors for proinflammatory chemokines [119]. Gradients of SLC/CCL19 produced by lymphatic ECs and ELC/CCL21 produced within the lymph node interact with CCR7 and direct the migration of mDCs from peripheral inflamed tissues via the lymphatics into the draining lymph nodes [118]. An analogous scenario results in the attraction of $CCR7^+$ T lymphocytes and $CXCR5^+$ B lymphocytes into the appropriate lymph node compartments [120]. There the mDCs and specific T lymphocytes find one another, resulting in T cell activation, which generates central memory and effector memory T cells, each with their characteristic combinations of chemokine receptors [121]. The T cells in turn produce "helper" cytokines that enable the B lymphocytes to respond to the intact antigens.

Chemokines, however, can also promote T cell activation and adaptive immunity by three additional non-chemotactic mechanisms as follows:

1. Chemokines based on their cationic charges can non-covalently bind to antigens provided they have a net anionic charge. The interaction of this chemokine-antigen complex, analogous to the chaperone functions of heat shock proteins, can facilitate the delivery and uptake of antigen based on interactions with chemokine receptors on APCs. Evidence in support of this scenario has been established by covalently linking chemokines or β defensins with antigen [122]. The fused antigens bind to the chemokine receptors of APCs and are internalized along with the ligand. Presumeably, the antigen is delivered to the antigen-processing pathway, because administration of these linked antigens to mice results in much better immune responses, than if they are delivered in an unlinked manner as a mixture of antigens and chemokines or defensins [123]. Furthermore, the nature of the chemokine or defensin determines whether Th1 or Th2 immune responses are generated. Thus, chemokines such as IP10/CXCL9 and

MIP1α/CCL3 or murine β defensin-2 linked to antigens favor induction of Th1 immune responses, whereas MDC/CCL22 (CCR4 ligands) and murine β defensin-3 favor polarization to Th2 humoral antibody responses [122].

2. A number of self antigens are chemotactic and have been shown to be capable of interacting directly with chemokine receptors such as CCR5, CXCR5, CXCR3 and CCR2 [124]. These chemotactic self antigens, although frequently intracellular in origin, are usually associated with the pathogenesis of various autoimmune reactions, or have been identified as differentiated tumor antigens associated with various neoplasias. Presumably, the capacity to interact directly with chemokine receptors also facilitates the uptake, processing and presentation of these antigens, accounting for their greater immunogenicity. A subsequent chapter in this monograph will document and discuss some of these issues more elegantly and in greater detail.
3. Finally, chemokines rapidly up-regulate the cell-cell interactions mediated by cell surface adhesion proteins. Induction of chemotaxis by chemokines and presumably defensins rapidly results in changing the configuration of cell surface integrins on target cells so as to increase their adhesion to other cell types [125]. This increase in adherence promotes the interaction between mDCs/APCs and T lymphocytes, and results in a chemokine-dependent comitogenic effect [125]. Furthermore, some chemokines (e.g., ligands for CCR7) have recently been reported to also promote the maturation of iDCs to mDCs [126]; another means of augmenting the immunostimulatory effect of APCs/DCs and promoting their comitogenic capabilities.

Consequently, ligand chemokine receptor interactions can dramatically promote adaptive immunity as follows:

1. by promoting the development and organogenesis of lymphoid tissues based on the directed migration of cells to appropriate tissue sites and the angiogenic induction of blood vessel formation in the lymphoid tissues;
2 by promoting the uptake and internalization of antigens by APCs;
3. by directing the migration of APCs and lymphocytes and promoting their interactions in lymphoid tissues;
4. by enhancing the adhesion of APCs to T cells resulting in comitogenic effects.

Unique contributions of chemokines to immunity

Since a number of chemokines may share a common surface receptor and one receptor may interact with multiple chemokines, it has been hypothesized that functional redundancy of chemokines may exist in the immune system. However, gene depletion studies thus far have failed to prove any single chemokine or receptor as being completely redundant. Virtually all chemokine ligand or receptor knockout mice exhibited either a defective spontaneous phenotype or abnormal

Table 2 - The impact of depletion of selected chemokine ligands on immune system

Eotaxin (CCL11)	Impaired eosinophil trafficking to the gastrointestinal tract Decreased eosinophil recruitment into intestine in a gastrointestinal allergy model [131, 132]
JE (CCL2)	Reduced peritoneal macrophage exudation after thioglycollate injection Reduced recruitment of macrophages (F4/80+) in a DTH model Reduced granuloma formation in a *Schistosoma* model Resistant to *Leishimania major* and unable to mount Th2 responses Protection from EAE, with decreased Th1, but increased Th2 responses Delayed wound healing [127, 133–135]
MIP-1α (CCL3)	Reduced myocarditis following coxsackie virus infection Reduced lung inflammation after influenza A infection Reduced liver inflammation after cytomegalovirus infection Reduced herpes simplex virus type I-induced herpes stromal keratitis [115, 136, 137]
ELC/SLC (CCL19, CCL21) (*plt/plt* mice)	Lack of CCL19 and CCL21 have impaired lymphoid organogenesis, impaired T cell adhesion to HEVs, reduced T cell entry into lymph nodes, decreased DC number in lymph nodes, and failure of T cells and DCs to organize normally in T cell zones in the spleen [59–61]

responses to challenge with microorganisms, antigens or other injurious insults. Tables 2 and 3 summarize some observation derived from mice depleted of selected chemokines or receptors. Studies of these mice have revealed many predictable as well as strikingly unexpected results. As predicted, mice lacking a certain chemokine or receptor, which specifically mediates the chemotaxis of certain leukocyte populations, show defects in the trafficking of such leukocytes either to their normal resident organs and tissues, or failure to infiltrate sites of microbial infection and inflammation. Such an impairment of leukocyte trafficking and infiltration in chemokine- or receptor-deficient mice inevitably results in compromised innate and adaptive immune responses mediated by phagocytic leukocytes, DCs and T or B lymphocytes (Tabs 2 and 3). Many such mice more readily develop, or are resistant to, artificially induced autoimmune diseases. In this regard, inflammatory chemokines and their receptors have been clearly shown to be essential for innate immune responses and also adaptive immune responses that require cross-talk between DCs and lymphocytes in secondary lymphoid tissues. On the other

Table 3 - Altered immune system in mice depleted of chemokine receptors

Receptors	Phenotype
CC receptors	
CCR1	Defective neutrophil chemotaxis Reduction of myeloid progenitors in the spleen and blood under steady state Reduction of myeloid progenitors in the spleen but increase in blood after LPS stimulation Impaired proliferation of myeloid progenitors in spleen after LPS treatment Increased susceptibility to *Aspergillus fumigatus* Resistant to pancreatitis-induced acute lung injury Decreased granuloma size and altered cytokine production in *Schistosonia mansoni* Susceptible to *Toxoplasma* with decreased blood and liver neutrophil influx Impaired neutrophil infiltration in pancreas and less lung injury in caerulein-induced pancreatitis Enhanced Th1 responses and severe nephrotoxic nephritis Decreased airway remodeling in *A. fumigatus*-induced allergic airway disease Suppression of acute and chronic cardiac allograft rejection [138–144]
CCR2	High cycle rate of bone marrow myeloid progenitors Reduced leukocyte firm adhesion, extravasation and impaired recruitment of macrophages to inflammatory sites Decreased clearance of *Listeria monocytetogenes* Protection and decreased macrophage recruitment in influeza A infection Defective monocytes trafficking and Th1/Th2 balance in *Crytococcus neoformans* infection Decreased number and size of liver and lung granuloma and altered cytokine production Defective skin DC migration and spleen DC relocation following *L. major* Protection from EAE Attenuated airway hyper-reactivity in cockroach antigen-induced allergic airway inflammation Reduced atheroma formation [113, 129, 130, 145–155]
CCR4	Resistance to LPS-induced endotoxin shock Decreased peritoneal macrophage recruitment and production of TNF-α, IL-1β, and MIP-1α after LPS treatment [67]
CCR5	Resistant to HIV-1 infection in humans with Δ32 mutation

Table 3 (continued)

Receptors	Phenotype
CC receptors	
CCR5	Susceptible to *Listeria*, influenza A, *C. neoformans* and enhanced DTH reaction Protection from colitis with Th2 cell activation Resistant to LPS-induced endotoxemia Lower early, but increased late, antigen-specific IFN-γ response to *Leishmania* [156–159]
CCR6	Impaired humoral immune response to oral antigen and rotavirus Increased T cell subsets in mucosa, absence of subsets of DCs in Peyer's patches Higher contact sensitivity [160]
CCR7	Abnormal architecture in secondary lymphoid organs Impaired T cell and DC trafficking and homing Impaired antibody production, DSH and DTH reaction [161]
CCR8	Defective Th2 responses [162]
CCR9	Impaired early T and B cell development Reduction in T cell receptor γδ(+) gut intraepithelial lymphocytes Reduced number of IgA^+ plasma cells in the lamina propria of the small intestine [163]
CXC receptors	
CXCR2	Increased neutrophils in blood Lymphadenopathy and splenomegaly Reduced neutrophil recruitment at inflammatory site Susceptible to candida and bacterial infection Impaired peripheral tolerance Delayed wound healing Decreased atherogenesis progression [128, 164–167]
CXCR3	Resistance to acute allograft rejection [168]
CXCR5	Lack of Peyer's patches Defective formation of primary follicules in spleen Increased B cell number in circulation [161]
CXCR6	Reduced influx of activated CD8 cells in GvHD-induced hepatitis [169]

hand, depletion of homeostatic chemokines and their corresponding receptors such as CXCR5, CCR6 and CCR7 severely disrupt lymphoid organ structures due to impaired or disrupted trafficking and homing of DCs, T and/or B lymphocytes. The resultant abnormalities in lymphoid organ structure become the basis for defects in T or B cell-mediated adaptive immune responses, which are dependent on synaptic contact of these cells with APCs in a normal lymphoid microenvironment (Tabs 2 and 3) as reviewed in [127].

Gene depletion studies also have revealed that a chemokine or receptor, may also mediate important unexpected cellular functions *in vivo*. For instance, in an anterior chamber-associated immune deviation model, CXCR2 was found to mediate immunologic tolerance of the mice [128]. In mice, the functional homologue of human CXCL8 is thought to be MIP-2 (KC or CCL1, 2 or 3), which is selectively up-regulated in tolerance-conferring APCs and serves to recruit NK T cells to the splenic marginal zone to form clusters with APCs and T cells. In the absence of the CXCR2, or in the presence of a blocking antibody to MIP-2, peripheral tolerance is prevented without generation of antigen-specific T regulatory cells. Another example of an unexpected role of a chemokine receptor *in vivo* is the active participation of CCR2 in the initiation and progression of experimental autoimmune encephalitis (EAE). $CCR2^{-/-}$ mice are resistant to induction of EAE with peptide 35–55 derived from myelin oligodendrocyte glycoprotein (MOG) [129, 130]. The mice have neither mononuclear phagocyte infiltrates, nor an increase in chemokines MCP-1 (CCL2), RANTES (CCL5) and IP-10 (CXCL10) as well as chemokine receptors CCR1 and CCR5 in the central nervous system. Additionally, T cells from immunized $CCR2^{-/-}$ mice showed decreased production of IFN-γ on antigen stimulation. In adoptive transfer experiments, $CCR2^{-/-}$ mice failed to develop EAE, despite the administration of MOG-specific $CD4^+$ T cells from wild-type or $CCR2^{-/-}$ donors. These observations suggest that CCR2 is necessary for the initiation of EAE, in addition to its role in directing infiltration of mononuclear phagocytes and, in the host, clearance of microbial infection. Thus, chemokines and receptors constitute the building blocks of the immune system, and lacking a single molecule can be sufficient to unbalance the immune system and impair responses.

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Lymphocyte-endothelial cell interaction

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The multistep paradigm of lymphocyte recruitment

Lymphocyte recruitment is a highly controlled process that is critical in immune system regulation. Lymphocyte tissue-specific vascular recognition and extravasation from blood into tissues relies on the functional interplay between adhesion molecules, expressed by lymphocytes and endothelial cells, and chemoattractants, presented by the endothelial cells to rolling lymphocytes [1–3]. Numerous molecular components of this system have been identified and their mode of action investigated in detail at molecular level (see next sections).

Leukocyte vascular recognition is commonly described to involve three steps, each mediated by a distinct protein family (Fig. 1) [4]. Selectins control initial tethering and rolling of free-flowing white blood cells on carbohydrate moieties present on endothelia [5]. Tethering or capture consists of the initial transient adhesion contacts of leukocytes with the endothelium or an adhered leukocyte or platelet. The slow motion of rolling leukocytes then facilitates sensing of chemoattractants exposed on the endothelial surfaces. Subsequently, chemoattractants rapidly deliver intracellular signals via seven transmembrane domain G protein-coupled receptors (GPCRs) which, in turn, promote firm leukocyte adherence and transendothelial migration by up-regulation of integrin's adhesiveness (avidity) to a family of immunoglobulin-like endothelial ligands [6]. Starting from this simple, and elegant, schema, in recent years a number of important advances has been made, leading to continuous revision of specific, but important, details of the model. For instance, a better distinction between tethering and rolling was clarified, with integrins, and not only selectins, involved in the rolling process. Notably, the dogma implying that rolling was an activation-independent process was challenged by the discovery that $\alpha 4$ integrins (and in certain condition also $\beta 2$ integrins) may also support rolling upon chemokine triggering [7, 8]. A clearer distinction between "fast" and "slow" rolling was also formulated, including the possibility that selectin-triggered, and not only chemokine-generated, signal transduction could also play a role in integrin activation under flow [9]. This observation led to the proposal that, during rolling,

Lymphocyte Trafficking in Health and Disease, edited by Raffaele Badolato and Silvano Sozzani

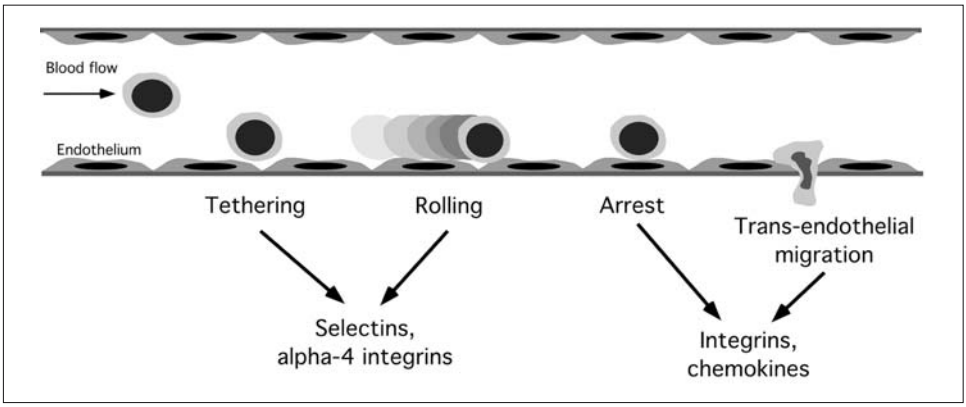

Figure 1
The three-step model of leukocyte recruitment
The diagram shows the phases of lymphocyte recruitment. Lymphocytes freely flow in the blood until the endothelium locally expresses adhesion molecules (for example E-selectin, P-selectin, PNAd or VCAM-1) able to support tethering and rolling of interacting lymphocyte expressing L-selectin, PSGL-1 or VLA-4. Activation by locally expressed chemokines triggers, in rolling lymphocytes, rapid intracellular inside-out signaling events, leading to integrin affinity triggering and subsequent immediate arrest. Finally, directional motility controlled by chemokine gradients and integrin outside-in signaling leads to trans-endothelial migration and extravasation.

selectin-triggered signaling events, although unable to fully trigger integrins, participate in priming integrins to a subsequent full activation induced by chemokines. Moreover, in the last few years the modality of integrin activation induced by chemokines and responsible of lymphocyte arrest under flow was definitively identified (see below). Finally, important advances in describing the signaling events controlling the entire process have been also achieved (see below).

As diverse members of selectins, integrins, chemoattractants and their receptors were identified, and found to possess leukocyte- as well as tissue-specific expression patterns, the concept of a tissue-specific 'area code' for leukocyte trafficking was developed [3, 10, 11]. In such a model, selectins, chemoattractants and integrins were proposed to generate a great combinatorial diversity depending on the type of selectin-carbohydrate, chemoattractant-receptor and integrin-immunoglobulin ligand pairs displayed on the leukocyte and on the endothelium, respectively. The combinatorial logic of the multi-step model illuminated how, by combining a relatively small set of address signals, it was possible to generate a variety of leukocyte area codes for different tissues. It was postulated that each leukocyte subtype would be equipped with a specific combination of receptors allowing its entry into those tis-

sues that displayed the appropriate counter-receptors. This mechanism would generate an unambiguous tissue-specific molecular code. Thus, the multi-step paradigm not only models the leukocyte extravasation process at mechanistic level, but also provides a conceptual framework for the exquisite specificity of leukocyte vascular recognition.

The adhesion molecules and their specificity

In lymphoid organs, PNAd and MAdCAM-1 adhesion molecules, together with their lymphocyte ligands and chemokine/chemokine receptors, create a specific area code for the migration of naïve lymphocytes [2]. However, no area code with specific molecules for the migration of lymphocytes in distinct sites of inflammation has yet been characterized. Some mucins, selectins, VLA-4 and LFA-1 integrins have been implicated in the migration of leukocytes in different inflamed organs. However, the combination of unique hemodynamic characteristics of the blood vessels (for instance high *versus* low shear stress) and the level (low or high) of adhesion molecule and chemokine receptors expression, as well as the specific time point of the inflammation process (early *versus* late) may specifically select lymphocyte subpopulations during inflammation. The main adhesion molecules implicated in the first steps of lymphocyte homing in lymphoid organs or in the recruitment of lymphocytes in extralymphoid organs and in sites of inflammation are introduced below.

Selectins and their ligands

Selectins are adhesion molecules involved in tethering and rolling of lymphocytes during the migration into lymphoid or non-lymphoid organs. Three selectins have been identified: L-, P- and E-selectin. All three selectins are type I transmembrane glycoproteins that bind sialylated carbohydrate structures in a Ca^{2+}-dependent manner. Each selectin has a lectin-like domain and various numbers of consensus repeat domains, which show homology to complement regulatory proteins. The lectin domains of the three selectins share about 60% homology, which results in subtle differences in carbohydrate binding and confers selectin specificity.

L-selectin (CD62L) is expressed on the microvillae of naïve lymphocytes and central memory T cells, and is important for lymphocyte homing and adhesion to high endothelial cells of post capillary venules of peripheral lymph nodes and Peyer's patches [3]. L-selectin is critical to the capture/tethering during the migration through the endothelial lining. It interacts with endothelial mucin MAdCAM-1 in Peyer's patches. In addition, L-selectin binds to endothelial ligands, most of which are characterized by MECA-79 reactivity and are collectively known as peripheral

node addressins (PNAds). The glycoprotein structure(s) that express the MECA-79 antigen are not completely known, but include CD34. It has been also reported that L-selectin might interact with mucin P-selectin glycoprotein ligand-1 (PSGL-1) expressed by adhered leukocytes, and this may help to deliver L-selectin-bearing lymphocytes in sites of inflammation [12].

P-selectin (CD62P) is constitutively expressed on the endothelium of lung and choroids plexus microvessels and on the platelet surface after activation, while E-selectin is constitutively expressed in normal skin vessels. Both E- and P-selectin are up-regulated by inflamed endothelium in most organs during inflammatory diseases [13–17]. PSGL-1 is considered the main lymphocyte ligand for P-selectin and is also able to bind E- and L-selectin. Although PSGL-1 mucin is expressed by all T cells, it is not always glycosylated properly for selectin binding, and this explains why naïve T cells cannot bind P- and E-selectins.

All selectin ligands are carbohydrate-containing molecules, and several glycosyltransferases have a role in the biosynthesis of selectin ligands [18]. These include two α1,3-fucosyltransferases, FucT-IV and FucT-VII, the O-linked branching enzyme core 2 β1,6-glucosaminyltransferase-I (C2GlcNAcT-I), a β1,4-galactosyltransferase-I (b1,4GalT-I), and at least two sialyltransferases of the ST3Gal family that add sialic acid to galactose in a 2-3 linkage. In addition, at least one of two tyrosine sulfotransferases must be active to produce high-affinity P-selectin binding, and the sulfated tyrosine residues of PSGL-1 directly participate in P-selectin binding.

PSGL-1 is a dimeric, mucin-type glycoprotein ligand originally identified as a ligand for P-selectin. PSGL-1 is expressed on the surface of all lymphocytes and is a ligand for E- P- and L-selectin [19]. Much attention has been given to the N-terminal region of PSGL-1 as it contains binding regions for the selectins. P-selectin binds to the extreme N terminus of PSGL-1 by interacting stereospecifically with clustered tyrosine sulfates and a nearby core 2 O-glycan with a sialyl Lewis x (sLex) epitope (C2-O-sLex). Similarly, L-selectin binds with high affinity to the N-terminal region of PSGL-1 through cooperative interactions with three sulfated tyrosine residues and an appropriately positioned C2-O-sLex O-glycan. E-selectin-PSGL-1 binding seems to be sulfation independent, requiring sLex and glycosylation of PSGL-1 by alpha-(1,3)-fucosyltransferases. Expression of cutaneous lymphocyte antigen (CLA), a fucosyltransferase VII (FucT-VII)-dependent carbohydrate modification of PSGL-1, is closely correlated with interactions between PSGL-1 and E-selectin. It has been previously demonstrated that FucT-VII expression is high in Th1 cells, while Th2 lymphocytes expresses high levels of FucT-IV, but not FucT-VII [8]. Moreover, Th1 cells, but not Th2 cells, are able to bind to P-selectin and E-selectin. Experiments performed in mice with targeted deletions of the FucT-IV and FucT-VII loci have established that absence of FucT-VII yields a severe attenuation of lymphocyte migration to secondary lymphoid organs and to sites of cutaneous inflammation [20]. Moreover, it has been recently shown that efficient

recruitment of activated lymphocytes to the brain in the contexts mimicking EAE is controlled by FucT-VII and its cognate cell surface antigen CLA expressed by PSGL-1 [21, 22].

PSGL-1-mediated tethering and rolling *in vivo* depends on the interactions with E- and P-selectin expressed by the endothelium or by P-selectin presented by adhered platelets on the vessel wall. Moreover, it has been shown that also the interactions between the leukocyte adhesion receptor L-selectin and PSGL-1 play an important role *in vivo* in regulating the inflammatory response by mediating leukocyte tethering and rolling on adherent leukocytes. In the last few years, it has been well documented that inhibition of PSGL-1 using different approaches has a beneficial effect in various pathologies in experimental animal models, thus PSGL-1 is emerging as a new promising therapeutic target to be explored in the future also in humans [23].

Integrins and their ligands

Integrins are a large family of heterodimeric transmembrane glycoproteins that attach cells to extracellular matrix proteins of the basement membrane or to ligands on other cells. Integrins contain large (α) and small (β) subunits of sizes 120–170 and 90–100 kDa, respectively.

$\alpha_L\beta_2$ integrin (CD11a/CD18, lymphocyte function-associated antigen-1 – LFA-1) is the most prominent member of the β_2 integrin family, and is expressed by all lymphocytes [1–3]. Although it has been implicated in some rolling interactions, LFA-1 mainly mediates firm adhesion/arrest of lymphocytes in blood vessels of lymphoid organs or in sites of inflammation by binding its ligands from the immunoglobulin family, intercellular adhesion molecule-1 (ICAM-1) and ICAM-2, expressed by the vascular endothelium [1–3, 8, 21].

The most important member of the β_1 integrin subfamily on lymphocytes is the very late antigen-4 (VLA-4, CD49d/CD29, $\alpha_4\beta_1$ integrin). VLA-4 binding to its ligand vascular cell adhesion molecule-1 (VCAM-1) expressed by the endothelium has been implicated in lymphocyte rolling and firm arrest in inflamed vessels [21].

A particular role in lymphocyte homing is ascribed to the interactions between $\alpha_4\beta_7$ integrin with its vascular ligand MAdCAM-1, which act as a brake during naïve lymphocyte interactions in the high endothelial venules (HEVs) of the Peyer's patches [4]. $\alpha_4\beta_7$ is expressed at low levels on naïve T cells, so that L-selectin is required for efficient tethering. In fact, $\alpha_4\beta_7$ integrin is required to slow L-selectin-initiated rolling sufficiently to allow activation and engagement of LFA-1 for firm arrest [4]. In contrast, on gut homing, memory/effector cells and plasmablasts, $\alpha_4\beta_7$ is highly expressed and may mediate tethering, rolling and arrest on its own.

Immunoglobulins are plasma proteins that include all antibody molecules. Members of the immunoglobulin superfamily share structural and genetic features with

immunoglobulin molecules and contain at least one immunoglobulin domain. An immunoglobulin domain is made of two β-pleated sheets held together by a disulfide bond. The vascular endothelium expresses molecules of the immunoglobulin superfamily, which act as counter-receptors for leukocyte integrins. Two immunoglobulins that are particularly important in the migration of lymphocytes are ICAM-1 and VCAM-1 [11].

ICAM-1 (CD54) is a member of the immunoglobulin superfamily of adhesion molecules, and contains five immunoglobulin-like domains. It is one of the principal ligands for the leukocyte β_2-integrins CD11a/CD18 (LFA-1). VCAM-1, or CD106, contains six or seven immunoglobulin domains and is expressed on both large and small vessels only after the endothelial cells are stimulated by cytokines. The sustained expression of VCAM-1 lasts over 24 h. Primarily, VCAM-1 is an endothelial ligand for VLA-4 of the β_1 subfamily of integrins and for integrin $\alpha_4\beta_7$. VCAM-1 promotes the adhesion interactions of lymphocytes and other leukocytes in inflamed vessels [21].

Chemokines and the modalities of integrin activation

The critical role of integrin activation in cell regulation is well established and a complete discussion of this topic is beyond the purpose of this chapter. In this section, we only focus on those aspects of integrin activation that are crucial to rapid lymphocyte arrest under flow conditions.

The importance of *in situ* rapid integrin triggering as a critical regulatory event of lymphocyte homing was originally established by a seminal work by Butcher's group [24]. In this study, pretreatment of lymphocytes with pertussis toxin, a specific inhibitor of Gαi-heterotrimeric GTP-binding protein, was able to completely prevent integrin-mediated lymphocyte adhesion to HEVs in secondary lymphoid organs. These data demonstrated that lymphocytes express specific receptors capable of fully triggering integrins through a Gαi-heterotrimeric GTP-binding protein-linked intracellular signaling pathway. Importantly, this observation clearly implied the *in situ* expression of pro-adhesive agonists, presented by HEVs and able to trigger, in rolling lymphocytes, rapid integrin activation. This study was a "big bang" in this field as it encouraged the research for the pro-adhesive agonists, expressed by HEV and other endothelia, that are able to selectively trigger integrins in different leukocyte subtypes. Furthermore, the definition of the intracellular signaling mechanisms controlling rapid integrin triggering became a major goal in the field. Nowadays, we know that a particular sub-family of chemotactic cytokines, called chemokines, is responsible of rapid integrin triggering (and of several other events) in circulating lymphocytes (see chapter 1) [25]. To date about 45 human chemokines (classified in four sub-families) have been identified, and more than 18 chemokine receptors, all Gαi-linked, have been cloned and variably associated to

different chemokines, thus generating a regulatory network of chemokine-chemokine receptor interactions characterized by specificity and robustness, and involved in regulating the diversity of leukocyte recruitment [26]. Although all were characterized by chemotactic activity on different leukocyte sub-types, and thus were shown to be involved in microenvironmental positioning, some chemokines have been clearly shown to be expressed by the endothelium [25, 26] and to be able to fully activate lymphocyte integrin under flow [27]. Considering that lymphocytes (and more generally leukocytes) operate in a high shear stress environment represented by the blood flow, pro-adhesive chemokines really accomplish an amazing task. Accurate studies have shown that chemokines can trigger full integrin activation and dependent lymphocyte arrest under flow within a few milliseconds [27]. De facto, chemokines are considered the most powerful physiological integrin activators.

However, what does "full integrin activation" mean? The capability of lymphocytes to modulate their own adhesiveness for a substrate by changing integrin avidity for the ligand was established a long time ago [28]. On the other hand, it was only recently that the modality of integrin activation induced by chemokines and responsible for almost all immediate lymphocyte arrest under flow was definitively identified. The most studied integrin involved in lymphocyte adhesion is the β_2 integrin LFA-1 (CD11a/CD18). Structural studies show that LFA-1 may exist in three different headpiece-conformational states, each corresponding to a different affinity for ICAM-1 (Fig. 2) [29–31]. The low-affinity state (folded conformation) is the resting state, a conformation basically incapable of mediating adhesion. Cell activation triggers the transition from low to intermediate state (extended conformation) and from intermediate to high-affinity state (extended but further rearranged conformation), with the last two able to support binding to soluble ligand and rapid adhesion under flow. Thus, "full LFA-1 activation" basically means modification of the equilibrium between the three different conformers, thus allowing LFA-1 to acquire the capability to bind the ligand with increasing energy. We now know that chemokines are able in milliseconds to trigger LFA-1 conformational changes corresponding to heterodimer intermediate- and high-affinity states for ICAM-1 [32, 33]. Importantly, studies focused on the definition of the signaling events controlling this complex phenomenon provided a formal demonstration of the critical role of triggering LFA-1 to a high-affinity state in lymphocyte homing to secondary lymphoid organs [34]. Interestingly, it seems that soluble chemokines may trigger the complete state transition from low to high affinity, whereas immobilized chemokines trigger only the transition from the low- to intermediate-affinity state, with high affinity induced upon ICAM-1 binding [33]. This finding allowed proposing a cooperative model between pro-adhesive signaling mechanisms, in which inside-out and outside-in signaling cooperate to trigger full lymphocyte arrest under flow [35]. Moreover, evidence show that chemokines may also trigger lateral mobility of LFA-1, leading to cluster formation and increase valency [32, 34] (although

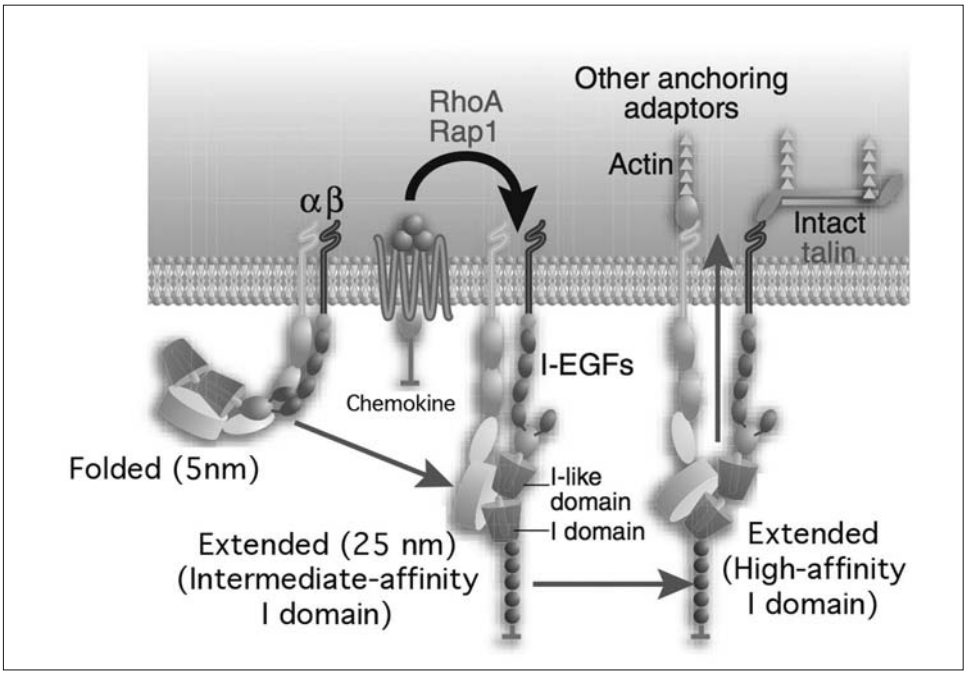

Figure 2

The equilibrium between LFA-1 conformers corresponding to different heterodimer affinity states

In resting state, LFA-1 is in a bent, folded, conformation corresponding to a low-affinity state. In resting state LFA-1, the affinity for ICAM-1 is in μM range. Upon chemokine activation, LFA-1 undergoes a transition to an extended conformation displaying an affinity for ICAM-1 in the mM range. A further transition to a high-affinity state corresponds to extended conformation with further heterodimer rearrangement and increased affinity in the nM range. The intracellular activity of RhoA and Rap1 small GTPases mediates chemokine-induced LFA-1 transition from low- to intermediate-, and from intermediate- to high-affinity state. Soluble chemokines may trigger the complete transition from low- to high-affinity conformers, whereas, in contrast, immobilized chemokines trigger only partial LFA-1 transition to intermediate affinity, with high affinity induced upon interaction with immobilized ICAM-1. Interaction with cytoskeleton components is essential to induce LFA-1 affinity triggering. The picture was modified from Laudanna C., Nat Immunol. 2005, 6: 429–430.

this possibility was recently challenged by studies showing that LFA-1 clustering may occur only in presence of immobilized ligand [36]). In summary, chemokines presented to rolling lymphocytes trigger, through Gαi-linked receptors, an intracellular signaling network capable of triggering LFA-1 conformational changes corre-

sponding to intermediate- or to high-affinity states, deepening on some, still undefined, quantitative signaling aspects. Immediate arrest under flow needs transition to a high-affinity state conformer, which allows binding of immobilized ICAM-1 with high energy.

Signaling mechanisms in integrin activation

A rather complex, still incompletely defined, aspect of this process is the nature of the intracellular signaling mechanisms triggered by chemokines and controlling rapid integrin activation. In recent years, a few important discoveries allowed a better definition of this biochemical mechanism (Fig. 3). Several signaling events triggered by chemokines have been implicated in the regulation of integrin activation, yet it appears that not every pathway is operative in every cell type and integrin. Here we describe only signaling events whose role has been validated under physiological conditions.

Small GTP-binding proteins play a central regulatory role in the modulation of both integrin affinity and mobility. The small GTPase, RhoA, was originally shown to be involved in rapid integrin activation by CXCL8 (IL-8) [37]. Pretreatment of lymphocytes with the Rho inhibitor C3 transferase was shown to prevent rapid leukocyte adhesion to VCAM-1, suggesting the involvement of Rho in the GPCR activation of the β_1 integrin VLA-4. More recently, RhoA was demonstrated to have a rather complex role in LFA-1 activation in lymphocytes. Using trojan peptides derived from three distinct effector regions of RhoA, able to block RhoA-dependent signaling in a domain-selective manner, it was shown that RhoA controls the triggering of both LFA-1 affinity as well as lateral mobility [34]. Importantly, it was demonstrated for the first time that RhoA-controlled triggering of LFA-1 to an increased affinity state is critical to lymphocyte *in vivo* homing to secondary lymphoid organs [34]. Moreover, the permeable RhoA peptide corresponding to the 23–40 domain of the GTPase, which was shown to block high LFA-1 affinity stimulated by soluble chemokines, also dramatically attenuates the ability of these chemokines, when surface bound, to trigger extended β_2 integrin conformations (corresponding to intermediate affinity) under shear flow (Shamri, Alon and Laudanna, unpublished). Thus, the RhoA-dependent signaling pathway is critically involved in triggering LFA-1 conformational changes required for abrupt lymphocyte arrest under shear flow.

Another small GTPase, the ras-like small GTPase Rap1, has recently emerged as an important regulator of rapid integrin activation by chemokine signals [38]. Transfection of lymphocytes with Rap1 dominant negative mutants prevents LFA-1- as well as VLA-4-mediated adhesion induced by chemokines. The role of Rap1 in integrin triggering also emerges from the recent description of a human genetic defect, called LAD III, in which a deficiency in lymphocyte adhesiveness correlates

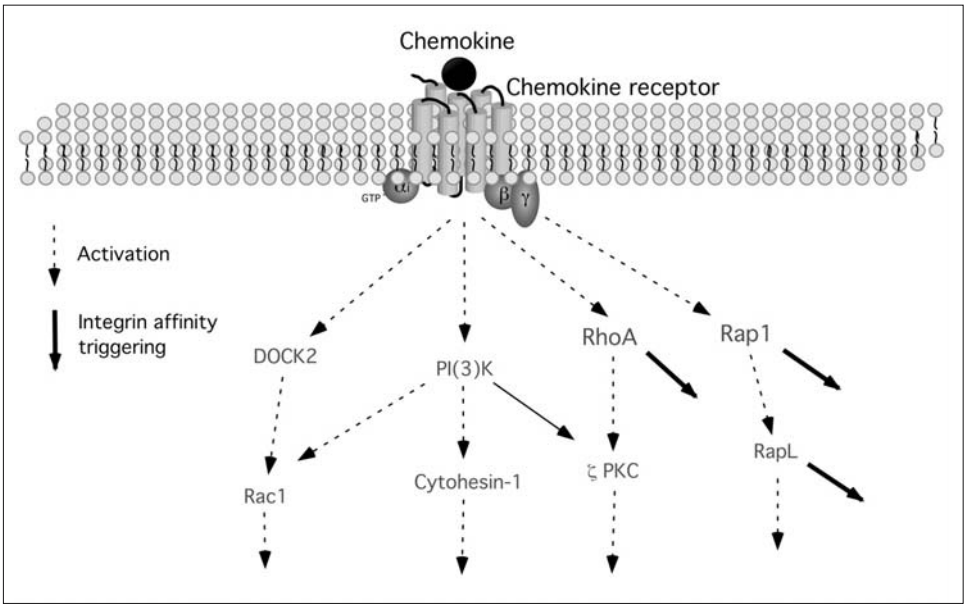

Figure 3
Signaling events triggered by chemokines and leading to integrin activation
The diagram shows the major signal transduction events involved in rapid integrin activation by chemokines. RhoA, Rap1 and RapL have been shown to regulate integrin affinity triggering. The role of Rac1, although suggested by DOCK2 involvement, has still not been formally demonstrated.

with impairment of Rap1 activation by chemoattractants [39]. Interestingly, a potential effector of Rap1 to LFA-1 activation was recently identified in a yeast two-hybrid screen, and called RAPL [40]. RAPL is highly expressed in lymphocytes and dendritic cells, and is able to bind Rap1-GTP and to the tail of the α chain (CD11a) of LFA-1. This suggests that RAPL may be a key effector linking activated Rap1 to LFA-1, and possibly to VLA-4 activation. This hypothesis is supported by recent data on RAPL-deficient lymphocytes, in which a partial reduction of chemokine-triggered integrin-mediated adhesion was observed [41]. Notably, Rap1 seems to control both affinity triggering and clustering of the β_2 integrin LFA-1, although a direct demonstration, under physiological conditions, of the capability of Rap1 to control distinct LFA-1 conformers changes is still lacking.

In addition to RhoA and Rap1, several other effectors triggered by chemokine signaling have been implicated in rapid integrin activation processes in various cellular and animal models. A more detailed description of these pro-adhesive molecular events has been already provided [6]; here, we only briefly describe them. The

ARF-guanine-nucleotide exchange factor (GEF), cytohesin-1, may induce LFA-1 activation by direct interaction with the β_2 chain [42, 43]. Chemokines may modulate rapid LFA-1 activation by triggering, via the cytohesin-1 PHc domain, membrane targeting of cytohesin-1, thus allowing a direct regulatory interaction with the β_2 chain of LFA-1 [44].

DOCK2, a hematopoietic specific member of the CDM family of proteins, has GEF activity for Rac1-2, and was recently shown to regulate lymphocyte recruitment *in vivo*. Interestingly, DOCK2 is involved in B but not in T lymphocyte integrin activation by chemokines as well as in *in vivo* recruitment to secondary lymphoid organs [45]. Although the role of DOCK2 suggests the involvement of Rac1 in rapid integrin triggering by chemokines, the formal demonstration of the involvement of this small GTPase in integrin affinity or lateral mobility triggering by chemokines in B cells is still lacking.

The lipid kinase phosphatidylinositol 3-OH kinase [PI(3)K] is also involved in lymphocyte integrin activation. PI(3)K inhibition prevents chemokine-induced rapid lymphocyte binding to immobilized ICAM-1, but only if ICAM-1 is expressed at a very low site density [20]. PI(3)K, whose activity is rapidly triggered by chemokines, is involved in the rapid induction of heterodimer lateral mobility induced by chemokines likely leading to increased valency. In contrast, PI(3)K activity is not required for the rapid induction of the LFA-1 high-affinity state. Together, the PI(3)K requirement for rapid adhesion at limiting ICAM-1 densities suggests that rapid PI(3)K activation by chemokines may function to facilitate integrin mobility in the cell membrane, and thereby enhance the probability of encounters between the activated heterodimers and the disperse ligand. PI(3)K activity is not implicated, however, in chemokine stimulation of VLA-4 avidity, suggesting that the lipid products of this kinase activate only specific subsets of integrins and under limited availability of ligand. Notably, the γ isoform of PI(3)K was shown to be involved in integrin activation in T but not B lymphocytes [45], further highlighting cell and integrin-type specificity in PI(3)K signaling to integrins.

Another example of a chemokine-triggered effector that regulates integrin valency rather than affinity is the atypical protein kinase C isoform, PKC ζ, apparently acting downstream of RhoA. The PKC family includes at least three categories of isozymes: the classical, novel and atypical sub-families. Neither classical (DAG- and Ca^{2+}-dependent) nor novel (DAG-activated but Ca^{2+}-independent) PKC isotypes play an essential role in rapid β_2 integrin activation by chemokines [32, 34]. In contrast, the atypical isoform, PKC ζ, which is not activated by DAG and Ca^{2+}, appears to be a critical participant in rapid β_2 integrin activation by chemokines. Indeed, PKC z-selective inhibitory peptides (myristoylated peptides with sequence identical to the pseudosubstrate inhibitory region of PKC ζ) efficiently block chemokine-induced rapid adhesion of lymphocytes to surfaces presenting a low density of ICAM-1 [34]. Importantly, PKC ζ inhibition does not prevent chemokine-induced triggering of LFA-1 to high-affinity state, but blocks the lateral mobility of the het-

erodimers [34]. Notably, PKC ζ is activated and rapidly translocated to the plasma membrane upon chemokine stimulation, and this is prevented by pretreatment with RhoA-derived trojan inhibitory peptides, indicating that PKC ζ may be a downstream effector of RhoA-stimulated integrin mobility [34].

Diversity generation: qualitative and quantitative control

Despite the increased knowledge gathered in the last two decades, the bewildering complexity and redundancy of the molecular system controlling lymphocyte-endothelial cell interaction still defeats our ability to accurately describe and predict the functional outcome of the lymphocyte recruitment process.

Accumulating evidence shows a rather high degree of promiscuity and redundancy in ligand-receptor interactions [46]. Most leukocytes possess largely overlapping patterns of receptors and multiple overlapping series of chemokines are often found expressed on endothelial cells from distinct tissues. Furthermore, at the signal transduction level, data are emerging suggesting the existence of cell- and integrin subtype-specific regulatory mechanisms [6]. These findings are blurring the ability of the original multi-step model to predict how a specific combination of pro-adhesive molecules may control selective leukocyte vascular recognition. Overall, it appears that a purely qualitative combination of different parameters controlling lymphocyte recruitment is no longer adequate to account for the observed diversity.

One of the most important aspects, not really accounted for by the original formulation of the multi-step model, is the regulatory significance of quantitative variations of pro-adhesive parameters, including variation in site density (expression level per area) of adhesion molecules and chemokines, and variation in the amount and topological distribution (compartmentalization) of intracellular signaling events. Recent studies show the importance of quantitative variation of pro-adhesive parameters regulating lymphocyte recruitment under flow. For instance, quantitative variation of the site density of ICAM-1 presented to rolling lymphocytes determines the relative importance of the distinct modalities of LFA-1 activation triggered by chemokines. Indeed, if affinity triggering seems always required for rapid arrest under flow [34], lateral mobility of the heterodimer, leading to increased valency, seems relevant only to facilitate adhesion to a disperse integrin ligand [32]. Thus, the amount of integrin ligand expression dictates the usage of a specific modality of integrin activation responsible for rapid adhesion. This dichotomy may assume a specific regulatory significance when endothelium expresses variable amount of integrin ligands during the immune responses.

Another example of the importance of quantitative variation of pro-adhesive parameters was recently provided in the context of the chemokine network. Here, different expression levels of chemokine receptors, combined with variation of the binding affinities of the chemokines for the cognate receptors, determine quantita-

tive variations in the intracellular signal transduction triggered by the chemokine presented to rolling lymphocytes. This variation in signaling efficiency was shown to affect the specificity of the lymphocyte recruitment process, altering the capability to direct specific recruitment of Th1 *versus* Th2 lymphocytes [47]. This observation clearly suggests that maintaining of stability in quantitative parameters controlling lymphocyte recruitment is critical to maintain diversity in the immune response. Thus, it appears that lymphocytes are able to decode quantitative, and not only qualitative, environmental information.

These preliminary findings emphasize the great importance of quantitative aspects in the overall regulation of lymphocyte recruitment and, finally, of the immune response. In this context, an updated version of the original three-step paradigm, including the mathematical concept of concurrency, was recently proposed. This model provides a conceptual framework necessary to the development of predictive computer modeling of lymphocyte recruitment process [48]. In this model a transition form of qualitative to quantitative area code for specific lymphocyte recruitment is envisioned. For such a vision, of course, experimental quantitative data need to be obtained to allow effective computer modeling and to improve our capacity to precisely predict the long-term dynamics of an immune response.

Acknowledgements

We wish to thank Ronen Alon for sharing unpublished data. C. Laudanna's research is supported by the Fondazione Cariverona and by the Fondo Incentivazione Ricerca di Base (FIRB). G. Constantin's research is supported by the National Multiple Sclerosis Society, New York, NY, USA, the Fondazione Italiana Sclerosi Multipla (FISM), Italy, and the Italian Ministry of Health (Ricerca finalizzata), Fondazione Cariverona, FIRB.

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Chemokine receptor expression in effector and memory T cell subsets

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T cell priming and generation of Th1 and Th2 responses

Clearance of infection involves the interaction of several cells of the immune system that, scattered throughout the body, have to be recruited and interact together in a precise place at a precise time. During a primary response, the rare antigen-specific naïve T lymphocytes continuously recirculate through secondary lymphoid organs. This strategy allows them to maximize their chance to encounter professional antigen-presenting cells, i.e., dendritic cells (DCs), presenting on their surface the antigen sampled at peripheral sites. Upon engagement of their T cell receptor (TCR) with antigenic peptides presented in association with molecules of the major histocompatibility complex (MHC) on the surface of DCs, naïve T cells proliferate and differentiate to effector cells [1]. While naïve T cells are functionally undifferentiated and capable of secreting mainly IL-2 upon stimulation [2], effector cells produce inflammatory cytokines such as IFN-γ and IL-4. Classically, two types of effector $CD4^+$ T cells can be distinguished in mouse and man based on the cytokines they produce [3, 4]. T helper 1 (Th1) cells are characterized by the secretion of IFN-γ and the induction of cell-mediated responses against intracellular pathogens whereas Th2 cells produce IL-4, IL-5 and IL-13 and mediate protection against extracellular parasites.

The balance between production of Th1 and Th2 cytokines determines whether specific immunity against invading microorganisms is successful, whereas unbalanced Th1 and Th2 responses can lead to pathological manifestations. In particular, excessive Th1-type cytokines have been associated with tissue destruction found in autoimmune diseases such as rheumatoid arthritis and multiple sclerosis [5], whereas overproduction of Th2-type cytokines has been implicated in atopy and allergic asthma [6, 7].

Th1 and Th2 differentiation is dependent on several factors. Besides DC type, antigen dose and costimulatory interactions between cell surface molecules, the most critical role is played by cytokines. The cytokines that control Th1 and Th2 differentiation are IL-12 and IL-4, respectively, which act primarily through the

Lymphocyte Trafficking in Health and Disease, edited by Raffaele Badolato and Silvano Sozzani

induction of the master transcription factors T-bet and Gata-3 [8–10]. Mature DCs represent the major source of IL-12, which is secreted at high amounts after stimulation with lipopolysaccharide (LPS) and CD40 ligand (CD40L) [11, 12]. Although several cell types can produce IL-4, the physiological source of this cytokine remains elusive. Mast cells produce IL-4, but they reside in peripheral tissues, while the T cell differentiation process takes place in secondary lymphoid organs. NK-T cells can also produce IL-4, yet the Th2 response is intact if NK-T cells are deleted [13]. There is evidence indicating T cells themselves as a possible source of IL-4. Indeed, it has been shown that naïve T cells express IL-4 at low levels upon activation [14, 15].

Regulation of cytokine gene expression in Th1 and Th2 cells

In the last few years, a big effort has been made to try to elucidate the mechanisms that lead to the cell type-specific production of the signature effector cytokines in Th1 and Th2 cells. The body of work that has been done in this field suggests that the regulation occurs at least partially at the chromatin level [16]. In naïve T cells, the IFN-γ and IL-4 genes are targeted by chemical modifications in the DNA and its associated proteins – the histones –, which leads to a compacted ("closed") conformation inaccessible to transcription factors. During differentiation, the Th1- and Th2-specific transcription factors T-bet and Gata-3 cause remodeling events at the relevant cytokine genes, such as DNA demethylation and histone acetylation, which lead to an "open" chromatin conformation followed by transcriptional activation and gene expression [17–19]. The chromatin modifications are transmitted epigenetically to the progeny, thereby ensuring a faithfully transmission of the imprinted phenotype through cell divisions in memory T cells [19]. Regulation of cytokine gene expression does not merely occur through a "closed" or "open" state of chromatin. Rather, distinct histone modifications (acetylation, methylation, phosphorylation) can generate synergistic or antagonistic interaction affinities for chromatin-associated proteins, which in turn dictate dynamic transitions between transcriptionally silent or transcriptionally active chromatin state. Thus, different histone modifications can be arranged in a combinatorial way that establishes a "histone code", thereby extending considerably the information potential of the genetic code [20].

Chemokine receptors in Th1 and Th2 cells

Naïve T cells recirculate within secondary lymphoid organs, while antigen-primed T cells exiting these organs are able to enter peripheral inflamed tissues, indicating that T cell differentiation is tightly coupled to the acquisition of new migratory

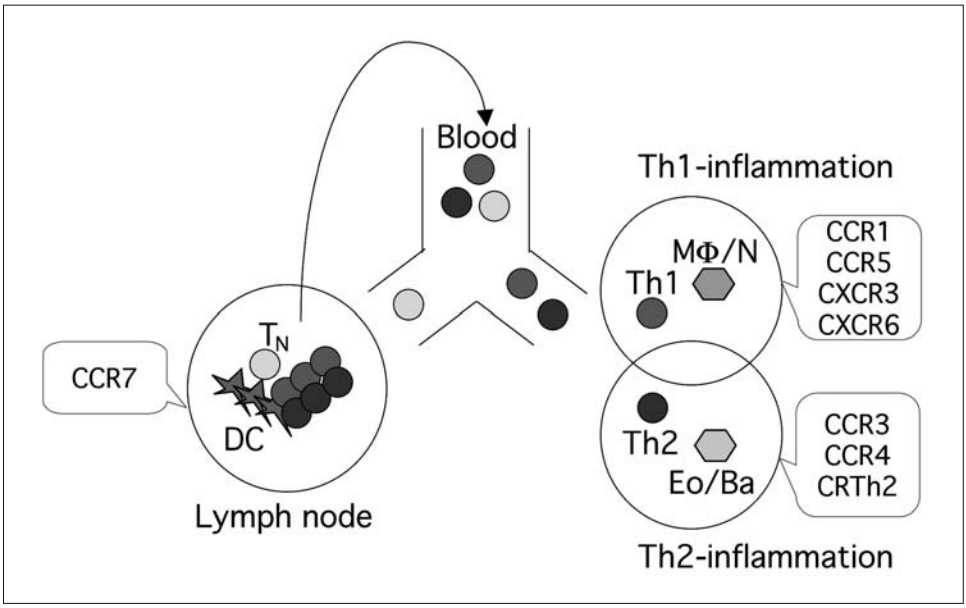

Figure 1
Naïve T cells exit the blood and enter into lymph nodes using the chemokine receptor CCR7. In lymph nodes they are activated by antigen-presenting DCs and differentiate to IFN-γ-producing Th1 (red) or IL-4-producing Th2 (blue) cells depending on environmental factors including polarizing cytokines. From the lymph nodes effector cells re-enter the circulation and, by virtue of expression of new chemokine receptors (as outlined in the red callouts), migrate to inflamed tissues. At these sites, Th1 and Th2 cells coordinate the effector response through activation of cells of the innate immune system such as macrophages (MΦ) and neutrophils (N) in Th1-type inflammation or eosinophils (Eo) and basophils (Ba) in Th2-type inflammation. The integrins and selectins, which are also involved in these migratory pathways, are not depicted.

capacities (Fig. 1). There is growing evidence that the accumulation of different types of effector cells in inflammatory lesions is a dynamic process orchestrated by the regulated expression of chemokines and chemokine receptor. The chemokines produced during an inflammatory process contribute to determine the extent, the quality and the duration of the cellular infiltrate. There are indications that this network may fit into a classification according to type 1/type 2 regulation. For instance, IL-4 and IL-13 stimulate the production of CCL11 and CCL22 (two chemokines involved in Th2-type immune reactions), an effect that is counteracted by IFN-γ [21, 22]. Conversely, IFN-γ induces CXCL9 and CXCL10 and up-regulates CCL5 (all of them major type 1-chemokines), and this effect is antagonized by

IL-4 [23]. On the other hand, TNF, a cytokine that is associated with both Th1 and Th2 responses, costimulates the production of both type 1 and type 2 chemokines.

A significant example of the role played by chemokines in the orchestrated recruitment of cells in polarized responses is shown by the C-C chemokine receptor CCR3 and its ligand CCL11. CCL11 is abundantly present in mucosal tissues undergoing allergic inflammation [21, 24–26]. CCR3 is expressed on eosinophils [27] and basophils [28] , as well as *in vivo* and *in vitro* polarized Th2 cells [29, 30]. The sharing of CCR3 expression may allow these different cell types to colocalize at sites of CCL11 production. Here the cells produce IL-4, IL-5 and IL-13, which in turn activate eosinophils and basophils and boost production of CCL11 and CCL22, thus amplifying the inflammatory reaction.

Besides CCR3, there are other inflammatory chemokine receptors expressed on Th2 cells. These include CCR4 [31–33], the receptor for CCL17 and CCL22, which is also expressed on basophils [34], and CCR8 [35], the receptor for CCL1 [36]. The relative importance of CCR3 and CCR4 as markers for Th2 cells has been debated. While CCR3 expression does not identify all IL-4-producing Th2 cells, CCR4 is also expressed on activated Th1 cells, on non-polarized T cells primed in the presence of TGF-β, and on skin-homing T cells (see below), which are mainly of the Th1 type. The presence of CCR4 on these different functional subsets may reflect the multiple roles of CCL17 and CCL22, which are expressed in a variety of lymphoid and non-lymphoid tissues and may behave as inflammatory, constitutive, or tissue-homing chemokines in different circumstances.

An unambiguous marker to identify T cells that produce IL-4 (as well as IL-5 and IL-13), but not IFN-γ, is the chemoattractant receptor CRTh2 [37], which binds prostaglandin D2 (PGD2), a prostanoid that has long been implicated in allergic diseases [38, 39]. CRTh2-expressing T cells are increased in the peripheral blood of patients suffering from atopic dermatitis [40]. Moreover, the vast majority of $CD4^+$ and virtually all $CD8^+$ T cells infiltrating the skin of atopic dermatitis patients are $CRTh2^+$. Thus, CRTh2 may represent a reliable marker for the identification of both $CD4^+$ and $CD8^+$ type 2 cells in health and disease. CRTh2 is also expressed on eosinophils and basophils, and all three cell types migrate in response to PGD2 [38], providing further evidence for a model of coordinate recruitment of cells participating to the same polarized immune response.

Four receptors are preferentially expressed on Th1 cells: CCR5, CXCR3, CXCR6 and CCR1 [31, 32, 41, 42]. In rheumatoid arthritis and multiple sclerosis, two diseases associated with type 1 responses, virtually all T cells in the lesions express CCR5 and CXCR3, in contrast to the 5–15% of T cells that normally express these markers in peripheral blood [43, 44]. CXCL10 (the ligand of CXCR3) is induced by IFN-γ and is expressed abundantly in Th1 lesions [23, 45]. The presence of CCR1 and CCR5 on macrophages and monocytes enables them to colocalize with Th1 cells. However, it should be pointed out that CCR5 and CXCR3 are expressed, although at lower level, on activated T cells and on Th2 cells, so that to

date a chemokine receptor associated with the selective expression of IFN-γ (i.e., the Th1 counterpart of CRTh2) is still missing.

Human T cells activated in the presence of IFN-α and mouse T cells stimulated with IL-23 up-regulate CCR1 [31, 46]. IL-23 is one of the essential factors required for the expansion of a pathogenic $CD4^+$ T cell population characterized by the expression of IL-17 (hence called Th17) and able to confer organ-specific autoimmune inflammation upon adoptive transfer [46–48]. Expression of IL-17 has been linked to a growing list of autoimmune and inflammatory diseases such as rheumatoid arthritis, lupus, asthma and allograft rejection. Mechanistically, IL-17 is believed to contribute to the pathogenesis of these diseases by acting as a potent pro-inflammatory mediator [49]. The molecular mechanisms that govern the development of Th17 cells have remained unclear. Two recent reports provide evidence that Th17 cells are a separate and early lineage of effector $CD4^+$ T cells derived directly from naïve cells and completely independent from Th1 and Th2 lineages [50, 51]. If a correlation between IL-17 production and CCR1 expression is confirmed, the presence of this receptor could represent a helpful tool to identify this novel population of pathogenic T cells involved in autoimmune inflammation.

The model that emerges from these data is a complex and flexible regulation of chemokine receptor expression, where several cytokines can influence the set of receptors that is present on the surface of T cells. In line with these observations, IL-2 (a critical survival factor for activated T cells) together with TCR triggering have been shown to modulate the expression of some chemokine receptors [52]. In addition, antigenic stimulation of Th1 and Th2 cells leads to a rapid switch in the set of chemokine receptors expressed [53]. Within the first hours after TCR triggering the receptors for inflammatory chemokines, i.e., CCR1, CCR2, CCR3, CCR5, CCR6, and CXCR3 are down-regulated, while CCR7, CCR4, CCR8 and CXCR5 are up-regulated. The original set of receptors is then regained when the cells go back to their resting state after a few days. As a result, T cells that are activated by antigen in tissues may either recirculate to draining lymph nodes or migrate to nearby sites of organized ectopic lymphoid tissues.

Regulation of chemokine receptor expression in Th1 and Th2 cells

The mechanisms underlying the coordinated expression of cytokines and chemokine receptors in developing Th1 and Th2 cells are beginning to be unraveled, at least in Th1 cells. Mice deficient for T-bet (the master Th1-differentiation factor) are resistant to a wide range of autoimmune diseases, including type I diabetes, inflammatory colitis and arthritis, lupus nephritis and experimental autoimmune encephalomyelitis *in vivo* [54–56]. While impaired cellular effector function due to the absence of T-bet is most likely playing a role, a common finding in the above models is the reduction in cellular infiltration to inflammatory sites, suggesting a

role for T-bet in cellular trafficking. Indeed, recent studies have elucidated a role for overexpressed T-bet in the induction of CXCR3 (a chemokine receptor preferentially present on Th1 cells) on polarized Th2 cells, and demonstrated an associated increase in chemotactic function [57, 58]. In addition, deletion of T-bet leads to a reduction in CXCR3 expression with the subsequent abrogation of multiple functions, including lymphocyte arrest on activated endothelium and chemotaxis [59]. These findings provide evidence that the transcription factor specifying the Th1 profile in developing T cells imprints a migratory program to ensure appropriate homing to inflammatory sites.

Chemokine receptors identify memory T cells with different functional properties

After pathogen clearance, the massively expanded effector T cells that have been generated become unnecessary, and their number must be reduced to provide some "space" necessary for the development of a subsequent immune response. To cope with this problem, the vast majority (>90%) of effector T cells are eliminated at the end of the primary immune response and only few cells persist for years as memory cells [60].

Memory T lymphocytes are heterogeneous, and two distinct subsets can be identified in the human $CD4^+$ and $CD8^+$ compartments by combining two distinct criteria, namely (i) the absence or presence of immediate effector function, and (ii) the expression of homing receptors that allow cells to migrate to secondary lymphoid organs as opposed to non-lymphoid tissues [61]. Human central memory T cells (T_{CM}) are $CD45RO^+$ memory cells that constitutively express CCR7 and CD62L, two receptors that are also present on naïve T cells, which are required for cell extravasation through high endothelial venules (HEVs) and migration to the T cell area of secondary lymphoid organs [62, 63].When compared to naïve T cells, T_{CM} have higher sensitivity to antigenic stimulation and are less dependent on costimulation, i.e., they possess a lower activation threshold. Also, they are able to up-regulate CD40L to a greater extent, thus providing more effective stimulatory feedback to DCs and B cells. Following TCR stimulation, T_{CM} produce mainly IL-2, but only little IFN-γ and IL-4.

Human effector memory T cells (T_{EM}) are memory cells that have lost constitutive expression of CCR7, are heterogeneous for CD62L expression and display characteristic sets of chemokine receptors and adhesion molecules that are required for homing to inflamed tissues. When compared with T_{CM}, T_{EM} are characterized by a rapid effector function, which is achieved through the prompt secretion of large amounts of cytokines such as IFN-γ, IL-4 and IL-5. Moreover, in the cytoplasm of $CD8^+$ T_{EM}, granules containing perforin – a protein that mediates killing of virus-infected target cells by forming pores in their membranes – can be visualized. These

granules are particularly visible in a population of T_{EM} cells that express CD45RA (defined as T_{EMRA}), representing a terminally differentiated subset. From these observations one can say that in humans the T_{EM} pool contains *bona fide* Th1, Th2 and cytotoxic T cells (CTLs).

The existence of T_{CM} and T_{EM} subsets has also been well documented in mice [64–66]. In this experimental system it has been possible to directly follow the kinetics of memory cell generation and the capacity of effector and memory populations to reconstitute long-term memory [67]. The indications that come from these studies support the idea that T_{CM} have higher reconstitution potential. Indeed, effector Th1 cells, defined by their secretion of IFN-γ, were shown to be short-lived and unable to reconstitute T cell memory. In contrast, a population of activated Th1 lineage cells, which did not secrete IFN-γ after primary antigenic stimulation, persisted for several months *in vivo* and developed the capacity to secrete IFN-γ upon subsequent stimulation [68]. Consistent with these results, the expansion potential of *in vitro* stimulated human memory subsets decreases from T_{CM} to T_{EM}, and is very low in $CD8^+$ T_{EMRA} [69].

The relative proportions of T_{CM} and T_{EM} in blood differ in the $CD4^+$ and $CD8^+$ compartment (T_{CM} are predominant in CD4 and T_{EM} in CD8). Within the tissues, however, T_{CM} and T_{EM} show characteristic patterns of distribution. Indeed, T_{CM} are enriched in lymph nodes and tonsils, whereas lung, liver and gut contain more T_{EM} [70].

In antigen-primed individuals, $CD4^+$ T cells specific for tetanus toxoid can be detected in circulating T_{CM} and T_{EM} up to 10 years after antigenic stimulation [61], and their frequencies increase in both subsets after booster immunization. Analogous examples can be found in the CD8 compartment, where antigen-specific T cells can be found in both T_{CM} and T_{EM} subsets [71]. A detailed TCR repertoire analysis was performed by spectra-typing memory $CD8^+$ T cells using CD62L to discriminate between T_{CM} and T_{EM} [72]. The analysis of six influenza-specific T cell clones showed that two clonotypes were shared between T_{CM} and T_{EM}, whereas four were detected only in the T_{CM} pool. These data indicate that the same expanded clone can be present in both T_{CM} and T_{EM} subsets, and support the model of intraclonal functional diversification [73].

One important issue to be understood is how these different memory subsets are maintained over several years. Proliferation of memory T cells can be driven not only by antigenic stimulation, but also by cytokines. Indeed, under steady-state conditions, memory T cells slowly turn over in the absence of antigen [74]. In particular, IL-7 and IL-15 have been shown to regulate mouse $CD8^+$ memory T cell survival and self-renewal in the absence of antigen, whereas naïve and $CD4^+$ T cells require IL-7 and TCR, but do not respond to IL-15 [75–77]. Conversely, human $CD4^+$ memory T cells proliferate in response to IL-15 in a TCR-independent fashion and with slow kinetics [78, 79], suggesting different roles for IL-15 in mouse and human $CD4^+$ T cell homeostasis.

Heterogeneity of T_{CM} and T_{EM}

Since the first description of T_{CM} and T_{EM}, it became evident that these two broad subsets were heterogeneous in expression of chemokine receptors, adhesion and costimulatory molecules. The T_{EM} pool can be subdivided according to the expression of chemokine receptors characteristic for Th1 and Th2 cells. Thus, within T_{EM}, CXCR3, CCR5 and CXCR6 discriminate Th1 cells and CTLs, whereas CCR3, CCR4 and CRTh2 identify Th2 cells. CXCR3 and CCR4 are, however, expressed also on some T_{CM} cells. These cells represent pre-Th1 and pre-Th2, respectively [80]. $CXCR3^+$ and $CCR4^+$ T_{CM} spontaneously differentiate to Th1 and Th2 effector cells in response to homeostatic cytokines IL-7 and IL-15 and independently of conventional Th1- or Th2-inducing stimuli. In contrast, T_{CM} lacking these receptors and expressing CXCR5 are uncommitted precursors, and their differentiation to Th1 or Th2 cells is dependent on TCR triggering and polarizing cytokines [19, 80]. Pathogen-specific $CD4^+$ T cells have characteristic distributions in the T_{CM} cell subsets, reflecting the Th1/Th2 polarization induced by the pathogen. Thus, tetanus toxoid-specific cells are detected in all subsets, consistent with the notion that vaccination to tetanus induces a mixed Th1/Th2 response. Conversely, cytomegalovirus and vaccinia virus promote Th1 polarization and virus-specific T cells are consequently detected in the $CXCR3^+$ but not in the $CCR4^+$ T_{CM} pool [80]. These results suggest that immune responses generate heterogeneous populations of memory cells that belong to different subsets and include a broad spectrum of differentiation stages.

In addition to circulating non-polarized $CXCR5^+$ T_{CM} cells, a population of tonsil-resident $CCR7^-$ $CD57^+$ T cells also express CXCR5 [81–83]. These cells, which have been defined as follicular B-helper T cells (T_{FH}), secrete IL-2 and IL-10, express CD40L and ICOS and provide spontaneous help to B cells.

Homing of memory T cells to skin and gut

The existence of subsets of memory T cells that preferentially migrate to gut or skin is well documented. Skin-homing T cells can be identified by their expression of cutaneous lymphocyte-associated antigen (CLA) [84]. CLA binds to E-selectin on endothelial cells of inflamed skin. However, E-selectin expression is not restricted to inflamed cutaneous endothelium, suggesting that there must be other elements determining skin tropism. This specifying element is CCR4. In fact, CCR4 is coexpressed together with CLA on a subset of memory/effector T cells, and its ligand CCL17 on endothelial cells of inflamed skin but not of inflamed gut [85]. CCL17 has been shown to induce integrin-dependent firm adhesion of CLA^+ T cells consistent with its role in the extravasation process. Besides its role in extravasation in skin vessels, CCR4 may drive cell migration within several types of inflamed tissues,

wherever CCL22 and CCL17 are produced by resident cells, for instance in lung or in liver [86, 87]. Another skin-expressed chemokine, CCL27 [88], may also promote migration of memory T cells to the skin through CCR10 binding [89]. CCR10 is expressed on skin-derived Langerhans cells, melanocytes, dermal fibroblasts and dermal endothelial cells, which suggests a broader role for this chemokine receptor in numerous dermal cell interactions. The majority of human T cells in healthy (non-inflamed) skin were shown to express CCR8 [90]. CCL1 (I-309, a ligand of CCR8) was found to be constitutively expressed in skin, principally in dermal microvessels and epidermal antigen-presenting cells, suggesting that CCL1/CCR8 may function in homeostatic T cell traffic through normal skin.

Gut-homing T cells express high levels of $\alpha_4\beta_7$ integrin that mediates L-selectin independent rolling on MAdCAM-1, a vascular addressin that is expressed on lamina propria venules and Peyer's patch HEVs, but also in the marginal zone of the spleen and in areas of chronic inflammation [91]. The crucial role of chemokine receptors in leukocyte migration has been demonstrated also for homing in the gut. In this case the receptor involved is CCR9, which binds CCL25, a chemokine selectively expressed in the endothelial cells of gut-associated tissues and in the thymus [92]. CCR6 is also expressed on subsets of memory T cells and may drive their migration in response to CCL20 to both skin and gut [93]. A significant conclusion can be drawn from these examples: selectivity of homing is achieved via the expression of different receptors that define a combinatorial code addressing cells to the right place.

Concluding remarks

We have seen examples of how chemokines allow the selective migration of functionally specialized subsets of T cells to particular tissues. The recruitment of cells plays a central role in the development of an immune response against microorganisms as well as under conditions of uncontrolled pathological immune responses. Given the high selectivity of this process, several attempts have been made to inhibit specific chemokine receptors with the aim to block one type of immune response, while leaving other unaffected. Indeed, there is experimental evidence showing that chemokine antagonists block inflammatory diseases in animal models [94]. However, while selective adhesion molecules and chemokine receptors expression have permitted the identification of novel T cell subpopulations, the signals that direct the differentiation of these subsets, their relationship and their development are still poorly characterized. Moreover, while T cell homing into tissues has been extensively studied, unraveling the mechanisms and regulation of T cell exit from tissues is equally important for a complete understanding of T cell trafficking, and may result in novel therapies for treating atopic, inflammatory and autoimmune disorders. Two recent studies addressing this issue suggest that CCR7 is an important

regulator of effector memory T cell exit from the periphery [95, 96], challenging the commonly held opinion of a passive T cell transit from the periphery into the afferent lymphatic vessels. In this view, T cells may constantly change the repertoire of chemokine receptors to migrate into specific areas. Clearly, a better understanding of the intricate relationship between T cell function and localization holds great potential for the therapeutic manipulation of T cell responses.

Acknowledgements
We thank Mariagrazia Uguccioni for critical reading of the manuscript. Research on human T lymphocyte subsets in the authors' laboratory is supported by the Swiss National Science Foundation (grant no. 3100-101962). The Institute for Research in Biomedicine is supported by the Helmut Horten Foundation.

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Migration of dendritic cell subsets

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Introduction

The original observation made by Ralph Steinman and coworkers in 1974 [1] has dictated the leading concept of dendritic cells (DC) as key elements in the afferent arm of the adaptive immune response. DC are hematopoietic, bone marrow-derived, professional antigen-presenting cells (APC), capable of both priming an immune response, and efficiently stimulating memory responses [2, 3]. DC are closely related to other cells of the myeloid lineage, but they are unique in term of morphology and functional properties and differ from other conventional leucocytes [4]. Within the past few years, it became possible to generate DC *in vitro* using defined growth factors, which has shown that DC are not a single cell type, but a heterogeneous population of cells [5–7].

In both mice and men, at least two major subsets of DC exist, myeloid-related DC (M-DC) and plasmacytoid DC (P-DC) [8]. In the peripheral blood, M-DC precursors express CD11c but lack CD123, while the P-DC precursors display the $CD11c^{-}CD123^{+}$ phenotype. Both subsets are immature, since they are negative for co-stimulation molecules CD80, CD86 and CD40 [9, 10]. Based on *in vitro* data, there are different pathways for the development of mature DC from bone marrow $CD34^{+}$ precursors. Each pathway differs in terms of progenitors and intermediate stages, cytokine requirements, surface marker expression and, most importantly, biological function [5–8, 11]. M-DC are distinguished by at least two distinct pathways of maturation from $CD34^{+}$ progenitors since, after 5 days in culture with granulocyte-macrophage colony-stimulating factor, stem cell factor and tumor necrosis factor (TNF)-α, cells are sorted into either $CD14^{+}CD1a^{-}$ or $CD14^{-}CD1a^{+}$ populations [5]. In addition, it is likely that under certain conditions mature monocytes migrate from blood into tissues and differentiate into DC

Lymphocyte Trafficking in Health and Disease, edited by Raffaele Badolato and Silvano Sozzani

[12–14]. Substantial diversity exists between M-DC and P-DC, supporting the possibility of different functional roles. M-DC have several features that allow them to capture antigens, exploiting a complex array of uptake mechanisms, including phagocytosis, micropinocytosis and receptor-mediated endocytosis [15], while P-DC have very limited phagocytic capacity [8, 11]. M-DC represent the classic T cell-priming subset, but this function in P-DC is less clear, although there is definite evidence that P-DC play an important role in the defense against pathogens and neoplasms [16]. Despite the experimental evidence that circulating and tissue P-DC can acquire the morphological and functional features of DC *in vitro* [17, 18], the existence of fully mature P-DC *in vivo* is still controversial [16, 19]. Furthermore, M-DC and P-DC show marked disparity in tissue distribution and migration pathways. Immature M-DC are constitutively distributed in peripheral tissues, especially in the skin and mucosal surfaces, which represent the areas of entry of exogenous antigens, where they are responsible for antigen capture and processing. Following antigen capture, M-DC undergo maturation into competent APC, bearing high levels of MHC and costimulatory molecules (e.g., HLADR, CD80, CD83, CD86, DC-LAMP/CD208), and migrate to lymphoid tissues, acquiring potent immunostimulatory activity [4, 6, 11, 20], to become mature APC (e.g., interdigitating DC, IDC). In contrast to M-DC, P-DC are scarce or totally absent in skin, mucosae and other non-lymphoid tissues, while they typically occur in lymph nodes and tonsils, in close association with high endothelial venules (HEV) [19, 20]. The topographical association between P-DC and HEV reflects the migration pathway of this subset of DC, which leave the circulation and enter lymphoid tissue through HEV [21–23]. Alternative ways, however, exist of migration of M-DC into lymph nodes. Even in the absence of inflammation, some DC are found in afferent lymph, suggesting that DC continuously traffic from normal tissues to lymph nodes [24]. These rare steady-state migrating DC from skin to lymph nodes are phenotypically mature [25] and might be important for immune tolerance, eliminating T cells with specificity for self antigens that have escaped the thymus during thymic selection [26, 27]. Recent evidence further suggests that under certain conditions a pathway may exist whereby M-DC can migrate into lymph nodes via HEV [28]. Finally, monocytes may undergo differentiation to DC upon migration to the lymph nodes [8].

It should be noted that during chronic inflammation, such as in autoimmune diseases, or tumors, an active recruitment of circulating M-DC and monocytes into inflamed tissues occurs [8]; the increased number of tissue M-DC supplies the continuous antigenic stimulation of regional lymph nodes by transporting antigens, but may also contribute to the maintenance of the local inflammatory process, undergoing local maturation and activation of T cells [20]. Similar to M-DC, P-DC are also recruited into inflamed peripheral tissues (such as allergic nasal polyps, skin in lupus erythematosus, lichen planus, and infections) [29–32] and tumors [33, 34], thus contributing to the local immune response.

Morphological identification of non-lymphoid tissue DC

M-DC include intraepithelial Langerhans cells (LC) and interstitial DC (IN-DC). The skin and mucosae contains a prominent supply of LC, which have typical DC morphology and contain characteristic Birbeck granules (BG) seen on electron microscopy. LC are easily recognizable on tissue sections due to their reactivity for HLA-DR, S-100 protein, CD1a, E-cadherin and the LC-specific marker langerin (CD207), while they lack CD68 and factor XIIIa, and most antigens expressed by dermal IN-DC (Fig. 1). Moreover, LC lack several maturation antigens, such as DCLAMP/CD208, while expression of CD83 can be variable [35–37]. The cutaneous lymphocyte activation (CLA) antigen recognized by the antibody HECA-452, which is expressed by LC precursors, is down-regulated by intraepithelial LC [38]. In conditions associated with an increase or activation of intraepidermal LC, such as contact dermatitis, cells expressing a hybrid monocyte-LC phenotype ($CD1a^+CD11b^+CD36^+CD68^+$) can be observed [36]; this observation supports the evidence that LC may derive from monocytes *in vivo* [12].

With the notable exceptions of the cornea and central nervous system [4], DC have been identified within the interstitial space of most human tissues, including the dermis and many solid organs (heart, lung, kidney, liver); these IN-DC express CD11c, CD68, factor XIIIa, macrophage-mannose receptor (CD206), along with the c-type lectin DC-SIGN (CD209) (Fig. 1). In analogy with LC, IN-DC lack DC maturation antigens [34] and, as with LC, dermal and mucosa IN-DC are strategically localized at the interface with the external surfaces where they can take up pathogens [39, 40] and transport them to lymph nodes.

The migration of DC from peripheral tissues to lymph nodes is associated with changes in their phenotype. LC adhere to keratinocytes via homophilic interactions with E-cadherins, and down-regulate this adhesion molecule to leave the epidermis [41]. In the lymph vessels and nodal sinuses migrating LC are identified as veiled cells, because of their sheet-like lamellipodia [41]; similar to LC, veiled cells express CD1a, S-100 protein and langerin/CD208, and are mostly immature [42] (Fig. 1).

Morphological identification of lymphoid tissues DC

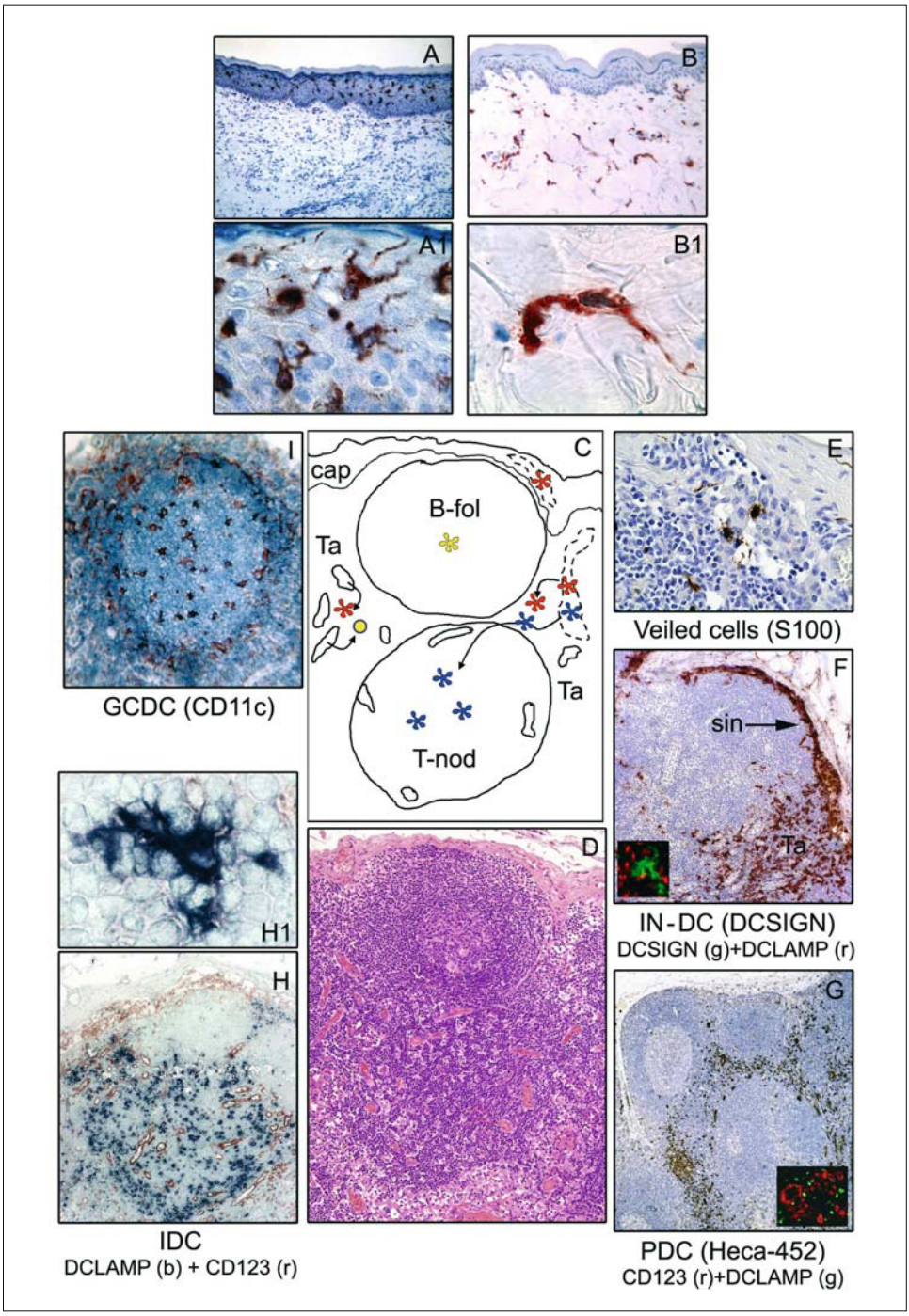
DC have been largely studied in lymph nodes and tonsils [42–45], where their distribution is rather complex, reflecting the occurrence of different subsets of DC, diversity of activation and maturation stages and pathways of migration (lymph *versus* blood borne). The lymph node parenchyma is compartmentalized into functional areas, which include cortical B follicles and paracortical T nodules. In addition, an indistinct area located between B follicles (and corresponding to the interfollicular area) and at the periphery of T nodules in the paracortex (where it has also been identified as the "outer paracortex") runs along intermediary lymphatic sinus-

es and surrounds HEV (Fig. 1). This area is partially delimited by fibroblastic reticulum cells, stromal cells expressing actin filaments and occasionally cytokeratins, which form conduits that facilitate the migration of cells [46]. The high rate of cell migration and cell-to-cell contacts occurring in this part of the lymph node justify the term 'traffic' area [19, 47]. M-DC-related IDC, represent the majority of mature DC within the lymph node, showing bright expression of HLADR and DC-LAMP/CD208 (Fig. 1). IDC occur in the traffic area, but they are predominantly found in paracortical T nodules, where they are intimately admixed with T lymphocytes [48]. IDC are considered to descend for the most part from LC, and maintain the positivity for S-100 protein (Fig. 1). However, antigens usually negative on LC (such as CD11c) are expressed by IDC [42, 43, 45], while langerin and CD1a are generally lost [42, 44]. Nevertheless, probably on the basis of different stages of maturation, minor subsets of IDC have been described, which either maintain CD1a, or lack CD11c, or do not display a full-blown mature phenotype [25, 43, 45].

In the special condition defined as to 'dermatopathic lymphadenitis', characterized by massive migration of LC into lymph nodes, resulting from inflammatory or neoplastic skin disorders, the IDC largely preserve LC antigens ($CD1a^+$, $S100^+$, $Langerin^+$) [34, 44]; moreover, they remain immature [49], but express *de novo* the interferon (IFN)-γ-dependent molecule class 6 semaphorin, which defines an activa-

Figure 1

Distribution and phenotype of DC subsets in peripheral tissues (skin) and lymph node
In the skin, langerin$^+$ LC are typically confined to the epidermis (A, A1), while IN-DC, here stained for macrophage mannose receptor, are found in the dermis (B, B1). In C and D, a drawing and the corresponding picture of a reactive lymph node is shown (cap: capsule; B-fol: B follicle; T-nod: paracortical T nodule; Ta: traffic area). Sinus vessels are illustrated with dotted lines, HEV as full lines; nodal DC are shown as star-shaped cells and include immature DC (red), mature DC (blue), and GCDC (yellow); P-DC are shown as round cells (yellow). In the marginal sinus some S-100 protein$^+$ veiled cells are present (E); F shows staining for DC-SIGN, which identifies macrophages within the marginal sinus (sin), as well as the sinus lining cells and numerous IN-DC along the traffic area (Ta); in the inset a double immunofluorescence for DC-SIGN and DCLAMP shows that the DC-SIGN$^+$ cells (green) do not express DCLAMP (red). In G, the traffic area at the periphery of B follicles and T nodules is clearly depicted by HECA-452 antibody, which stains P-DC and HEV; in the inset a double immunofluorescence for CD123 and DCLAMP shows that the CD123$^+$ P-DC (red) do not express DCLAMP (green). The paracortical T nodule shown in H contains large numbers of DCLAMP$^+$ mature IDC (blue), that form close contacts with surrounding T cells (H1); CD123 in red (H) stains the HEV and scattered P-DC mainly localized at the periphery of the T nodule. In a secondary B follicle, CD11c stains GCDC (I).


A
B
A1
B1
I
GCDC (CD11c)
C
cap
B-fol
Ta
Ta
T-nod
E
Veiled cells (S100)
F
sin
Ta
IN-DC (DCSIGN)
DCSIGN (g)+DCLAMP (r)
H1
H
IDC
DCLAMP (b) + CD123 (r)
D
G
PDC (Heca-452)
CD123 (r)+DCLAMP (g)

tion status [50]. It has been postulated that the IDC in dermatopathic lymphadenitis may also derive from langerin$^+$ cells migrating from blood, since not all cells positive for langerin are epithelial LC [51, 52]. These data indicate that IDC in dermatopathic lymphadenitis differ from IDC observed in common reactive conditions, and should be utilized with caution as a study model of normal IDC.

In the 'traffic area' of lymph nodes, several spindle cells or DC can be found; most of them are CD11c$^+$ and express DCSIGN/CD209, macrophage mannose receptor or factor XIIIa [34, 53–55]. The vast majority of these DC do not express DC-LAMP (Fig. 1) [34], and there is controversy as to whether these cells derive from IN-DC along lymph vessels, or if they derive from circulating DC precursors or monocytes [8]. Recently, the true "dendritic" nature of the DC-SIGN$^+$ cells of lymph nodes was questioned, and it has been suggested that they may represent macrophages [56]. This hypothesis is supported by the observation that DC-SIGN is strongly expressed on sinus macrophages [34, 55], and that it is found on macrophages but not on DC generated from monocytes by Toll-like receptor (TLR) activation triggers [56].

The enigmatic cell type occurring in lymph nodes and previously referred as to plasmacytoid T cells or plasmacytoid monocytes [45] represents the major subset of DC (P-DC) that under the influence of various stimuli, such as viruses, IL-3, CD40L, and non-methylated bacterial DNA (CpG-ODN) [16, 19] secrete high amounts of IFN-α [18]. P-DC are typically found in the 'traffic area' of lymph nodes [47] (Fig. 1). Despite they typical morphology (medium-sized cells, with round-oval nucleus and moderately abundant eosinophilic cytoplasm), they are better identified with the help of immunostains that show strong reactivity for CD68, CLA/HECA-452, CD123, BDCA2 and TCL-1 [19] (Fig. 1). In addition to IFN-α, P-DC also express granzyme B [57]. A direct role of granzyme B as cytotoxic mediator in P-DC seems unlikely, since they do not express other cytotoxic molecules such as perforin or TIA-1 [57, 58], and also contain the granzyme inhibitor PI-9 [59]. Recently, however, it has been found that P-DC are capable of inducing apoptosis via TRAIL [60]. In addition, in mice, a hitherto unrecognized cell secreting IFN-α and showing both potent anti-tumor cytolytic capacity and antigen-presenting functions has been described [61, 62]; it remains to be investigated if similar cells exist in humans and if they belong to a P-DC subset.

Cortical B follicles contain two main DC, the germinal center DC (GCDC) and the follicular DC (FDC). GCDC express CD4, CD13 and CD11c (Fig. 1), are strong APC for T cells [63], and can directly regulate B cell responses, producing IL-12 and inducing germinal center B cell expansion, plasma cell differentiation, and IL-10-independent isotype switching toward IgG1 [64]. The origin of GCDC is poorly studied; they might be related to the subset of dermal DC that express CXCR5 and traffic to B cell zones in lymph nodes [65]. FDC do not represent *bona fide* DC, since they are non-hematopoietic in origin, but mesenchymal. In addition, they are not capable of activating naïve T cells, do not display a capacity for antigen capture

and presentation, but do express preformed antigen-antibody complexes (antigen-carrying cells) on their surface. FDC are typically located within primary and secondary B follicles, and interactions between CXC ligand 13 (B lymphocyte chemoattractant; CXCL13) expressed on FDC and CXCR5 expressed by B cells and activated T cells play a role in B follicle development and organization [66, 67]. FDC express complement and Fc receptors, as well as a series of antigens that are useful for revealing them on sections, such as CD21, CD23, CD35, CNA.42, KiM4p, DRC1, nerve growth factor receptor, and clusterin [68] (Fig. 1). The functional role of FDC is still controversial [69–71]. The close association with germinal center B cells has fostered the idea that B cell recognition of retained antigen on the surface of FDC is important for affinity maturation and memory B cell development. However, it is possible that FDC support B cell proliferation and differentiation in a non-specific manner [70].

The major subsets of M-DC and P-DC observed in lymph nodes are also found in the spleen, along the route of blood-borne antigens, and in the thymus. In the spleen, $CD11c^+$ DC accumulate in the marginal zone at the periphery of the periarteriolar lymphoid sheath and in the T cell area (represented by IDC), as well as in the germinal center (as GCDC) [72, 73]; in contrast to M-DC, splenic P-DC are usually scant and are placed in the marginal zone area of both human [19] and mouse spleen [74].

In the thymus, DC are predominantly found in the medulla or at the corticomedullary junction; they include immature and mature M-DC, the latter corresponding to IDC, and P-DC [75, 76]. In addition, $CD11c^+CD11b^+$ DC related to GCDC have been described [77]. The different subsets of thymic DC may have a diverse functional potential, including induction of central tolerance (for IDC), transport and presentation of peripheral antigens (for GCDC), and IFN-α production (for P-DC) [78].

Chemokines and chemokine receptors

During their life span, DC migrate form the bone marrow through blood to peripheral tissues and to lymphoid tissues. DC migration and their capacity to orchestrate the migration of other effector leukocytes are fundamental for the induction of adaptive immunity. Optimal immune response requires the proper localization of DC to the sites of inflammation and subsequently to secondary lymphoid organs [79, 80]. Migration of DC into tissues depends on a cascade of discrete events that include the activation of chemokine receptors and the regulation of adhesion molecules [20].

Chemokines are a superfamily of small proteins that play a crucial role in immune and inflammatory reactions and in viral infection [81, 82]. Based on a cysteine motif, CXC, CC, C and CX3C subfamilies have been identified. Chemokines

interact with seven-transmembrane-domain, G protein-coupled receptors. At least ten CC (CCR1–10), seven CXC (CXCR1–7), one CX3C (CX3CR1) and one XCR (XCR1) receptors have been identified. Receptor expression is a crucial determinant of the spectrum of action of chemokines, and dictates most of the differences observed in the chemotactic response of immature *versus* mature DC [20]. Emerging evidence indicates that regulation of receptor expression during cellular activation or deactivation is as important as regulation of chemokine production for tuning the chemokine system.

Immature DC express a unique repertoire of inflammatory chemokine receptors (e.g., CCR1, CCR2, CCR5, CCR6) that are responsible for the recruitment of immature DC, or their precursors, to the inflamed tissues [8, 20]. These receptors bind a pattern of 'inflammatory' chemokines, including CCL2, CCL3, CCL4, CCL5 and CCL20. DC also express a wide variety of receptors for chemotactic agonists different from chemokines (Fig. 2). These include receptors for bacterial components, bioactive lipids and for signals of 'tissue danger'. These chemotactic stimuli are rapidly produced (within minutes) at the site of inflammation and represent an early signal for the recruitment of DC, or their precursors, that can precede chemokines action.

For instance, myeloid immature DC, but not mature DC, express functional receptors for formylated peptides (fMLP) and for chemotactic components of the complement cascade (i.e., C5a) [83]. The formyl peptide receptor family includes multiple proteins, two of them FPR and FPRL2 were found to be expressed by immature DC [84]. FPR is the fMLP receptor, whereas FPRL2 is activated by the WKYMVm hexapeptide and F2L, a highly conserved acetylated 21-amino acid peptide derived from the cleavage of the N terminus of the intracellular heme-binding protein (HBP) [85, 86]. DC express functional receptors for platelet-activating factor (PAF), a bioactive phospholipid that derives from the activation of phospholipase A2 [87, 88]. PAF plays a crucial role in the retention of DC in peripheral tissues, and may thus be relevant in the accumulation of DC observed at pathological sites, such as in atherosclerotic plaques [88].

Recent work has also shown that DC may have a pivotal function in the induction of autoimmunity [89]. Histidyl-(HisRS) and asparaginyl-(AsnRS) tRNA synthases, two cytoplasmic proteins involved in protein synthesis that function as autoantigens in myositis, were shown to induce the migration of immature DC through the interaction with CCR5 [90]. Furthermore, S-antigen and the interphotoreceptor retinoid binding protein (IRBP), two self antigens involved in autoimmune uveitis, were shown to bind and activate CXCR3 and CXCR5 on immature DC [91]. Therefore, self antigens may promote autoimmunity also through the recruitment of APC at sites of tissue injury. In systemic lupus erythematosus, a sustained production of IFN-α represents the 'cytokine signature' of the disease [92, 93], and supports the pivotal role played by the P-DC/IFN-producing cells in this autoimmune disorder [94]. Circulating immunocomplexes containing unmethylated

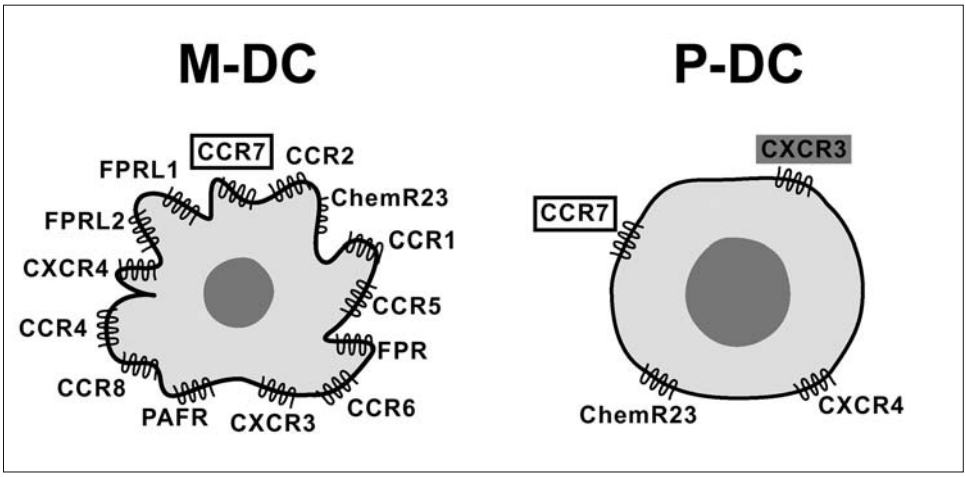


Figure 2
Chemokine receptor distribution in circulating M-DC and P-DC
Functional receptors for DC subsets. CXCR3 in P-DC is active in synergism with CXCR4 and when engaged by substrate-bound ligands. CCR7 is expressed at low levels also by circulating immature P-DC, but it becomes active only after DC maturation.

CpG motifs trigger IFN-α release from P-DC [95, 96] through the binding to TLR9 and CD32 [97]. Furthermore, apoptotic cells containing hypomethylated CpG DNA might be recognized and taken up by P-DC, using the scavenger receptor CD36 [45, 98, 99].

A dramatic change in the repertoire of chemokine receptors is promoted by DC activation. This change is functional for the migration of DC from the periphery to the draining lymph nodes. The signals that promote this process include a variety of maturation factors, such as IL-1, TNF and LPS [100–102]. DC activation is associated with the acquisition of a mature phenotype comprising an up-regulation of costimulatory and MHC molecules. Activation of DC is also associated with down-regulation of inflammatory chemokine receptors and the *de novo* expression of CCR7, the receptor for CCL19 and CCL21, two chemokines that are expressed at the luminal side of high endothelial cells and in the T cell-rich areas of secondary lymphoid organs, like tonsils, spleen and lymph nodes [101, 103, 104]. The crucial role of CCR7 and its ligands is documented *in vivo* in mice deficient for these proteins [80, 105]. CCR7 expression by DC is also required also for the entry of DC into lymphatic vessels at peripheral sites both in steady state and inflammatory conditions [106, 107]. During inflammation, the entry of DC into lymphatic vessels is boosted by the up-regulation of CCL21 on lymphatic endothelial cells. Therefore, inflammatory stimuli not only promote the recruitment of immature DC into tissues, but

also initiate their maturation process and boost the recruitment of maturing DC into lymphatics [107]. The relevance of chemotactic receptors in DC migration *in vivo* has been clearly documented in mice lacking the gamma isoform of phosphoinositide-3 kinase (PI3Kγ) [79]. PI3Kγ is located downstream of seven-transmembrane chemotactic receptors and plays a non-redundant role in cell migration in response to chemotactic agonists [108]. DC generated from PI3Kγ-null mice show a profound defect in the migration in response to both inflammatory and constitutive chemokines. A defect of DC migration was also observed *in vivo* in *PI3Kγ*$^{-/-}$ mice, and most importantly, this defect was associated with a defective ability of *PI3Kγ*$^{-/-}$ mice to generate a specific immune response [79].

Interaction of DC with endothelial cell barriers

Migration is a multistep process that involves the adhesion of DC with endothelial cells and the interaction with physical obstacles, such as basement membranes and collagen meshwork. Subsequently, DC leave peripheral tissues to migrate into secondary lymphoid organs through the lymphatics [109]. As mentioned above, CCR7 expression of maturing DC is required for their efficient entering into lymphatic vessels [107]. In addition, a recent study proposed that CCR8 and its cognate ligand CCL1 are involved in the emigration of mouse monocyte-derived DC from the skin [110].

Circulating DC first need to tether to endothelial cells through the interaction of E- and P-selectins with their respective ligands [111]. Firm adhesion between DC and endothelial cells is dependent on the engagement of chemotactic receptors and subsequent integrin activation on DC [112–114]. *In vitro*, DC express CD31, the b2 integrins LFA-1, Mac-1 and p150,95, the β_1 integrins VLA-4 and VLA-5 that mediate their binding to both resting and activated endothelial cells and to endothelial cell-derived extracellular matrix [113]. Transmigration of DC across an endothelial cell monolayer, unlike adhesion, involves the engagement of CD31. Activation of endothelial cells by oxidized low-density lipoprotein, TNF-α, or hypoxia strongly increases DC adhesion and transmigration [113, 115]. Of interest, endothelial cell apoptosis also markedly enhance DC adhesion [116]. *In vivo*, mice defective in β_2 integrin function [117] and α_6 integrin [118] showed a reduced ability in the migration of cutaneous DC to the draining lymph nodes. An accumulation of DC was reported in atherosclerotic areas [119] and in vascular regions prone to develop atherosclerosis [120, 121]. Furthermore, modulation of the endothelial nitric oxide synthase (NOS) is involved in DC-endothelial cell interaction [122]. Release of NO by activated endothelial cells inhibits DC adhesion and transmigration, whereas inhibition of NOS increases DC-endothelial cell interaction [115]. This evidence provides new insight into the DC-endothelial cell interaction, which plays an emerging role in inflammation and atherogenesis.

Recent work has outlined a crucial role for junctional adhesion molecule A (JAM-A) in DC-endothelial cell interactions. JAM-A is a 32-kDa transmembrane glycoprotein, which belongs to an immunoglobulin superfamily of proteins expressed at the intercellular junctions of epithelial and endothelial cells in close proximity to the tight junction components [123, 124]. The extracellular domain of JAM-A binds several ligands including JAM-A itself [125, 126], the leukocyte integrin $\alpha_L\beta_2$ [127] and the reovirus protein σ-1 [128]. Being localized at tight junctions, JAM-A may have a role in binding leukocytes and in directing their transmigration through endothelial junctions, both by homophilic binding and by linking integrin $\alpha_L\beta_2$ [127]. Recent work as shown that JAM-A is involved in DC transmigration across lymphatic endothelial cells. JAM-A$^{-/-}$ mice showed increased localization of skin DC to lymph nodes, and an exaggerated response in a contact hypersensitivity model, which directly related to an increased migration of DC [129]. No difference in DC migration across blood endothelial cells was observed. One possible reason for the different DC response across lymphatic and blood endothelia may be ascribed to the fact that lymphatics present weak intercellular junctions with a specific molecular organization, compared to blood vessels [130, 131].

Selective recruitment of DC subsets

Blood DC includes two main subsets, M- and P-DC. The expression of chemokine receptors on blood M-DC and P-DC is, in general, fairly similar [132]. Both subsets express relatively high levels of CC chemokine receptor CCR2 and CXCR4. Whereas CCR1, CCR3, CCR4, CCR6, CXCR1, CXCR2, and CXCR5 are very weakly, or not expressed, on both circulating M-DC and P-DC. Conversely, CCR5 and CXCR3 expression is clearly divergent in the two subsets, being low on blood M-DC, but high on P-DC [132, 133] (Fig. 2). In contrast with the overall similar pattern of chemokine receptor expression, circulating M-DC and P-DC exhibit a profound difference in their capacity to migrate in response to chemokines, with CXCL12 being the only chemokine active in a classic chemotaxis assay [132] or in transmigration assays across an endothelial cell monolayer [134]. In classic chemotaxis assays, the ligands of CXCR3, i.e., CXCL9, CXCL10 and CXCL11, are inactive in inducing P-DC migration, but can promote P-DC migration in response to CXCL12 [135, 136].

DC subsets also differ for their ability to interact with endothelial cells, *in vitro*. M-DC were shown to vigorously migrate across endothelium in the absence of any chemotactic stimuli, whereas spontaneous migration of P-DC was limited [134]. In contrast, the interaction with an endothelial cell monolayer greatly favored transmigration of P-DC in response to CXCL1 and CCL5 [134], ChemR23 (see below) [30] and in response to CXCR3 ligands [133].

P-DC are normally absent from peripheral tissues and they are believed to migrate constitutively from the blood into lymph nodes through HEV [21-23].

This migration is mediated by L-selectin and is increased by an E-selectin-dependent mechanism when lymph nodes are exposed to inflammatory conditions [18, 23, 137]. Accordingly, P-DC express high levels of CD62 ligand and the HECA-452$^+$ isoform of P-selectin glycoprotein ligand-1 (PSGL1), the counter ligands of P- and E-selectins [21, 47, 133]. Recruitment of P-DC to non-lymphoid tissues is observed in some pathological conditions, such as autoimmune diseases (i.e., lupus erythematosus disease, psoriasis and rheumatoid arthritis) [19, 29, 138], allergic diseases (i.e., contact dermatitis and in nasal mucosa polyps) [139] and in tumors [34, 140, 141]. However, the mechanisms leading to the recruitment of P-DC to inflammatory sites remain unresolved. Recently, chemerin, a new chemotactic factor was proposed as a key signal for the recruitment of P-DC into pathological tissues [30]. Chemerin is a novel chemotactic protein identified as the natural ligand of ChemR23, a previously orphan G protein-coupled receptor expressed by immature DC and macrophages [142]. Chemerin is expressed by many tissues, including spleen and lymph nodes, and is secreted as prochemerin, a poorly active precursor protein. Extracellular proteases involved in the coagulation cascade [143] or released by leukocytes convert prochemerin into a full agonist of ChemR23 by proteolytic removal of the last six amino acids [144]. ChemR23 is expressed by blood P-DC, and chemerin was found active in inducing their transmigration across an endothelial cell monolayer. *In vivo*, ChemR23 has been shown to be expressed by P-DC localized in reactive lymph nodes and in skin lesions of lupus erythematosus patients. Of note, chemerin was selectively expressed by HEV in lymph nodes and by dermal blood vessels in lupus skin lesions. These results strongly suggest that the ChemR23/chemerin axis is likely to play a key role in regulating the trafficking of P-DC to lymph nodes and to pathological tissues [30].

Concluding remarks

DC are professional APC. To accomplish their biological functions, they need to go through a complex pattern of migration, which includes their localization to both peripheral non-lymphoid tissues and secondary lymphoid organs. In the absence of correct tissue localization, DC fail to promote proper immune responses. DC trafficking includes the interaction with both blood and lymphatic endothelium and the response to chemotactic signals. In the past few years many chemokines have been reported to regulate DC migration *in vitro* and *in vivo*; however, more recent findings strongly support the role of a considerable array of non-chemokine chemotactic signals and adhesion molecules in this complex process. A better understanding of the signals involved in the migration of DC subsets *in vivo* constitutes a valuable basis for the development of new strategies for the control of DC migration and function under pathological conditions.

Acknowledgements
This work was supported by AIRC (Associazione Italiana per la Ricerca sul Cancro), MIUR (Ministero dell'Istruzione Università e Ricerca), Association for International Cancer Research (grant no. 04-223), Istituto Superiore di Sanità (Grant I.S.S, no. 40F.37) and by Fondazione Berlucchi.

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Migration of NK cells

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Introduction

Natural killer (NK) cells belong to a distinct lineage of lymphocytes that play an important role in the early phase of immune responses against certain microbial pathogens by exhibiting cytotoxic functions and secreting a number of cytokines and chemokines.

NK cells develop from a common lymphoid precursor resident in the bone marrow (BM) that is considered the main site of their generation. The BM microenvironment provides a rich source of cytokines and growth factors and allows an intimate contact between developing NK cells and stromal cells, which is required for their full maturation [1]. However, final maturation of BM-derived NK cell precursors has been suggested to occur also at the periphery [2].

Mature NK cells mainly circulate in the peripheral blood, but are also resident in several lymphoid and non-lymphoid organs such as spleen, tonsils, liver, lungs, intestine and uterine decidua. In addition, homing to lymph nodes of a particular subset of activated NK cells has been described both in human and mouse [3, 4].

During viral infections, inflammation, tumor growth and invasion, NK cells are rapidly recruited from the blood and accumulate in the parenchyma of injured organs [1, 5, 6], where activated NK cells can kill target cells and release inflammatory cytokines and chemokines, thus participating in the recruitment and activation of other leukocytes and in the modulation of dendritic cell (DC) function.

Unlike B cells and T cells that express a single antigen-specific receptor, NK cells are endowed with a multiple cell surface receptor system encoded by genes that do not undergo recombination or sequence diversification. This complex receptor system is acquired during NK cell development, and consists of both activating and inhibitory receptors [7, 8].

The best studied among the activating receptors is the low-affinity Fc-receptor γ IIIA (CD16) that is responsible for antibody-dependent cellular cytotoxicity (ADCC) and allows NK cells to participate in the elimination of antibody-coated target cells [1]. Among the receptors capable of triggering natural killing, recent evi-

Lymphocyte Trafficking in Health and Disease, edited by Raffaele Badolato and Silvano Sozzani

dence underscores the relevance of the C-type lectin family NKG2D receptor that recognizes the MHC class I-related A and B proteins (MICA and MICB) and the members of a family of proteins named UL16-binding proteins (ULBP) [9]. These ligands are mainly expressed on the surface of tumor cells of different histotypes, and infected or stressed cells, and are induced in response to DNA damage [10]. Other activating receptors, i.e., NKp46, NKp44, and NKp30, are Ig-like molecules and belong to the natural cytotoxicity receptor family, but their ligands are still unidentified [11].

In addition, NK cells express a number of receptors acting as activating or costimulatory molecules such as CD2, CD244 (2B4), NKp80, β_1 and β_2 integrins and DNAM-1 (CD226). Interestingly, DNAM-1 is associated with the β_2 integrin LFA-1 and binds to the poliovirus receptor (PVR, CD155) and the nectin-2 (CD112), two members of the nectin family involved in the regulation of cell-cell interaction and leukocyte extravasation [12, 13].

NK cell functions are tightly regulated by inhibitory receptors that specifically interact with MHC class I antigens. In the human, they belong to two distinct groups: the killer cell Ig-like receptor (KIR) family that comprise molecules binding to groups of human leukocyte antigen (HLA)-A, -B, -C alleles, and the C-type lectin receptors (i.e., CD94/NKG2A) specific for the widely expressed non-classic HLA-class I molecule, HLA-E. Both receptor families include activating counterparts with similar specificity, but different ligand affinity. The functional role of these activating receptors as well as the identity of their ligands are at present, quite obscure.

Based on the receptor complexity, NK cell functions are thus the result of concomitant engagement of various activating and inhibitory receptors by the particular set of ligands on target cells. However, in most instances the inhibitory signals override the triggering ones [14].

All the receptors expressed by NK cells are not unique to this cell type, but are also present on cells of other lineages such as T cells or myeloid cells. The expression on NK cells is highly regulated, and some receptors are oligoclonally distributed or expressed on subsets of NK cells. Unlike peripheral blood human NK cells, some tissue-resident NK cells do not express CD16, but show high levels the NCAM adhesion molecule, CD56; in addition, CD16 and CD56 receptors can be expressed at different density on circulating blood NK cells.

Based on the receptor repertoire and surface receptor levels, phenotypically distinct NK cell populations have been identified, and suggested to represent specialized subsets capable of performing different functions and endowed with distinct migratory properties. Two major subsets of human peripheral blood NK cells have been described: the majority (about 90%) are $CD56^{low}CD16^{high}$, whereas about 10% of NK cells are $CD56^{high}CD16^{low}$. It has been proposed that $CD56^{high}$ NK cells have a unique functional role in the innate immune response as primary source of NK cell-derived immunoregulatory cytokines, whereas the $CD56^{low}CD16^{high}$ subset represents the principal cytotoxic population [15].

It is still matter of debate as to whether these different NK cell populations represent functionally distinct subsets of mature NK cells, or whether $CD56^{high}$ NK cells are terminally differentiated cells indistinguishable from mature NK cells recently activated in response to cytokines such as IL-12 [16].

NK cell adhesion molecules and chemokine receptors

The ability of leukocytes to traffic coordinately throughout the body is an essential requirement for the maintenance of immunosurveillance. NK cell migration across endothelium, as for other leukocytes, is a spatially and temporally integrated multistep process regulated by a plethora of chemoattractants and adhesive molecules belonging to the selectin, integrin, and Ig families, as well as chemokines [17, 18].

Among adhesion molecules, both selectins and integrins contribute to the initial leukocyte tethering and rolling along vessel endothelium, while firm adhesion of the leukocyte to vascular endothelium and subsequent diapedesis into the underlying extravascular tissue is mainly mediate by integrins. The various steps of migration are tightly regulated; in fact, for migration to be effective, adhesion receptors must undergo cycles of attachment and detachment from their endothelial ligands.

Chemokines are a superfamily of inflammatory mediators that properly guide leukocyte recruitment and positioning into healthy or diseased tissues by interacting with seven-transmembrane-domain receptors and initiating complex signaling events that govern leukocyte migration, not only by eliciting a chemotactic response but also through a dynamic regulation of integrin adhesiveness for endothelial and extracellular matrix ligands [19–21]. Integrins can also regulate cell migration by initiating similar intracellular signal transduction pathways [22, 23].

Adhesion molecules

In regard to selectin receptor family, human NK cells express L-selectin (CD62L), a molecule involved in the initial adhesion of leukocytes to peripheral lymph node high endothelial venules (HEV) [24, 25]. L-selectin has been found to be uniquely expressed on the $CD56^{high}$ subset of peripheral blood human NK cells at a density higher than that of all other peripheral blood leukocytes, including $CD56^{low}$ NK cells. NK cell activation results in modulation of L-selectin expression depending on the stimulus: phorbol esters, IL-2, IL-15, and TGF-β down-regulate L-selectin on $CD56^{high}$ NK cell subset, whereas increased levels can be observed on both NK cell subsets in response to IL-12, IL-10, and IFN-α. In accordance with these observations, $CD56^{high}$ NK cells bind to the physiological L-selectin ligands on peripheral lymph node HEVs with higher efficiency as compared to the $CD56^{low}$ subpopulation, thus resulting in a selective advantage of this population in extravasation

across HEV [26]. There is also evidence from Uksila et al. [25] showing that a portion of $CD16^+$ NK cells express L-selectin, and that IL-2 treatment diminishes the expression of this molecule and concomitantly increases the levels of α_4 integrin and CD44, two major receptors involved in lymphocyte binding to mucosal HEV. Thus, IL-2 activation of NK cells decreases adherence to peripheral LN HEV, while increasing adherence to mucosal HEV.

NK cells can also express selectin ligands such as the sialyl stage-specific embryonic antigen 1, sialyl-Lewis^x (sLe^x) ligand and P-selectin glycoprotein ligand-1 (PSGL-1), which can bind to E- and P-selectin under static and flow conditions; this binding is up-regulated by IL-12 [27–30]. There is also evidence indicating that the sulfated lactosamine epitope expressed selectively on $CD56^{low}CD16^+$ NK cells, PEN5, is a carbohydrate decoration of PSGL-1 that confers to PSGL-1 the ability of binding to L-selectin [31]. These results suggest that PEN5-L-selectin pair may promote cell-cell interactions and amplify the accumulation of NK cells at site of inflammation.

On the NK cell surface, another carbohydrate modification of PSGL-1, CLA has been also found, which is a marker for tissue infiltrating leukocytes. Notably, expression of PEN5 and CLA on NK cells is mutually exclusive, suggesting that distinct NK cell subsets exhibit different trafficking properties [31].

In regard to integrins, human NK cells express various members of the β_1, β_2 and β_7 families. Among the β_1 integrins, freshly isolated peripheral blood NK cells express $\alpha_5\beta_1$ and $\alpha_4\beta_1$ as fibronectin and VCAM-1 receptors, and $\alpha_6\beta_1$ as laminin receptor [32]. The pattern of β_1 integrin expression changes upon NK cell activation, in that activated NK cells acquire $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins, and down-regulate the expression of $\alpha_6\beta_1$ [24, 33–35]. $\alpha_4\beta_1$-VCAM-1 adhesive pathway is involved in the adhesion and migration of resting or IL-2-activated NK cells across IL-1β-, IFN-γ-, TNF-α-activated, but not resting, endothelial cells. Integrin-mediated NK cell interaction with endothelial cells is characterized by a peculiar structural feature: the formation of podosomes that represent dot-shaped protrusions of the cellular ventral membrane provided with adhesive properties and formed by particular cytoskeletal architecture [36]. Recent evidence indicates that the interaction of $\alpha_4\beta_1$ integrin with VCAM-1 on porcine endothelial cells is also required for both rolling and firm adhesion of human NK cells to porcine endothelial cells [37].

α_4 integrin subunit can also associate with another β chain, the β_7, to give a functionally distinct integrin receptor capable of binding the mucosal vascular addressin MAdCAM-1. $\alpha_4\beta_7$ is expressed on NK cells and mediates NK cell binding to mucosal HEV [25, 38, 39]. Functional evidences indicate, however, that NK cells expressing both $\alpha_4\beta_7$ and $\alpha_4\beta_1$ bind well to VCAM-1 but poorly to MAdCAM-1, suggesting that regulation of MAdCAM-1 *versus* VCAM-1 expression might critically control the recruitment of NK cell subsets to distinct tissues [40].

NK cells express all members of the β_2 integrin family (CD11a–d/CD18), which are leukocyte-associated adhesion molecules mainly involved in the regulation of

cell-cell interactions [41]. The leukocyte function-associated antigen 1 (CD11a/CD18 also known as LFA-1) is the receptor for the intercellular adhesion molecules (ICAM-1, 2, 3), and plays a crucial role in mediating NK cell adhesion to target cells as well as NK cell binding and transmigration across endothelial cells [36]. The expression and function of β_2 integrins on NK cells is highly regulated. In this regard, it has been shown that the levels of LFA-1 are higher on the $CD56^{low}$ subset compared with the $CD56^{high}$, while $\alpha_M\beta_2$ (CD11b/CD18 also known as Mac-1) and $\alpha_X\beta_2$ (CD11c/CD18) integrins are expressed on all and one-half of NK cell population, respectively [25, 26]. NK cell activation by cytokines, such as IL-2 or IL-12, results in up-regulation of LFA-1 expression and function, while CD11b and CD11c are down-regulated [24, 42] (Tab. 1).

The differential expression of adhesion molecules on NK cells together with quantitative and qualitative regulation of integrin expression and function occurring following NK cell activation, can be responsible for the recruitment of specialized NK cell subsets during inflammation.

Chemokine receptors and chemokine-induced *in vitro* NK cell migration

A large body of evidence indicates that NK cells can express several receptors for CXC, CC, C and CX3C chemokines, with a great heterogeneity in the chemokine receptor repertoire among different NK cell populations and between resting *versus* activated NK cells.

With respect to the CXCR and CX3CR families, it has been previously reported that human peripheral blood NK cells express both CXCR1 and CXCR2 as CXCL8 (IL-8) receptor [43–45] and CX3CR1 as CX3CL1 (fractalkine) receptor [46, 47]. These observations have been further extended by Campbell and colleagues [48] who provided the first evidence that distinct ($CD56^+CD16^+$ and $CD56^+CD16^-$) peripheral blood NK cell subsets have a unique repertoire of chemokine receptors. $CD16^+$ NK cells uniformly express high levels of CXCR1 and CX3CR1, low levels of CXCR2 and CXCR3 and no detectable levels of CXCR5. By contrast, $CD16^-$ NK cells express high levels of CXCR3, low levels of CX3CR1, and are negative for CXCR1, CXCR2 and CXCR5; moreover, both NK cell subsets express high levels of CXCR4, the receptor for CXCL12 (SDF-1α/β). With respect to the CC chemokine receptor family, these authors found that the majority of NK cells lack the expression of CCR1-7 and CCR9, and only the $CD16^-$ NK cell subset expresses high levels of CCR5 and CCR7, the latter molecule mainly involved in the homing of lymphocytes to secondary lymphoid organs [48].

Consistent with this expression profile, CXCL8 (IL-8) and soluble CX3CL1 (fractalkine) preferentially attract the $CD16^+$ NK cell subset which can also respond moderately to the CXCR3 ligands, CXCL11 (I-TAC) and CXCL10 (IP-10); by contrast, $CD16^-$ NK cells respond more dramatically to the CCR7 ligands, CCL19

Table 1 - Adhesion molecules and chemokine receptor expression on peripheral blood NK cell subsets

	$CD56^{low}CD16^{high}$	$CD56^{high}CD16^{low}$
CD62L (L-selectin)	+[a]	++[b]
PSGL-1/PEN5	+	–[c]
PSGL-1/CLA	–	+
CD49dCD29 (α4β1)	+	+
CD49eCD29 (α5β1)	+	+
CD49fCD29 (α6β1)	+	+
CD49dβ7 (α4β7)	+/–[d]	+
CD11aCD18 (αLβ2)	++	+
CD11bCD18 (αMβ2)	+	+
CD11cCD18 (αXβ2)	+/–	+/–
CXCR1	++	–
CXCR2	+	–
CXCR3	+	++
CXCR4	++	++
CCR1	–	–
CCR2	–	–
CCR3	–	–
CCR4	–	–
CCR5	–	++
CCR6	–	–
CCR7	–	++
CCR9	–	–
CX3CR1	++	+

[a]*indicates intermediate levels of expression*
[b]*indicates high levels of expression*
[c]*indicates undetectable levels of expression*
[d]*indicates low levels of expression*

(ELC/MIP-3β) and CCL21 (SLC), as well as to the CXCR3 ligands, CXCL11 (I-TAC) and CXCL10 (IP-10), and poorly to a CCR2 ligand, CCL2 (MCP-1), or CCR5 ligands, CCL4 (MIP-1β) and CCL5 (RANTES). Both NK cell subsets strongly migrate in response to the ligand for CXCR4, CXCL12 (SDF-1α/β) [48, 49].

Moreover, Kim et al. [50] have found that $CD56^{high}$ $CD16^{-}$ cells respond more than $CD56^{low}$ $CD16^{+}$ cells to CCL21 (SLC) and CCL19 (ELC) when used at high

concentrations, although they observed that the two NK cell subsets express equal levels of CCR7mRNA.

As above mentioned, the expression of chemokine receptors on NK cells may be modulated upon cytokine stimulation. A significant decrease of CXCR3 expression on NK cells treated for 6 or 24 h with IL-2 and IL-12 alone or in combination has been reported, and the decreased expression was associated with reduced chemotaxis to CXCL10 (IP-10). The same treatment did not affect the expression of other chemokine receptors such as CCR1, CCR2 or CXCR4 [51]. However, previous reports have shown that short-term exposure of freshly isolated NK cells to IL-2 can positively modulate CCR2 mRNA expression [52], and long-term (8–10 days) stimulation of NK cells with IL-2 results in increased expression of CCR1, CCR2, CCR4, CCR5 and CCR8 [53]. In agreement with these observations, IL-2-activated NK cells can migrate in response to many CC chemokines, such as CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), and CCL22 (MDC) [53–56].

A recent report indicates that NK cell treatment with IL-18, differently from IL-2, results in selective induction of CCR7 expression on the $CD56^{low}$ NK cell subset but not affects CCR7 expression on the $CD56^{high}$ subset; increased expression of CCR7 on $CD56^{low}$ NK cell subset is associated with reduced levels of CD16 and enhanced capability to migrate in response to the lymph node-associated chemokine CCL21 (SLC) [57] (Tab. 1).

Signaling events controlling chemokine-induced integrin-supported NK cell migration

Despite the increasing evidence on the prominent role of chemokines and integrins in the dynamic regulation of leukocyte adhesion and migration, the signaling pathways responsible for the integrin-supported leukocyte migration elicited by chemokines are not yet completely defined. The propagation of the migratory signals depends on a complex interplay among molecules that regulate actin, myosin and other cytoskeleton components, and results in the formation of protrusive structures at the front of migrating cell and retraction at cell rear [58, 59].

Thus, NK cell migration, as for all leukocytes, depends on a highly integrated signaling network culminating in coordinate activation and functional cooperation between different pathways triggered by integrin and chemokine receptors.

Activation of protein tyrosine kinases (PTK) is a prerequisite event for leukocyte migration, controlling both integrin adhesiveness and chemotactic response. The involvement of tyrosine kinases belonging to the Src and Syk/Zap families in cell migration have been largely documented for T lymphocytes and cells of myeloid lineage.

In regard to NK cells, it has been reported that LFA-1 engagement results in both Src and Syk kinase activation but these events have been associated with the cyto-

toxic function rather than with the migratory ability of NK cells [60, 61]. Using PTK inhibitors such as the general tyrosine kinase inhibitor herbimycin A, the specific Lck inhibitor damnacanthal, and the Syk inhibitor piceatannol, a role for the Src kinase Lck but not for Syk in CXCL12 (SDF-1α/β)-induced NK cell chemotaxis has been described. In accordance with these results, NK cell stimulation with CXCL12 (SDF-1α) leads to tyrosine phosphorylation and activation of Lck [62].

More recently, a role for the focal adhesion kinases as cytoplasmic mediators of motility events in multiple cell types has been reported. The focal adhesion kinase family comprises two members that share an amino acid identity of almost 50%, the p125 focal adhesion kinase (p125Fak) and the proline-rich tyrosine kinase 2 (Pyk-2) also known as cell adhesion kinase-β (CAK-β), or related adhesion focal tyrosine kinase (RAFTK). They are non-receptor PTK capable of coupling several receptors including integrins and chemokine receptors, with a variety of downstream effectors, such as small GTP-binding proteins belonging to the Ras and Rho families, MAPK, PKC and inositol phosphate metabolism [63, 64].

The expression of Fak family members on NK is controversial. Rabinowich and colleagues [65] have reported that p125Fak is expressed on NK cells and that β_1 integrin engagement results in activation of this kinase and its association with Fyn and Zap-70 PTK. By contrast, we demonstrated that human peripheral blood NK cells express Pyk-2 that is constitutively associated with the cytoskeletal protein paxillin, but not p125 FAK. Engagement of β_1 or β_2 integrins on human NK cells results in rapid tyrosine phosphorylation of both Pyk-2 and paxillin. Moreover, we demonstrated that Pyk-2 acts as an upstream mediator of β_1 and β_2 integrin-triggered MAPK cascades, and controls the development of NK cell-mediated natural cytotoxicity [66–68]. More recently, we have reported that NK cell binding to endothelium activates Pyk-2 and the small GTP-binding protein Rac, a key regulator of actin cytoskeleton dynamics. Both Pyk-2 and Rac activation are coupled to integrins and chemokine receptors. Using recombinant vaccinia viruses encoding dominant negative mutants of Pyk-2 and Rac, we demonstrated that both Pyk-2 and Rac are functionally involved in chemokine-induced NK cell migration through endothelium or ICAM-1 or VCAM-1 adhesive proteins. We also found that Pyk-2 is associated with the Rac guanine nucleotide exchange factor Vav, which undergoes tyrosine phosphorylation upon integrin triggering, but not with PIX, another exchange factor for Rac that is associated with paxillin through p95 PKL. Collectively, these results indicate that Pyk-2 acts as a receptor-proximal link between integrin and chemokine receptor signaling, and the Pyk-2/Rac pathway plays a pivotal role in the control of NK cell transendothelial migration [68]. These results are consistent with a report by Sancho et al. [69] indicating that Pyk-2 can colocalize with the microtubule-organizing center at the trailing edge of migrating NK cells and in the area of the NK cell membrane that faces target cells.

PI3K and its products are other signaling intermediates that play a crucial role in cell migration. In this regard, evidence is available on the involvement of PI3K on

chemokine-mediated NK cell chemotaxis. In particular, it has been reported that wortmannin as well as antibody to PI3K-γ, but not PI3K-α, can inhibit C, CC, and CXC chemokine-induced NK cell chemotaxis, suggesting that PI3K IB plays a crucial role in chemokine-induced activation of NK cells. In agreement with these results, recruitment of PI3K-γ into NK cell membranes in response to RANTES stimulation has been reported [70].

In vivo NK cell migration

Although NK cells express several adhesion molecules and chemotactic receptors that are involved in the control of NK cell migration across endothelium, and in their correct positioning into different lymphoid and non lymphoid organs, very little is known about the molecular events that govern NK cell trafficking *in vivo* under physiological or pathological conditions.

It has been reported that during murine cytomegalovirus infection, NK cells migrate through a CCL3 (MIP1-α)-dependent mechanism to the site of liver infection, where they contribute to antiviral defense [71]. The involvement of CCL3 (MIP1-α) in the recruitment of NK cells in the liver is further supported by the demonstration that CCL3 (MIP1-α)-deficient mice show decreased resistance to cytomegalovirus infection that is associated with a dramatic reduction of NK cell accumulation and IFN-γ production in the liver [72].

A role for CCL3 (MIP1-α) in recruitment of NK cells has been also demonstrated by intrapulmonary transient transgenic expression of this chemokine that resulted in increased *Klebsiella pneumonia* lung clearance associated with NK cell activation and accumulation in this organ [73]. Accumulation of NK cells in the lungs has also been observed in mice with invasive aspergillosis. In this model, however, NK cell recruitment was mediated by CCL2 (MCP-1), as neutralization of this chemokine resulted in reduced NK cell numbers in the lungs and impaired clearance of the pathogen from this organ [74]. Among CXC chemokines, CXCL10 (IP-10) has been reported to promote innate defense mechanisms following coronavirus infection in the central nervous system by recruiting and activating NK cells [75]. The involvement of CX3CL1 (fractalkine) in supporting NK cell migration *in vivo* has been provided by the evidence that CX3CL1-transfected tumor cells exhibit a reduced growth capability that is mediated by an increased recruitment of activated NK cells [76, 77]. In addition, using an *in vivo* model of NK cell-mediated lung tumor cell clearance and blocking antibodies against CX3CL1 or CX3CR1, it has been demonstrated that decreased clearance of tumor cells following perturbation of CX3CL1/CX3CR1 interaction is attributable to defective NK cell recruitment to the lung [78].

Defective recruitment of NK cells has been described in mice deficient for chemokine receptors. Using a model of *in vivo* pulmonary injury in CXCR3-defi-

cient mice, Jiang et al. [79] have reported that NK cells fail to migrate to the lungs. Using CXCR3-knockout mice, recruitment of NK cells in the lungs has also been shown to participate to the pulmonary host defense against *Bordetella bronchiseptica* [80]. In addition, a specific defect of NK cell recruitment to pulmonary granulomas has observed in CCR1-deficient mice [81]. Recently, using selective depletion and adoptive transfer experiments, Martin-Fontecha et al. [4] have reported that DC-induced recruitment of NK cells into lymph nodes occurs in a CXCR3-, but not CCR7-dependent manner.

These data strongly support the *in vivo* relevance of number of chemokine receptor-ligand interaction, including CX3CR1-CX3CL1, CXCR3-CXCL10/CXCL11 (IP-10/I-TAC), CCR5-CCL3/CCL4 (MIP-1α/β), CCR5-CCL5 (RANTES), shown to mediate human NK cell chemotactic response.

Uterine NK cells

NK cells are the predominant lymphocyte population present in the uterus. Their number increases drastically in the late secretory phase during the menstrual cycle and early pregnancy of humans, and at the implantation site in rodents [82, 83]. They accumulate as single cells or aggregates around endometrial glands and vessels playing a crucial role for the normal development of placenta and/or its vasculature [84].

Uterine NK cells exhibit a particular transcriptional profile [85], but their origin is still unknown. It is debated whether they are recruited from blood and/or arise from an NK cell progenitors found in the uterus or recruited from other tissues.

The analysis of the molecules potentially involved in the control of NK cell accumulation in the uterus has shown that first trimester human decidual NK cells, which are characterized by high levels of CD56 but fail to express CD16 ($CD56^{high}$ $CD16^{-}$), express a distinct repertoire of adhesion molecules and chemokine receptors as compared to their peripheral blood counterpart [82, 85, 86]. In particular, they exhibit high levels of $\alpha_E\beta_7$, $\alpha_1\beta_1$, $\alpha_X\beta_2$, $\alpha_D\beta_2$, whereas they do not express $\alpha_6\beta_1$ laminin receptor. In addition, uterine NK cells also display the β_5 integrin subunit and selectively express high levels of tetraspan 5, CD151, and CD9 tetraspanins that are constitutively associated with integrins and modulate integrin function [87].

In regard to chemokine receptors, first trimester human decidual NK cells express higher levels of CCR1, CCR3, CXCR3, CXCR2, and lower levels of CCR7, CXCR4, CX3CR1 as compared with $CD56^{high}CD16^{low}$ peripheral blood NK cells [88]. This receptor profile is consistent with evidence showing the ability of uterine NK cells to migrate in response to CXCL9, CXCL10 and CXCL12 that have shown to be produced by the trophoblast or by the endometrial cells [88–90].

Evidence so far available in the mouse models has not allowed the identification of a particular chemokine receptor-ligand system involved in the control of uterine

NK cell accumulation. Indeed, no changes in NK cell localization and activation have been observed in mice genetically ablated for CCR2, CCR5, and MIP-1α or mice doubly deleted for CCL3 (MIP-1α) and CCR5 [91]. This may be attributable to the known redundancy of the chemokine system as well to differences in the human *versus* mouse pregnancy.

By contrast, at day 11 of gestation at implantation sites, altered size and frequency of uterine NK cells have been observed in mice either lacking adhesion molecules such as P-selectin or β_7-integrin or treated with blocking monoclonal antibodies against MAdCAM-1 or $\alpha_4\beta_7$ integrin [92, 93]. Whether this finding is related to the absence $\alpha_4\beta_7^+$ leukocytes other than NK cells involved in uterine NK cell differentiation/accumulation is presently unknown.

Conclusions

NK cells were initially thought to be endowed with a particular migratory pattern and to mainly circulate in the blood. The recent findings reviewed here highlight that NK cells can exhibit different predilictions for tissue compartments, i.e., lymph nodes, inflamed tissues, etc., where they can play an important role as active participants in directing DC maturation and T cell response polarization and/or as cytotoxic effector cells.

The mechanisms regulating tissue-selective NK cell homing and functional specialization are just starting to be unraveled, but the importance of tissue microenvironment is becoming increasingly clear. An example is given by decidual NK cells whose differentiation, accumulation and functional program are under hormonal influence.

Acknowledgements

This work was supported by grants from Istituto Pasteur Fondazione Cenci Bolognetti, MIUR-PRIN, Centro di Eccellenza BEMM, EC NoE EMBIC contract no. 512040.

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Immunopathology of lymphocyte trafficking

Lymphocyte trafficking and chemokine receptors during pulmonary disease

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Introduction

The localization of lymphocytes to tissue during immune/inflammatory responses involves a series of complex mechanisms, including activation of integrin binding, adhesion molecule expression, and tissue-based chemokine production. The regulation of specific molecules and the expression of certain receptors on lymphocytes during the progression of disease determine the type of T lymphocytes, Th1 or Th2, which migrate into the tissue. Although there have been studies that have outlined tissue-specific expression of certain chemotactic molecules, a more logical view may be that the type of immune/inflammatory response induced within the affected tissue would dictate the mediators that are expressed. The trafficking of naive lymphocytes from the blood to lymph nodes is pivotal to the maintenance of effective immune surveillance; however, deciphering the mechanisms involved in lymphocyte recruitment during inflammation may be more pharmaceutically attractive for regulation of chronic debilitating diseases. Functional diversity of T cells has been demonstrated by the observation that naive T lymphocytes are activated and differentiate into Th0 type cells that produce different combinations of cytokines. Subsequently, these cells can further differentiate into either Th1 type cells (IL-2 and IFN) or Th2 type cells (IL-4, IL-5, and IL-13) depending upon the cytokine environment to which the Th0 cells are exposed [1, 2]. Over the years, it has become clear that certain diseases are characterized by the T helper (Th) cytokine phenotype that is produced. In particular, allergy and asthma responses have been identified as a largely Th2-type disease [3, 4]. The following review outlines some of the recent concepts that may dictate how and why certain Th lymphocyte subsets migrate into inflamed tissues and what contributions other disease mediators provide to worsen the disease process.

Lymphocyte Trafficking in Health and Disease, edited by Raffaele Badolato and Silvano Sozzani

Chemokine and chemokine receptor patterns during Th1- or Th2-type responses

The migration of lymphocytes into tissues appears to be dependent upon the expression of specific chemokines during the progression of the inflammatory disease. Chemokines are a family of small molecular weight cytokines that are important for localization of particular leukocyte populations during immune/inflammatory responses [5–7]. Chemokines are primarily divided into two main groups, CxC and CC, based upon the juxtaposition of the first two-cysteine residues in their sequence (Tab. 1). The responses induced by the chemokines are initiated via specific G protein-coupled receptors on the surface of cells. Although not entirely characterized, it appears that there are no less than six different CxC family receptors and ten different CC family receptors. In the context of allergy, members of the CC subfamily have been implicated as potential mediators of the inflammatory response through their ability to induce migration of eosinophils, T cells and monocytes. In addition to playing a prominent role in the localization of leukocytes to tissue sites, these activating factors are also involved in important biological events, such as eosinophil and mast cell degranulation, differentiation of Th lymphocyte phenotypes, and regulation of antibody isotype switching. Thus, these molecules have important functions in multiple phases of the developing immune response.

Some of the initial studies in chemokine biology outlined the role of early response cytokines, such as TNF and IL-1, for the activation of chemokines [8]. These early studies reported that chemokines could be induced in nearly every cell type. Subsequently, investigators have begun to define the association of certain chemokine profiles with particular types or phases of immune responses. In fact, the preferential expression of certain chemokines during immune responses likely dictates their function [9–11]. For example, the CC chemokine family members, CCL3 and CCL5, are induced by IFN and TNF, but regulated by IL-4, and appear to be closely associated with Th1-type responses. Likewise, the production of CxCR3 ligands, CxCL9, CxCL10, and CxCL11, are specifically activated by IFN and may have critical roles in enhancing Th1-type lymphocyte recruitment and activation. Along with the preferential expression of chemokines during Th1-type responses, there is also the preferential expression of the associated chemokine receptors on Th1-type lymphocytes. A number of studies have shown the preferential expression of CCR1 and CCR5 (which binds CCL3 and CCL5) as well as CxCR3 (which binds CxCL9, CxCL10, and CxCL11) on Th1-type lymphocytes. Thus, the chemokine expression during a Th1-type response correlates directly with the specificity of the chemokine receptors that are expressed on Th1-type lymphocytes.

As there are chemokines associated with Th1-type responses, there also appears to be certain chemokines that are closely associated with Th2-type responses [9, 12, 14]. An extensive amount of work was performed on CC chemokines, and particular members of this family are specifically activated by IL-4 and IL-13 [15–19]. The

Table 1- Chemokine receptors, their ligands and diseases

Chemokine receptor	Ligands	Disease association
CC chemokines		
CCR1	CCL3, CCL5, CCL6, CCL7, CCL14, CCL15	viral and fungal disease
CCR2	CCL2, CCL7, CCL12	asthma, viral, autoimmune
CCR3	CCL5, CCL7, CCL11, CCL24, CCL26	asthma, parasitic
CCR4	CCL17, CCL22	asthma, sepsis
CCR5	CCL3, CCL4, CCL5	HIV, MS
CCR6	CCL20	asthma, RA
CCR7	CCL19, CCL21	IPF, neoplasia, IBD
CCR8	CCL1	asthma, atopy
CCR9	CCL25	IBD
CCR10	CCL27	atopic dermatitis
CxC chemokines		
CxCR1	CxCL1, CxCL6, CxCL8	sepsis, pneumonia, RA
CxCR2	CxCL2, CxCL3, CxCL5, CxCL6, CxCL7, CxCL8	sepsis, pneumonia, COPD
CxCR3	CxCL9, CxCL10, CxCL11	viral, autoimmune, transplant
CxCR4	CxCL12	HIV, asthma, metastasis
CxCR5	CxCL13	lymphoma
CxCR6	CxCL16	sarcoidosis, RA

MS, multiple sclerosis; RA, rheumatoid arthritis; IPF, idiopathic pulmonary fibrosis; IBD, inflammatory bowel disease.

CC chemokines that are preferentially up-regulated by Th2-, but not Th1-, type cytokines include CCL1, CCL2, CCL11, CCL17, and CCL22. Interestingly, these IL-4- and IL-13-induced chemokines bind to a single chemokine receptor (see Tab. 1), which is fairly unusual among chemokine family members that tend to have a promiscuous binding pattern to multiple chemokine receptors. Studies have indicated that CCL2 is involved in allergen-induced T lymphocyte accumulation in the lungs of sensitized mice, whereas CCL11 is most closely associated with eosinophil accumulation during allergic responses. Thus, the Th2 activation pathway, which has been associated with allergen-induced airway hyperreactivity, likely induces preferential chemokine production that is associated with allergic cell recruitment. This area will be of particular interest since studies have previously shown that these Th2-associated chemokines play significant roles in allergen-induced airway inflam-

mation and airway hyperreactivity. Furthermore, analysis of *in vitro*-derived Th2-type cells indicates preferential expression of CCR3 (CCL11), CCR4 (CCL17, CCL22) and CCR8 (CCL1) [12, 20–23]. This receptor expression pattern correlates well with the type of chemokines that are induced by Th2-type responses discussed above. In addition to lymphocyte migration, there is also preferential chemokine receptor expression on effector cells that migrate into the airways and can induce damage, leading to airway hyperreactivity. Overall, the recruitment of multiple cell populations into allergic tissue is mediated by a combination of preferential chemokine production within the inflamed tissue and the receptors expressed on the marginated leukocyte populations.

Preferential patterns dictate chemokines utilized during asthmatic disease

The above correlations continue as researchers examine chemokines expressed in samples from asthmatic patient populations. Chemokines previously identified in the airways of asthmatics include CCL5/RANTES, CCL11/eotaxin, MIP-1a/CCL3, CCL7/MCP-1, CCL13/MCP-4, CCL24/Eot-2, CCL17/TARC, CCL22/MDC, CCL28 and CxCL10. The importance of individual chemokines and chemokine receptors in allergic airway inflammation has been investigated using knockout mice sensitized and challenged with allergens including ovalbumin (OVA) and cockroach allergen (CRA) or with infectious challenges including *Aspergillus fumigatus* [24–36]. The preferential expression of these chemokines within the airways is believed to regulate recruitment and activation of a range of leukocyte subtypes including eosinophils and Th2 lymphocytes to the lungs.

$CD4^+$ T cells recruited to the lungs following allergen challenge secrete additional Th2 cytokines such as IL-4 and IL-13, and these cytokines are known to modulate chemokine expression in the lungs, resulting in elevated levels of CCL11, CCL13, CCL22, CCL1 and CCL17 via regulation of signal transducers and activators of transcription 6 (STAT6)-mediated transcription pathways [15, 37, 38]. The chemokine receptors expressed on lymphocytes skewed toward a Th2 phenotype correspond to the expression of ligands that have been implicated in the pathogenesis of allergic airway disease. Recent studies have begun to elucidate the role of individual chemokine receptors in directing Th2-type cell trafficking. Lloyd et al. determined that recruitment of these cells in the initial stages of an allergic response is dependent on expression of CCR3 ligands, but that repeated antigen stimulation results in the predominant use of CCR4 pathways possibly due to a progressive increase in recruitment of $CCR4^+$ cells [39]. Studies utilizing neutralizing CCR3 antibody or $CCR3^{-/-}$ mice have demonstrated that there is a significant defect in not only eosinophil accumulation but also the induction of airway hyperreactivity that may be related to T cell accumulation [40, 41]. CCR4 has also been detected on the majority of Th2 cells found in endobronchial biopsies collected from asthmatic

patients after allergen challenge, while <30% of the cells co-expressed CCR8 [12]. CCR3 expression was detected only on eosinophils. In these patients, CCL17 and CCL22 expression was strongly up-regulated in the airway epithelial cells following allergen challenge further indicating a key role for CCR4 in mediating T cell trafficking to the airways. Although the authors were unable to detect elevated levels of the CCR8 ligand CCL1, CCL17 has been shown to induce migration of CCR8 transfectants in addition to CCR4. As indicated above, CCR8 is expressed predominantly on Th2 cells and a recent study in $CCR8^{-/-}$ mice showed that, while the development of a peripheral Th2 response was normal, the response to a localized allergen challenge in the lungs was altered [14]. Allergen-challenged $CCR8^{-/-}$ mice exhibited reduced levels of Th2 cytokines in the lungs possibly due to an inability to recruit Th2-type lymphocytes to the lungs. The resulting alterations in the immune environment also led to attenuation of eosinophil recruitment. Although the cytokines and eosinophil accumulation was altered in allergic airway responses, no alteration in airway physiology was observed. This latter observation has been confirmed in two independent studies in different $CCR8^{-/-}$ mice [42, 43]. Other studies have shown that $CCR8^{+}$ $CD4^{+}$ T cells are directly associated with IL-10 production and appear to be phenotypically similar to T regulatory cells [44, 45]. Thus, these cells may have a significant role in maintaining or skewing the immune response toward a Th2 environment. It is clear that T cells play a critical role in modulating the immune environment within the lungs, and strategies that exploit the role of chemokines in Th2 cell recruitment may prove extremely beneficial in the development of new treatments for asthma.

Other chemokine receptors have also been implicated in mediating Th2 lymphocyte accumulation in the lungs of mice. In addition to their presence on naïve lymphocytes, receptors, such as CxCR4, CxCR5, CCR6 and CCR7, have now been identified on T lymphocytes that are of memory/activated phenotype as well as skewed helper cell populations [46–52]. These receptors may therefore have a role in recruitment of lymphocytes to the airways of asthmatic patients and lead to exacerbation of disease. This notion can already be supported in the existing literature where targeting CxCR4 or CCR6 have had a beneficial effect within models of allergic airway responses. In the case of blocking CxCR4, studies show that there is a beneficial effect regardless of whether the receptor or ligand (CxCL12) is blocked [24, 53]. In contrast to the CxCR4 results, studies with $CCR6^{-/-}$ mice demonstrated an altered migration of $CD4^{+}$ lymphocytes to the lung, suggesting that tissue-specific migration was altered [32, 54]. There may be multiple explanations for results from the CxCR4 and CCR6 studies including interruption of normal trafficking patterns of memory/activated lymphocytes to lymphoid organs or target tissue as well as reduced pulmonary recruitment and activation of eosinophils that express these receptors. Further studies with other homeostatic receptors indicate that CCR7 expression on T cells in both human and mouse studies is also important for development of asthmatic responses [55, 56].

Role of dendritic cells for lymphocyte activation

Chemokines influence the immune response at multiple levels. The presentation of foreign antigen to T cells is the initiating step of the adaptive immune response, and a number of leukocytes are effective antigen-presenting cells (APC). The dendritic cell (DC) is particularly indispensable in this regard, expressing high levels of MHC class II on its surface [57]. Thus, it is not surprising that work has been undertaken to identify chemokines that mediate the trafficking of these highly motile cells from the bone marrow to non-lymphoidal tissues and, following encounter with antigen, to regional lymph nodes. Although differential expression of chemokine receptors during their maturation has not been fully characterized, mature DC express detectable levels of CCR1, CCR2, CCR5 and CCR6, as well as CXCR1, CXCR2 and CXCR4, and the respective ligands for these receptors are effective chemoattractants [6, 58–60]. In particular, immature DC express CCR6, which is down-regulated during the maturation process as the DC migrates to the lymph node to participate in its APC function. Using CCR6-deficient mice, studies have demonstrated defects in DC positioning in the gut mucosa as well as defects in DTH responses centered on T lymphocyte accumulation [61–63]. Thus, CCR6 has the potential to participate in a vast range of immunological responses and, therefore, may not be segregated to only a homeostatic function. Recent data using $CCR6^{-/-}$ mice during allergen-induced airways disease demonstrated a defect in the accumulation of DC subsets in the lungs of challenged animals [54]. These latter data, which suggest that CCR6 is important for DC accumulation in the lungs during allergic responses, have been supported by previous studies. CCR6 is preferentially displayed on myeloid DC populations, and allergen challenge invokes an influx of circulating myeloid DC into the lung [64]. As CCL20 has been shown to be produced by airway epithelial cells [65, 66], CCR6 might be required for localization of DC subsets to the airway, become activated, and acquire antigen for transport back to the draining lymph node. Furthermore, there may be specific defects in defined subsets of DC within the airway and/or decreased accumulation within the draining lymph nodes attributed to the CCR6 deficiency.

A number of chemokines have also been suggested to have a role in determining DC function. DC can produce a number of chemokines that may aid in preferentially recruiting specific T cell subsets to the DC for antigen activation. One of the first chemokines described to be produced, CCL22, which binds to CCR4, has been shown to have a role in allergen-induced airway disease [67–69]. Interestingly, stromal cell-derived factor-1 (SDF-1), which binds CXCR4 and selectively attracts naive T cells, is also an attractant for DC and may be an important mediator to attract these cells for antigen presentation [70–72]. The macrophage is probably the best-studied APC with respect to chemokines. Although they are highly phagocytic, they also express MHC class II and can participate in antigen presentation. A number of the CC chemokines (MCP 1-5, RANTES) were originally described as chemoat-

tractants for monocytes/macrophages, and most CC chemokine receptors (with the notable exception of CCR3) are found on the surface of these cells. Thus, chemokines and their receptors likely play a crucial role in the recruitment and activation of APC, as well as in providing a source of chemokine for recruiting T cells for antigen presentation.

Viral infection, chemokine production, and exacerbation of the lung disease

The causes of severe asthma exacerbations are poorly understood. While it is clear that environmental levels of allergen can play a role, it is unlikely that this is sole cause of exacerbated asthma. Clinical studies have shown that a decrease in the CD4:CD8 T cell ratio in the bronchoalveolar lavage (BAL) of asthmatic patients is correlated with increased asthma severity. In stable asthmatics the ratio of CD4:CD8 T cells is 3:1; however, this ratio decreases to 1:1 in patients experiencing acute asthma attacks. Additionally, in cases of asthma death the CD4:CD8 T cell ratio is reversed to 1:2–1:6. A possible explanation for the increased recruitment of $CD8^+$ T cells to the BAL of exacerbated patients may be viral infection [73–75]. It has been reported in adults that 80% of asthma exacerbations, as characterized by a decrease in the peak expiratory flow rate, are associated with viral infection. There are a number of respiratory viruses that may play an important role in the exacerbation of the viral responses. The clinical data clearly indicate that one of the common features of most pulmonary viral infections is the early and intense production of chemokines [76–78]. The viruses that have been ascribed to producing chemokines upon infection of target cells include rhinovirus (RV), adenovirus, influenza virus, respiratory syncytial virus (RSV), as well as parainfluenza and SARS. The one characteristic that these viruses have is an often intense inflammatory response that initiates damage in the lungs of susceptible patients. Those most at risk usually have underlying pulmonary diseases, such as asthma, chronic obstructive pulmonary disease (COPD), or are transplant recipients, premature infants, etc., and suffer the most severe disease from the initiation of the anti-viral inflammatory responses [79, 80].

There are a number of chemokines that are initiated by respiratory viruses that appear to be commonly induced, including CCL2, CCL3, CCL5, CxCL8, CxCL9, and CxCL10. Although some of these chemokines are produced during Th2-type allergic responses, many of these have primarily been associated with Th1-type responses, such as those needed for anti-viral responses. A number of studies have identified production of chemokines after viral infection of isolated cell populations via PAMPs, which activate innate molecules such as Toll-like receptors [81–84]. The early and intense up-regulation of these chemokines would normally play an important role in the anti-viral responses. However, during asthmatic responses the overproduction of these chemokines leads to increased leukocyte recruitment and exac-

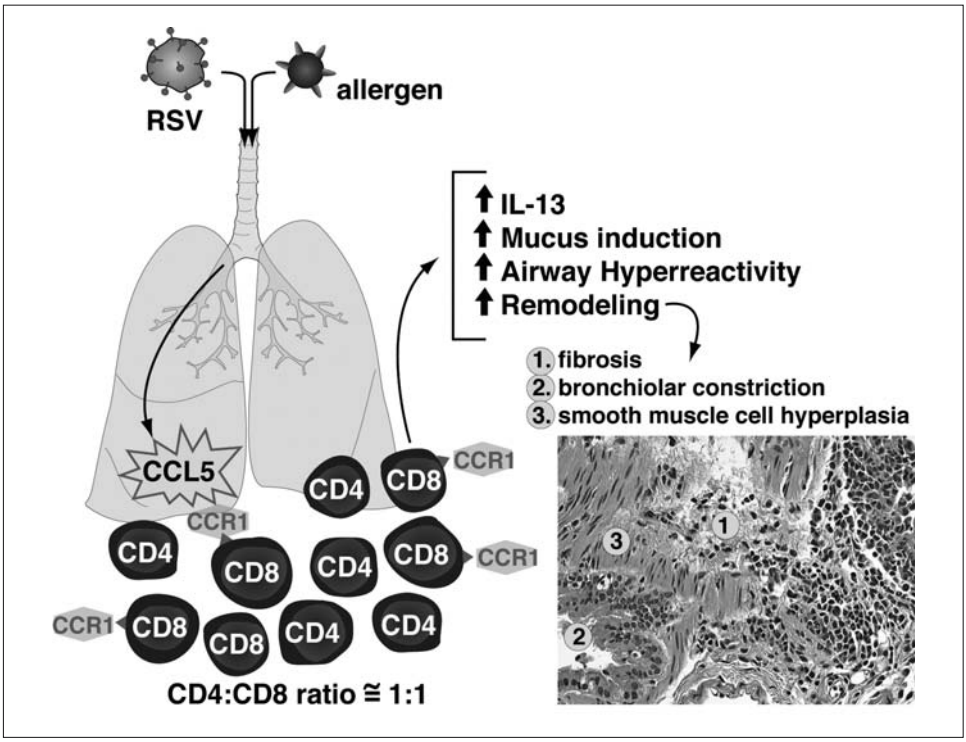

Figure 1
Viral exacerbation of asthma due to Respiratory virus infection.

erbated disease. As indicated above, the overproduction of these virus-associated chemokines leads to the accumulation of CD8 T cells. The identification of chemokine receptors on CD8 T cells has not been thoroughly investigated. Recent data would indicate that CCR1 appears to be one of the receptors that mediate CD8 T cell recruitment ([85] and unpublished data). Other studies have indicated that CCR2, CCR5, and CxCR3 may individually allow the accumulation of CD8 T cells to a site of viral infection [86–89]. The overall effect of producing specific chemokines that are induced during viral responses may be to elicit certain T cell subsets during disease. Data from our laboratory using RSV infection has shown that CCR1 specifically regulates CD8 T cells that produce Th2-type cytokines, especially IL-13 (unpublished data). This effect would bias the entire pulmonary immune environment toward ineffective clearance of the viral response and lead to enhanced Th2-mediated asthmatic reactions, especially mucus hypersecretion, as illustrated in Figure 1. This mechanism may also operative in other diseases such as COPD, where mucus overproduction is related directly to the intensity of the $CD8^+$

Table 2- Potential chemokine receptor targets for asthma

Receptor targets	Cellular distribution	Aspect of asthmatic response altered	Ligands
CCR1	Monocytes, T cells eosinophils, neutrophils	Chronic stage remodeling, infectious organism response, mucus hypersecretion, IL-13 production	CCL3, CCL5, CCL6, CCL7, CCL14, CCL15
CCR3	Eosinophils, basophils, mast cells, Th2 cells	Eosinophil accumulation, basophil recruitment, development of AHR	CCL5, CCL7, CCL8, CCL11, CCL13, CCL24, CCL26
CCR4	Dendritic cells, Th2 cells	T cell recruitment, airway remodeling, clearance of fungal spores	CCL17, CCL22
CCR6	Dendritic cells, T cells, B cells, eosinophils	$CD4^+$ T cell recruitment, eosinophil accumulation, development of AHR, IgE	CCL20
CCR7	T memory Cells, DC, naive T cells	Unknown	CCL19, CCL21
CCR8	Th2 cells, eosinophils, dendritic cells	Th2 cytokines, eosinophil accumulation	CCL1
CxCR4	Naïve and Th2 cells, eosinophils, mast cells	Th2 cytokines, eosinophil accumulation, development of AHR, T cell accumulation	CxCL12

AHR, airway hyperresponsiveness

T cell recruitment [90–92]. In contrast, other recent publications have identified an important role for CxCR3 for the recruitment of cytotoxic $CD8^+$ T cells for clearance of MHV-68 during lung infection [93, 94]. While this subset may be important with clearance of virus, it was also associated with development of chronic cough in non-asthmatic children [95] and may be associate with post-viral syndromes. Thus, there may be a preferential use of chemokine receptors during viral responses that dictate the phenotype of the $CD8^+$ T cell that is recruited to the airways.

Conclusion

Although a number of chemokine receptors that have been described that mediate the accumulation of T lymphocytes to the lung during disease progression, there

continues to be a paucity of data regarding their specific function during certain pulmonary diseases. Questions persist on whether the differential receptor display on T cell subsets described in *in vitro* experiments, represent those that cause accumulation during disease. The diversity of chemokine production and the promiscuous pattern of chemokine-chemokine receptor interactions have made the identification of individual chemokine or chemokine receptor targets for therapeutic intervention extremely difficult. Interestingly, several of the "lead" candidates for targeting during chronic asthmatic disease are receptors that appear to bind a single or at most two chemokines, including CxCR4, CCR4, CCR6, and CCR8 (Table 2). Choosing the proper targets for specific disease phenotypes will only occur after careful coordinated animal modeling experiments coupled with translational research efforts in human disease.

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Lymphocyte migration to the brain

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Introduction

The central nervous system (CNS) comprises the brain, spinal cord and cerebrospinal fluid (CSF). Neurons are vulnerable non-renewing cells and require tight regulation of their external milieu. Any event causing a major disturbance in CNS homeostasis might result in neuronal cell death. Thus, inflammatory reactions in the CNS, involving infiltration of leukocytes and production of inflammatory mediators, and, particularly, edema, are potentially disastrous. In fact, the CNS has been regarded an immune-privileged because of a large spectrum of experimental results and descriptive phenomena, including: tissue graft survival [1]; lack of constitutive major histocompatibility complex molecule expression and antigen-presenting cells (APC); lack of lymphatic vessels; and the presence of barriers between the blood and CNS. However, it has become clear that certain immunological events do occur in the brain: CNS tissue grafts induce peripheral immune responses, are rejected eventually and delayed-type hypersensitivity as well as autoimmune reactions do take place in the CNS. In addition, it is now more or less accepted that, like all other organs, the CNS is also subject to immunosurveillance [2]. Here, the mechanisms of lymphocyte migration into the CNS and its implication under normal and pathological conditions are discussed. Although the basic principles for lymphocyte migration are similar, the molecular details differ for the various inflammatory stimuli and affected CNS compartments. Most information on this subject is derived from studies on multiple sclerosis (MS) and one widely studied animal model, experimental autoimmune encephalomyelitis (EAE), both of which are characterized by lymphocyte and monocyte infiltration in the CNS. In this review, we focus primarily on lymphocytes, the focus of the majority of experimental studies.

Lymphocyte Trafficking in Health and Disease, edited by Raffaele Badolato and Silvano Sozzani

Routes for lymphocytes to enter the CNS

The CNS is protected from a massive influx of inflammatory substances by various barriers, present at different anatomical sites. Consequently, three key routes to enter the brain can be identified based on CNS anatomy (Fig. 1). First, lymphocytes can enter the brain parenchyma directly by crossing the endothelium of the blood-brain barrier (BBB) into the perivascular spaces. Secondly, lymphocytes extravasate across postcapillary venules at the pial surface into the subarachnoid space and Virchow-Robin perivascular spaces, which are in direct contact with the CSF. Finally, lymphocytes might simply enter the CSF directly at the place where CSF is being produced, at the choroid plexus (CP) [3]. This summary represents a 'minimalist' view and the situation is more complex than implied by the 'three pathways' formulation: it is evident that cell infiltration of the spinal cord parenchyma utilizes specific mechanisms, and that cell entry directly into the CSF can occur across meningeal spinal vessels.

The blood-CNS barriers

Anatomically, the BBB consists of capillary endothelial cells surrounded by astrocytic endfeet and a basal lamina. In addition, pericytes can be found at irregular intervals within the basal lamina (Fig. 2A) [4]. The BBB is a low-permeable and selective diffusion barrier, which normally prevents blood-borne elements from entering the brain due to low pinocytic activity and the presence of inter-endothelial tight junctions (TJs) [5]. It is assumed that selective diffusion across this barrier is primarily determined by the endothelial features, while astrocytes are probably critical in development of the BBB. Pericytes are suggested to play a role in blood flow regulation and induction and maintenance of barrier properties [4]. Microvascular TJs are comprised of a complex of molecules including: junction adhesional molecule (JAM)-A, occludin, accessory proteins like zonula occludins (ZO-1 and 2), and claudins. The TJs at the BBB respond to various stimuli, regulated by multiple intracellular signaling pathways [4, 5]. Although the characteristics of the brain and spinal cord BBB endothelium appear similar, subtle differences between meningeal (no astrocytic ensheathment) and parenchymal (no P-selectin storage) microvessels have been reported [6–8].

Gross BBB disruption accompanies catastrophic events such as stroke or traumatic brain injury. Alternatively, more subtle and focal opening of the BBB coincides with lesion formation in MS. Studies addressing TJ alterations in neuropathology are rapidly accumulating. Hypoxia increases rat BBB permeability *in vivo* and decreases occludin expression [9]. Inflammatory mediators affect BBB permeability and MS lesions are associated with loss of occludin and ZO-1 [10]. Peripheral processes such as shock may also alter BBB permeability [11].

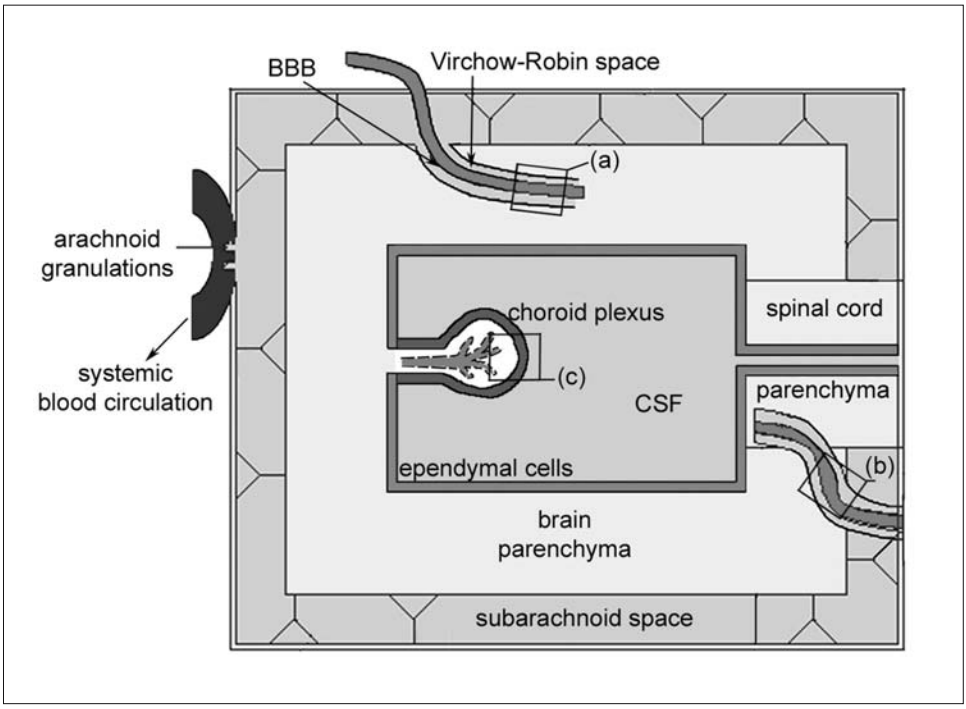
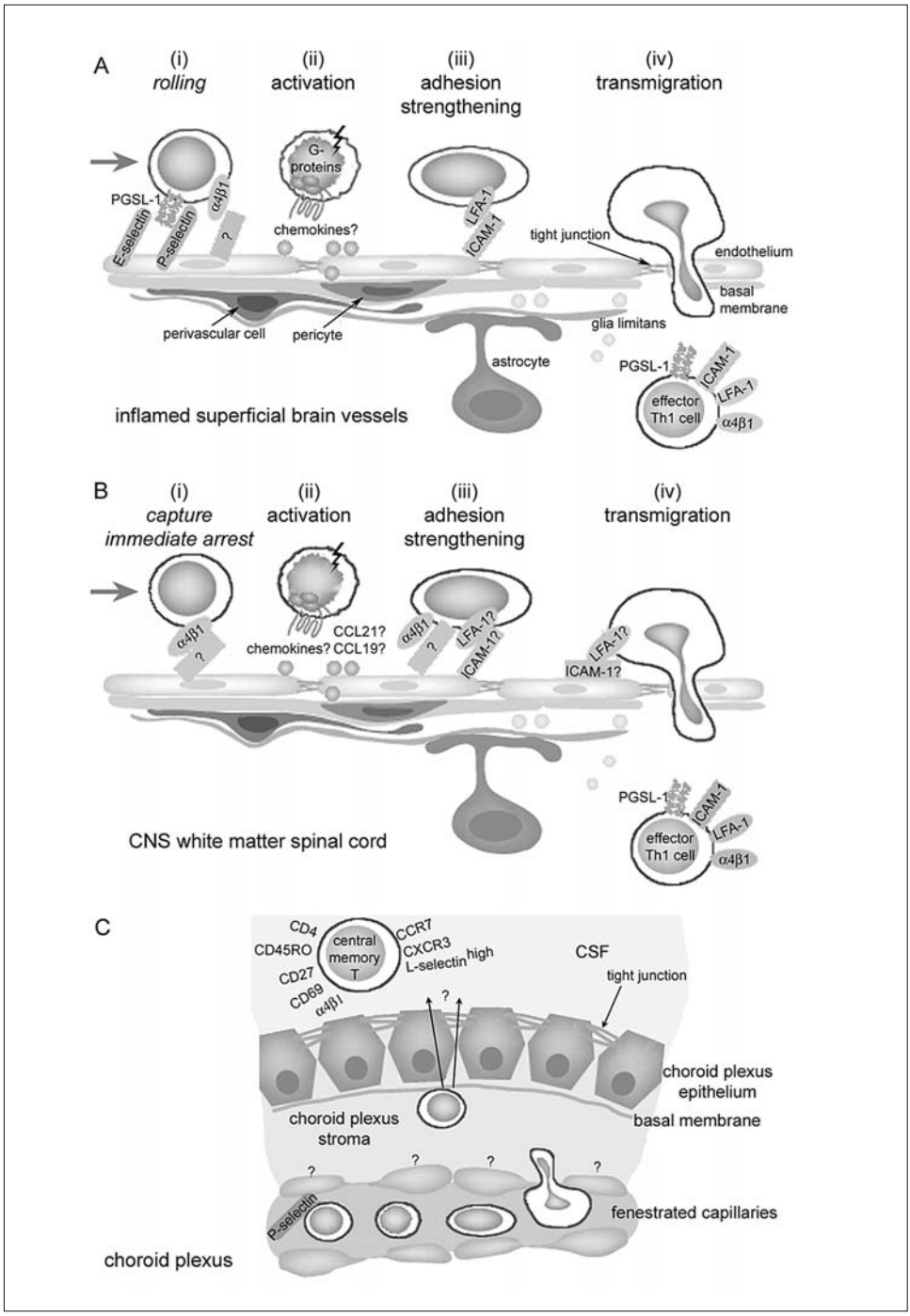


Figure 1
The CNS compartments and blood-CNS barriers
Lymphocytes enter the brain or spinal cord parenchyma by crossing the BBB into the perivascular spaces. Via postcapillary venules at the pial surface and the Virchow-Robin perivascular spaces, lymphocytes extravasate into the subarachnoid space and CSF. At the CP, lymphocytes may enter the CSF-filled ventricles by passing the fenestrated capillaries in the CP stroma and the CP epithelium. Insets a, b and c are depicted in more detail in Figure 2 (adapted from [2]).

Similar to other circumventricular organs (characterized by absence of the BBB), fenestration and gap junctions exist between the vascular endothelial cells at the CP (Fig. 2C). The actual blood-CSF barrier is, therefore, at the level of the epithelial cells and primarily created by the TJs between CP epithelial cells.

Mechanisms involved in crossing the vascular endothelium

Crossing vascular endothelium is a multi-step process supported by experimental evidence from studies on peripheral vasculature [12]. Although different in details,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transmigration
PGSL-1
E-selectin
P-selectin
α4β1
?
G-
proteins
chemokines?
LFA-1
ICAM-1
tight junction
endothelium
basal
membrane
glia limitans
perivascular cell
pericyte
astrocyte
PGSL-1
ICAM-1
LFA-1
α4β1
effector
Th1 cell
inflamed superficial brain vessels
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(i)
capture
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adhesion
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CCL21?
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LFA-1?
ICAM-1?
PGSL-1
ICAM-1
LFA-1
α4β1
effector
Th1 cell
CNS white matter spinal cord
C
CD4
CD45RO
CD27
CD69
α4β1
central
memory
T
CCR7
CXCR3
L-selectin high
CSF
tight junction
?
choroid plexus
epithelium
basal membrane
choroid plexus
stroma
?
?
?
?
?
P-selectin
fenestrated capillaries
choroid plexus

the basic principles of crossing vascular endothelium in the CNS are similar and thus include the following steps: tethering and rolling, activation and tight adhesion of leukocytes to the endothelium and finally diapedesis (Fig. 2A, B). Recent applications in neuroimmunology of techniques such as intravital microscopy (IVM) using a cranial or spinal window or through the intact skull, have made it possible to visualize the interactions of labeled lymphocytes with the cerebrovascular endothelium, and study the different steps in more detail *in vivo*. Furthermore, the importance of specific molecules in each step of the extravasation process has been explored by antibody-inhibition studies and experiments using knockout mice.

Tethering and rolling: selectins

IVM studies using specific antibodies demonstrated that tethering and rolling of lymphocytes in cerebral vessels is mediated by platelet (P)- and endothelial (E)-selectins expressed on the activated endothelium and their carbohydrate ligands, which decorate the P-selectin glycoprotein ligand-1 (PGSL-1) scaffold on lymphocytes. In addition, murine lymphocytes can roll *via* interactions of α_4-integrins with endothelial VCAM-1. Interestingly, injected fluorescence-labeled encephalitogenic and activated T lymphoblasts were observed by IVM to cross the microvessels with-

Figure 2
Cellular and molecular players in the multi-step recruitment of T lymphocytes across the blood-CNS barriers. (A) For the superficial vessels of inflamed brain, E- and-P-selectin, PSGL-1 and α_4-integrin are involved in lymphocyte tethering and rolling. G protein-dependent activation of LFA-1 on T lymphocytes, probably by chemokines (yet to be identified), leads to their firm adhesion on endothelial ICAM-1. Lymphocytes migrate transcellularly through the BBB endothelium, leaving TJs intact. In EAE, the inflammatory lymphocytes present in brain (and spinal cord) parenchyma are Th1 effector memory cells with a characteristic surface-marker phenotype. (B) In the spinal cord white matter, T lymphocytes arrest immediately through α_4-integrin. G protein-dependent increase in α_4-integrin avidity on the T lymphocytes is required for firm adhesion to endothelial counter-receptors. LFA-1 supports T lymphocyte diapedesis, adjacent to TJs. (C) Molecular mechanisms in lymphocyte transit across fenestrated CP microvessels, and subsequent migration across the CPE into the CSF are relatively unknown, although α_4-integrin is required. Endothelial P-selectin mediates T lymphocyte recruitment into the CP stroma. Pathways of T lymphocyte traversal across CP endothelial and epithelial barriers are equally mysterious. T lymphocytes in the CSF, both of healthy individuals and MS patients, are predominantly central-memory $CD4^+$ T lymphocytes, about half of which express the recent activation marker CD69 (adapted from [2]).

out rolling in the spinal cord [13]. Instead, these cells were captured immediately and adhered tightly to the endothelium in an α_4-integrin-dependent fashion (Fig. 2B).

Activation: chemokines

Canonically, activation of rolling leukocytes and strong adhesion to the endothelium is mediated by chemokines. Chemokines are released into the bloodstream or they are, in association with glycosaminglycans on endothelial cells, presented to leukocytes in the microvessel [14]. Specific binding sites for the chemokine CCL2 on the abluminal side of human brain microvessel *in vitro*, and the functional importance of CCR2 (on microvascular, not leukocytic elements) in transmigration across murine cerebrovascular microvessels *in vitro*, indicate a possible specific mechanism for transportation of chemokines to the lumen [15, 16], similar to CXCL8 in the skin [17]. Nevertheless, the mechanism of chemokine translocation across endothelium in the CNS *in vivo* remains largely unexplored. Chemokines activate leukocytes after binding to specific – pertussis toxin-sensitive – $G_{\alpha i}$ protein-coupled chemokine receptors (GPCRs) expressed on the leukocyte cell surface. Activated T cells undergo programmed expression of cell surface molecules including chemokine receptors [18] *in vivo*, enabling them to sense chemokine concentrations and recognize sites for extravasation. It is clear from IVM studies that events during extravasation are dependent on pertussis toxin-sensitive signaling [13, 19]. Contingent on the experimental design and vascular bed, these events may include firm adhesion, strengthening of adhesion, arrest or transmigration. Despite heroic efforts, unequivocal assignment of such functions to individual chemokines, for lymphocyte entry into the CNS, has not occurred. In various neuropathologies, expresssion of chemokines is induced in glial cells, endothelial cells, perivascular cells and neurons [20]. Thus, many potential sources of chemokines are available within the CNS.

Adhesion

Stimulation of leukocytes by chemokines or other GPCR-activating molecules induces activation of integrin molecules like leukocyte function-associated molecule-1 (LFA-1) and α_4 integrins. This is accompanied by an increase in the affinity for their endothelial ligands: intercellular cell adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule (VCAM), respectively. These interactions are necessary for tight adhesion and subsequent diapedesis [12, 21]. The tissue-specific display of adhesion molecules and chemokines by vascular endothelial cells provides an excellent mechanism for targeting different leukocyte populations to different organs [12]. It should be noted that there remains uncertainty about the human cerebrovascular ligand for $\alpha_4\beta_1$ integrin. This ligand is likely to be VCAM-1 in

rodents, but detection of VCAM-1 in human CNS has not been consistent, and alternatives, such as the CS1 domain on fibronectin, need also to be considered.

Diapedesis

There are two potential routes, paracellular and transcellular, by which lymphocytes might cross the BBB. Both have been reported for BBB diapedesis. The first entails of lymphocyte passage between adjacent endothelial cells, crossing the integrins and molecules of the aherens/TJ complex [12]. The transcellular pathway involves migration of cells through the endothelial cell body, leaving the TJs intact [22]. Although both routes seem to be applicable in peripheral tissues, *in vitro* and *in vivo* studies on the BBB have demonstrated that lymphocytes cross this endothelium by a transcellular pathway [23].

Lymphocytes trafficking into the healthy CNS

T lymphocytes in healthy CNS parenchyma and CSF

Initially, tracing studies showed that a limited number of encephalitogenic T cell blasts migrate across the BBB into the non-inflamed CNS. In contrast, resting T cells failed to enter the CNS, indicating that the activated state is a prerequisite [24, 25]. However, IVM studies have reported that non-activated brain endothelium does not support lymphocyte interaction, irrespective of activation state and, although lymphocytes are detected in the meninges and CP, they remain absent in the parenchyma 2 h after injection [26]. Moreover, activation of endothelium and subsequent up-regulation of selectins and adhesion molecules, by proinflammatory cytokines such as TNF-α, appears to be necessary for lymphocytes to cross [19]. The contradiction is probably due to experimental approach: Injection of T lymphoblast possibly activated and preconditioned the microvascular endothelium and, therefore, altered barrier function. After passing the BBB these cells reside in perivascular spaces and, for persistence, they need to encounter cognate antigens [27], which are most likely presented by dendritic cell (DC)-like macrophages present in the same space [28].

T lymphocytes in the CSF

Normally, absolute numbers of T lymphocytes are higher in murine CP when compared to brain tissue. Moreover, relative increases in T cells after peripheral immune activation are many times higher than in the rest of the brain [29], suggesting pref-

erential T cell migration to the CP, from which they enter the CSF. Entry of murine T lymphoblasts into the meninges and CP is P-selectin dependent, as antibody treatment or P-selectin-knockout mice show a reduced number of lymphoblasts in these CNS compartments [26]. Yet, the presence of P-selectin on meningeal or CP endothelial cells has not been demonstrated unambiguously by immunohistochemistry, possibly due to expression below detection level [26, 30, 31].

Healthy human CSF contains approximately 150 000 cells, of which more than 80% (compared to <5 % in blood) are $CD4^+CD45RO^+CD27^+CXCR3^+/CCR7^+/L\text{-}selectin^{hi}$ central-memory T lymphocytes. About 50% percent of these cells are $CD69^+$ (as contrasted with <5% in peripheral blood), indicating selective accumulation of recently activated cells in the lumbar CSF. Consistent with the finding that P-selectin is involved in lymphocyte entry into the meninges and CP in mice, is the observation that P-selectin immunostaining is localized in stromal veins of CP and in the bridging meningeal veins in brains of human individuals who died without neurological disease [32]. Although the exact migration mechanisms remain to be determined, these cumulative data suggest that $CD4^+$ central memory T lymphocytes preferentially enter the CNS across the CP. Direct evidence supporting this hypothesis came from demonstrating that the cellular composition of ventricular and lumbar CSF in patients without CNS inflammation is similar, containing primarily $CD4^+$ central-memory T lymphocytes [33]. Moreover, these cells re-enter the bloodstream or lymphoid organs, and are replaced by newly immigrating lymphocytes about twice a day [34], indicating there is a continuous flow of lymphocytes with different specificities, making them good candidates for executing CNS immunosurveillance [2, 35].

Under physiological conditions the number of extravasated lymphoblasts in the CNS is small (estimated to be 10^5 per 24 h at maximum), and it is very unlikely that they would encounter cognate antigen in the perivascular spaces containing single DCs [28]. However, it is not unlikely that this represents the mechanism by which inflammation in adoptive transfer EAE (AT-EAE) is initiated [2], since large numbers of cells are injected and a reasonable chance for encountering highly abundant self antigens presented by perivascular DCs is present [36]. T lymphocytes can induce neuroinflammatory disease without the presence of lymphoid tissue [28], and perivascular DCs are responsible for re-activation and retention of neuroantigen-specific T cells in the CNS [36, 37]. It is thus clear that leukocyte trafficking mechanisms during immunosurveillance (into the CSF) and disease/host defense (into the parenchyma) are different.

Lymphocyte trafficking into the inflamed CNS

Massive infiltration of leukocytes into the CNS is observed during neurodegenerative and neuroinflammatory conditions. This is associated with increased expression

of adhesion molecules and chemokines [38]. Despite a leaky BBB, T cell recruitment remains tightly controlled during inflammation, as evidenced by expression of a specific adhesion molecule repertoire on infiltrating lymphocytes, distinct from lymphocytes infiltrating other organs [39].

Tethering and rolling during inflammation

Tethering and rolling is observed with IVM in inflamed superficial brain and meningeal microvessels. IVM through the intact skull demonstrated that activated brain endothelium expresses E- and P-selectins at the luminal side of venules, but not arteries [19]. Interestingly, differential preference for molecules used to roll was observed for $CD8^+$ (P-selectin) and $CD4^+$ T (α_4-integrin) lymphocytes from MS patients when injected into inflamed murine vessels [40]. Both α_4-integrin and PGSL-1 are involved in rolling during EAE [41], and lymphocyte rolling in inflamed superficial brain vessels is mediated by E- and P-selectin [19].

EAE displays inflammation primarily localized in the spinal cord and here, as mentioned previously, lymphocytes are captured immediately. Accordingly, L-selectin is not involved in the development of EAE [42], and PGSL-$1^{-/-}$ mice or PGSL-1 antibody-treated mice do not differ in developing EAE [43, 44], indicating that selectin mediated rolling is not required. Instead, α_4-integrin and CD44 seem to play a more important role in EAE [42] (Fig. 2B, C).

Chemokine-mediated activation during inflammation

Chemokine-mediated signaling leading to integrin activation is required for firm arrest of lymphocytes in CNS microvessels [13, 19]. Numerous chemokines (or functionally equivalent GPCR agonists) are produced in inflamed CNS tissue and activated lymphocytes express various chemokine receptors. EAE studies focusing on CCL2 and its receptors CCR1 and CCR2 showed that mice lacking CCR1 or CCR2 are relatively resistant to active-immunization EAE, mainly due to impaired monocyte recruitment as $CCR2^{-/-}$ T cells still mediate AT-EAE [45]. Antibody-inhibition and IVM showed CCL2 and CCL5 to be involved in leukocyte adhesion during EAE [46], providing a consistent body of data regarding these receptors. Conversely, CXCL10 antibody inhibition studies in AT-EAE provided contradictory results [47, 48]. Furthermore, CXCL10-deficient mice exhibited a reduced threshold of EAE susceptibility [49]. Additionally, constitutive over-expression of astrocytic CXCL10 resulted in CNS leukocyte infiltration, primarily of neutrophils and not lymphocytes, without neurodegenerative consequences [50].

Two chemokines, CCL19 and CCL21, were reported in BBB endothelial cells during EAE [51, 52]. Although both chemokines trigger adhesion of $CCR7^+$

encephalitogenic T cells to inflamed brain vessels *in vitro* [52], their role in BBB transmigration *in vivo* is unknown. Over-expression of CCL21 but not CCL19 in murine CNS resulted in development of neurological disease associated with leukocytic infiltrates consisting primarily of neutrophils and eosinophils, but not lymphocytes [53]. Further, immunohistochemical analysis of human MS tissue has failed to identify CCL19 and CCL21 in lesional vasculature [54]. Although it is regarded as axiomatic that chemokines should play a role in immune-mediated CNS inflammation by promoting lymphocyte infiltration, the specific 'players' in this scenario remain undefined.

Adhesion molecules: roles in CNS inflammation

Studies in EAE consistently demonstrated up-regulation of endothelial adhesion molecules ICAM-1 and VCAM-1 [55, 56]. Ligands for these adhesion molecules, LFA-1 and $\alpha_4\beta_1$-integrin, respectively, were identified on perivascular inflammatory cells surrounding ICAM-1$^+$ and VCAM-1$^+$ murine vessels. Ultrasound using targeted microparticles demonstrated ICAM-1 and VCAM-1 in the BBB of rats with AT-EAE. Strong ICAM-1 signals were observed in periventricular regions, the brainstem, cerebellum and spinal cord, whereas VCAM-1 expression was of lower intensity and confined to major sites of inflammation [57]. Findings using human tissue have been inconsistent, with one study showing vessel-associated VCAM expression [58], while others detected VCAM expression on activated microglia [32, 59]. Similarly, contradicting results were reported for mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in BBB endothelium during EAE [55, 60].

Antibody-inhibition studies [56, 61] and (frozen section) adhesion assays [55, 62] clearly implicated $\alpha_4\beta_1$-VCAM-1 and probably also LFA-1-ICAM-1 interactions during EAE. The involvement of $\alpha_4\beta_7$-MAdCAM-1 in EAE is controversial since $\alpha_4\beta_7$ antibodies do not [61], while MAdCAM-1 antibodies do [63], inhibit EAE, even though MAdCAM-1 is not detected on CNS endothelium in C57BL/6 mice. Moreover, β_7-integrin$^{-/-}$ mice exhibit mild EAE [64].

In clinical MS trials, anti-α_4 integrin antibodies were very successful in reducing disease activity [65]. Unfortunately, ongoing trials were recently suspended, due to an increased risk of developing progressive multifocal leukoencephalopathy [2, 66].

Due to contradictory results [67–70], the contribution of LFA-1-ICAM-1 in leukocyte recruitment during EAE remains to be elucidated. Nonetheless, *in vitro* studies demonstrated LFA-1 and ICAM-1 to be crucial in lymphocyte-cerebrovascular endothelial interactions and migration across brain endothelium [71–73]. Moreover, ICAM-1 is consistently found to be up-regulated on inflamed vessels in MS lesions and essentially all infiltrated leukocytes here are LFA-1$^+$ [74].

Inflamed CP epithelium (CPE) expresses ICAM-1 and VCAM-1 [75], which mediate adhesion of inflammatory cells [31]. In EAE, up-regulation of VCAM-1 and

ICAM-1 mRNA and protein and *de novo* expression of MAdCAM-1 was reported on the apical surface of CPE, but not on fenestrated endothelial cells [76]. Extending the proposed immune functions of the CP [2], DCs are recruited across the CPE during EAE [77].

Diapedesis during inflammation

LFA-1 and ICAM-1 were recently shown to be involved in transendothelial migration of leukocytes *in vitro*. Although TJs appear to remain intact, the role of junctional molecules like platelet-endothelial cell adhesion molecule-1 (PECAM-1) and JAMs need to be addressed as PECAM-1$^{-/-}$ mice have larger CNS infiltrates and prolonged exaggerated BBB permeability during EAE [78]. Conflicting results have been reported on the involvement of JAM-A in leukocyte migration across CNS endothelium [79, 80].

Besides, CD99, an unique highly O-glycosylated protein expressed on leukocytes and in endothelial cell-cell contacts [81] was found to be important in encephalitogenic T cell migration across endothelial cells *in vitro*. Since CD99 antibodies inhibit this migration, involvement in BBB diapedesis *in vivo* is plausible [82].

Conclusion

Lymphocyte migration to the CNS is a tightly regulated process, which involves many cellular signaling cascades and molecules. Under normal conditions, lymphocyte migration to the CNS is limited and restricted to the perivascular spaces and CSF, but is thought to be important for CNS immunosurveillance. Here, the CP is most likely the "port of entry" for CD4^{+} central-memory T cells. Under pathological conditions, traffic signals are altered and expression of many adhesion molecules and chemokines permit lymphocytes to enter the CNS parenchyma where they need interaction with APCs to persist. The specific molecular mechanisms differ, depending on CNS site and stimuli, and need further exploration for better understanding and novel treatments of neuroinflammation.

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Lymphocyte migration to the kidney

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Introduction

The interactions of activated infiltrating cells and resident renal cells are actively involved in the pathogenesis of renal inflammation. The trafficking of immune competent cells from peripheral blood into the kidneys is an indispensable inflammatory event that is encountered in the early phases of most renal diseases [1]. Proinflammatory molecules, through their interactions with receptors expressed on lymphocytes and resident renal cells, are capable of inducing a number of cytokines, chemokines, adhesion molecules and growth factors, both by autocrine and/or paracrine loops, during acute and chronic phases of various renal diseases [2]. Some of these secreted molecules are not only involved in the inflammatory phase of the disease, but also contribute to the development of subsequent renal fibrosis. Here we review lymphocyte migration to the kidney, especially based on the pathophysiological roles of chemokines. Further, we discuss the intervention of chemokine/chemokine receptor systems as the particular immunotherapeutic strategies to combat progressive renal diseases.

Cell infiltration to the kidney

Infiltration of immune competent cells to the kidney from peripheral blood is a hallmark of almost any kind of renal diseases [1] (Fig. 1). The main inflammatory cells actively involved in renal diseases are neutrophils, monocytes/macrophages, and lymphocytes. Neutrophils that have infiltrated into diseased kidneys via activation of adhesion molecules induce the release of lysosomal enzymes and generation of superoxide anions to initiate inflammatory events and subsequent tissue damage [3]. Monocytes/macrophages also have a scavenging role in the clearance of non-self and/or altered-self materials, including glycated proteins and oxidized lipoproteins [4]. Macrophages exert dual effects in renal injury, i.e., damaging and protective effects. Therefore, infiltrated and activated macrophages play a crucial role in the

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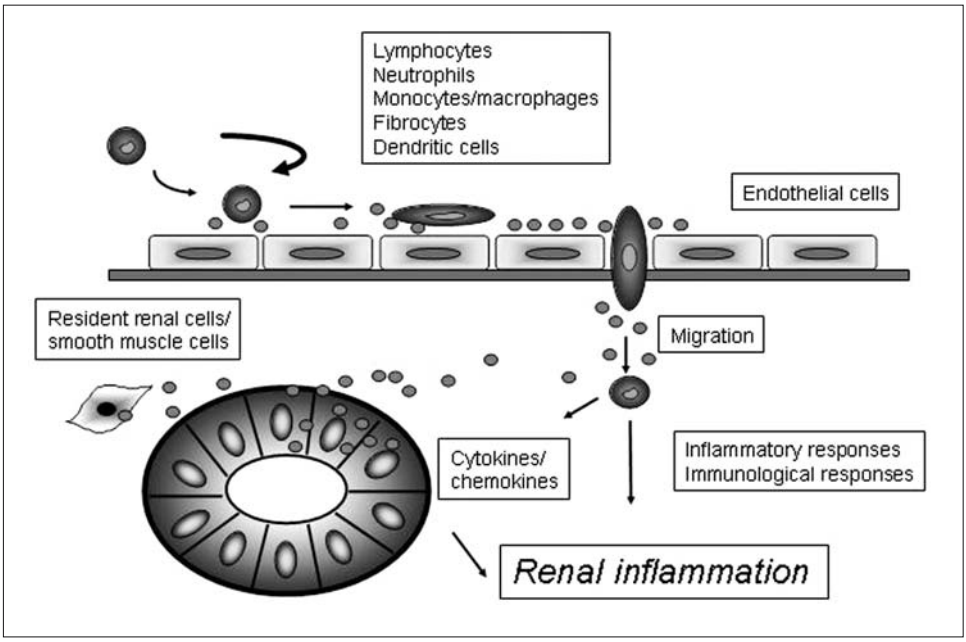

Figure 1
Leukocyte migration to the kidney and renal inflammation

pathogenesis of both inflammatory and fibrotic phases of the disease process. Macrophage colony-stimulating factor (M-CSF) is actively involved in monocyte and macrophage survival, proliferation, and chemotaxis [5]. Overexpression of M-CSF by tubular epithelial cells is closely associated with the interstitial accumulation and proliferation of macrophages, as demonstrated in experimental anti-glomerular basement membrane nephritis and unilateral urethral obstruction models [6]. A correlation between overexpression of M-CSF and accumulation of macrophages has also been demonstrated in various human and experimental diseases, including glomerulonephritis [7, 8]. Similarly, increased expression of macrophage migration inhibitory factor (MIF) has been shown to be involved in human and experimental models of tubulointerstitial injury, possibly by facilitating the interstitial accumulation of macrophages and lymphocytes [9, 10]. Infiltration of inflammatory cells including lymphocytes into kidneys exerts inflammatory responses by secreting certain proinflammatory cytokines and chemokines [11]. For instance, intrarenal M-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α) were absent in T cell receptor (TCR) αβ- and CD4-deficient MRL-*Fas*^{lpr} strains, and drastically reduced in class I-deficient MRL-*Fas*^{lpr} compared with wild-type mice. In addition, the decrease in M-CSF, GM-CSF and

TNF-α was associated with a reduced kidney lymphocytic infiltrates and spontaneous autoimmune nephritis. Intrarenal *ex vivo* retroviral gene transfer of M-CSF and GM-CSF failed to elicit nephritis in these T cell-deficient MRL strains (TCR αβ, CD4, CD8/double negative) as compared with wild-type mice. Therefore, these results suggest that MRL-*Fas*^{lpr} kidney disease is driven by a T cell-dependent amplification feedback loop dependent on multiple T cell populations [11].

Interaction of resident renal cells with infiltrates

Interactions between activated infiltrates and resident renal cells are thought to play a crucial role in the pathogenesis of renal diseases from acute to chronic phases [2] (Fig. 2). Cell-cell interaction has an enormous impact on lymphocytes migration to the kidney, resulting in renal inflammation (Fig. 1). There are at least two groups of resident renal cells that actively participate in the inflammatory phase of various renal diseases; glomerular cells (mesangial cells, endothelial cells and epithelial cells), and tubulointerstitial cells (tubular epithelial cells, interstitial cells and peritubular capillary endothelial cells). These intrarenal cells are capable of proliferation, differentiation, and synthesis of proinflammatory cytokines, chemokines, and growth factors in response to various stimuli, which in turn augment inflammatory responses.

Recent studies report that bone marrow cells migrate to the kidney, suggesting a reservoir of repopulating mesangial cells during glomerular remodeling [12, 13]. Imasawa et al. [12] recently reported the potential of bone marrow-derived cells to differentiate into glomerular mesangial cells. Similarly, Ito et al. [13] reported an increased number of bone marrow-derived Thy1$^+$ cells constituting about 7–8% of glomerular cells during the remodeling phase of anti-Thy1 nephritis. Although the role of lymphocytes among these bone marrow-derived cells remains unclear, these reports are suggestive of the presence of bone marrow-derived mesangial cells in nephritis. Further studies are required to determine the pathogenic role of these marrow-derived mesangial cells in various renal diseases.

Chemokine-mediated lymphocyte migration to the kidney

It is of note that detailed mechanisms of lymphocyte recruitment and activation via chemokine/chemokine receptor systems have shed light on better understanding of the pathogenesis of renal diseases [14, 15]. Chemokines expressed on the surface of endothelial cells interact with their cognate receptors on specific infiltrates including lymphocytes, a process that triggers activation of adhesion molecules and result in firm adhesion of lymphocytes to the surface of endothelial cells. Once lymphocytes migrate to the kidney, chemokines and proinflammatory cytokines produced

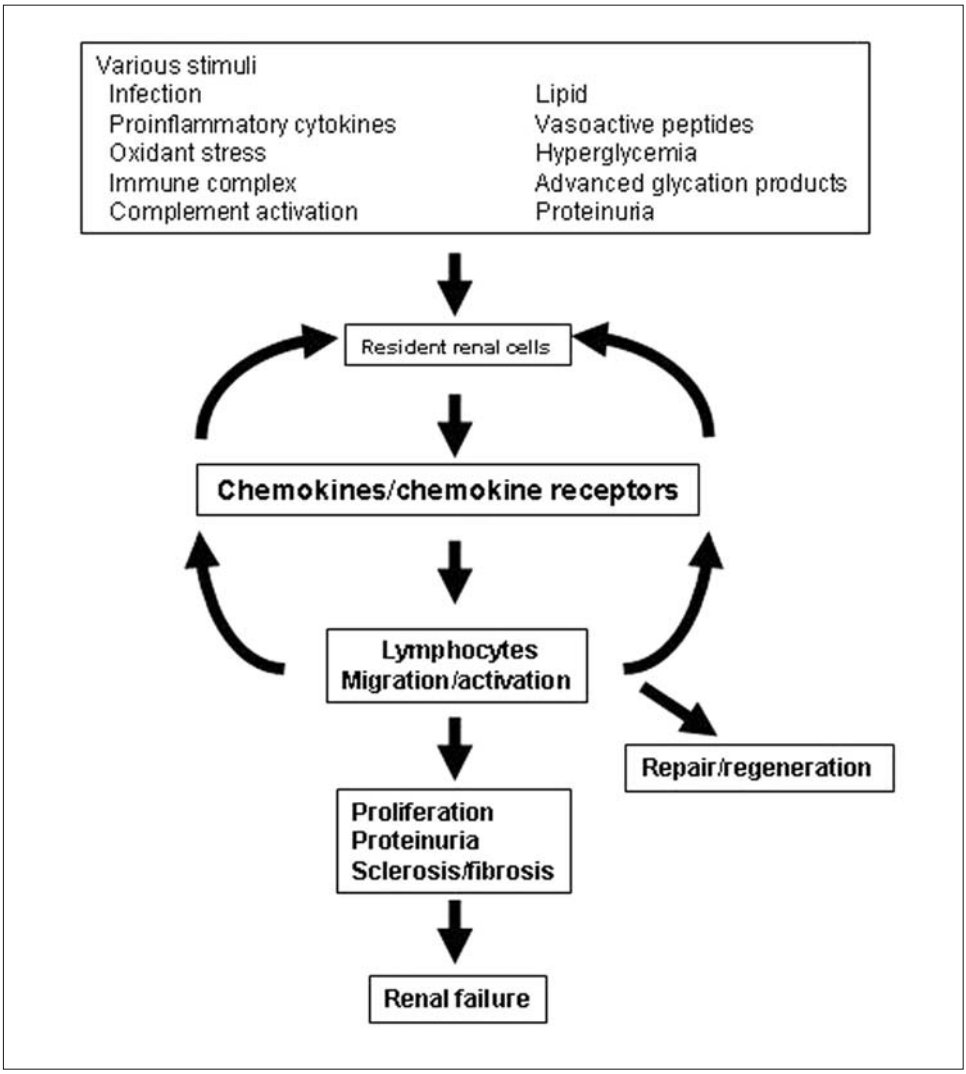

Figure 2
Interactions between activated lymphocytes and resident renal cells in the pathogenesis of renal diseases from acute phases to chronic phases

by both resident cells and infiltrated inflammatory cells exert a wide range of biological activities at the inflammatory sites. Selective expression of chemokine receptors and adhesion molecules on specific cell populations determines the specific types of infiltrating cells in inflamed kidneys.

Recent studies have documented a direct link between locally and systemically produced chemokines and the infiltration and activation of lymphocytes in the kidneys. For instance, the infiltration of Th1 T lymphocytes in the interstitium in human renal diseases is partly regulated by RANTES/CCL5, through its interaction with its cognate receptors CCR1 and CCR5 [16]. RANTES is up-regulated in the kidneys of a murine lupus nephritis model, MRL-*Fas*^{lpr} mice, prior to renal injury and increased with progressive injury [17]. Moore et al. [17] explored whether locally produced RANTES could cause renal injury with T lymphocyte infiltration, and showed that tubular epithelial cells, genetically modified to secrete RANTES, infused under the renal capsule were capable of inciting interstitial nephritis in MRL-*Fas*^{lpr} mice. RANTES promoted the accumulation of a distinct subset of T cells (CD4$^+$ T lymphocytes), which is compatible with clinical findings. Supporting this, circulating components, including CD4$^+$ T lymphocytes, are required to incite renal injury in MRL-*Fas*^{lpr} mice via both cellular and humoral immune responses [11]. Further, Furuichi et al. [16] reported that the cellular infiltration, including that of Th1 T lymphocytes, in interstitium in human renal diseases can be attributed to the action of RANTES via its cognate receptors, CCR1 and CCR5. These findings suggest that locally expressed chemokines are capable of inciting lymphocyte migration to the kidney.

Chemokine cascade, lymphocyte migration to the kidney, and renal fibrosis

In renal inflammation, the types of leukocytes that migrate to the diseased kidneys depend on the types of insult, and include neutrophils in acute inflammation, and macrophages, lymphocytes and plasma cells in chronic inflammation, resulting in renal sclerosis/fibrosis. The presence of chemokine amplification has been reported in which CXC chemokines induce both CC chemokines and CXC chemokines in mesangial cells *in vitro* [18]. In this report, treatment of mesangial cells with low levels of CXC chemokines macrophage inflammatory protein-1α (MIP-1α)/CCL3, MIP-1β/CCL5 and interferon-inducible protein-10 (IP-10)/CXCL10 resulted in higher levels of these CXC chemokines, suggesting that the subsequent expression of CC chemokines followed by CXC chemokines may be responsible for switches from acute inflammation to chronic inflammation, including lymphocyte migration to the kidney.

Migration of mesenchymal stem cells to the kidney

A recent report suggests that mesenchymal stem cells, but not hematopoietic stem cells, are renotropic, helping to repair the kidney and improve renal function in a mouse model of acute renal failure [19]. Intravenously injected mesenchymal stem

cells migrated to the injured kidney, underwent differentiation, and promoted structural and functional repair. However, the detailed mechanisms involved in migration to the kidney are still ununknown. Stromal cell-derived factor-1 (SDF-1)/CXCL12 and its receptor, CXCR4, which are responsible for lymphocyte migration, proliferation and survival, may play a role in this mechanism. Although the relationship between $CD34^+$ cells and mesenchymal stem cells remains unclear, mobilization of $CD34^+$ cells into the circulation and their homing to the kidney in an acute renal failure model may have renoprotective effects, which are dependent of SDF-1 and CXCR4 [20]. Since it has been shown that mesenchymal stem cells can be encouraged to differentiate and contribute towards the formation of functional kidney structures [21], further studies are needed to clarify the migration of mesenchymal stem cells, with or without lymphocytes, to the kidney, where they could exert their renotropic property and renal regenerative potential.

Lymphocyte migration and renal diseases

Crescentic glomerulonephritis: Th1-type immune responses

Crescentic glomerulonephritis is usually associated with clinical features of anemia and morphological features of crescent formation in glomeruli as well as tubulointerstitial nephritis, which eventually lead to renal insufficiency. Macrophages and T lymphocytes preferentially migrate to both glomeruli and interstitium in crescentic glomerulonephritis. MIP-1α was mostly undetectable in urine samples collected from healthy control subjects and in patients with renal diseases lacking crescent formation [22]. However, urinary MIP-1α levels in patients with crescentic glomerulonephritis correlated well with the percentage of cellular crescents and the number of $CD68^+$ infiltrating cells, and $CCR1^+$ and $CCR5^+$ cells in the glomeruli [16]. Moreover, elevated urinary levels of MIP-1α and the number of $CCR5^+$ cells dramatically decreased during glucocorticoid therapy-induced convalescence. MIP-$1\alpha^+$ cells were mainly detected in crescentic lesions. $CCR1^+$ and $CCR5^+$ cells, preferentially expressed on Th1 T lymphocytes, were detected in diseased glomeruli and interstitium [16]. It is therefore likely that MIP-1α plays a significant role in crescentic glomerulonephritis, by recruiting and activating macrophages and T lymphocytes. It has been recently shown that the p38 mitogen-activated protein kinase (MAPK)-chemokine cascade affects lymphocyte migration to the kidney and contributes to progressive crescentic glomerulonephritis in humans [23]. Therefore, this cascade may be an appealing therapeutic target for combating crescentic glomerulonephritis via the administration of p38 MAPK inhibitor(s) [24, 25].

The beneficial effects of neutralizing antibodies and chemokine/chemokine receptor antagonists in crescentic glomerulonephritis have been demonstrated. Anti-

MCP-1 or anti-MIP-1α antibodies or MCP-1 deficiency resulted in less glomerular accumulation of macrophages and lymphocytes (e.g., $CD4^+$ lymphocytes), reduced crescent formation, decreased interstitial damage, and, most importantly, less proteinuria [26, 27]. In contrast, aggravated renal dysfunction and increased proteinuria were detected in CCR1-disrupted mice, compared to wild-type mice [28]. The inhibitory effects of the antagonist, Met-RANTES on crescentic glomerulonephritis in CD1 mice was shown by Lloyd et al. [29]; although reduction in urinary protein excretion, cell infiltration of T lymphocytes and mononuclear cells was seen, crescentic formation was not affected. In addition, the beneficial effects of blocking the bioactivity of CXC chemokines have been reported in crescentic glomerulonephritis. Neutralizing antibodies against cytokine-induced neutrophil chemoattractant (CINC) ameliorated the cell infiltration, including neutrophils, and reduced urinary protein excretion [30].

Lupus nephritis

The recruitment and activation of macrophages and T lymphocytes were augmented by locally produced cytokines and/or chemokines both in human lupus nephritis [31] and in mice, including New Zealand Black × New Zealand White (NZB/W) F1 mice [32] and MRL-*Fas*^{lpr} mice [11]. These findings were, at least in part, confirmed by evidence that MRL-*Fas*^{lpr} kidney is not defective, but rather a circulating stimulant in the MRL-*Fas*^{lpr} mouse can induce cytokines/chemokines and renal injury in a normal MRL-++ kidney *via* an experimental renal transplantation system [33, 34]. Further, modulation of the biological activities of MCP-1 dramatically reduced the recruitment of macrophages and T lymphocytes that not only reduced pathological alterations in the kidney, lung, skin, and lymph node, but also diminished proteinuria, and prolonged survival [35]. A very recent study demonstrated that MCP-1 activates a regional Th1 immune response in nephritis of MRL-*Fas*^{lpr} mice [36]. Mononuclear cell infiltration has been demonstrated in the kidneys of MRL-*Fas*^{lpr} mice by weeks 10–12. At week 12, the expression of certain chemokines and chemokine receptors, including CCR1, CCR2, and CCR5 was up-regulated in the mouse kidneys, associated with morphological features of renal injuries and proteinuria. These results are in accord with the notion that chemokine-mediated leukocyte infiltration precedes proteinuria and renal damage in MRL-*Fas*^{lpr} mice [37].

Recently, it was shown that aberrant high expression of B lymphocyte chemokine (BLC/CXCL13) by myeloid dendritic cells in the target organs in aged BWF1 mice may play a pivotal role in breaking immune tolerance in the thymus and in recruiting autoantibody-producing B lymphocytes in the development of murine lupus [38]. From the perspective of Th1/Th2 balance, $CCR4^+$ but not $CCR5^+$ T lymphocytes in peripheral blood, which represent Th2 cells, preferentially migrate into the kidneys of patients with lupus nephritis. It is likely that the disproportionate dis-

tribution of CCR4+ T lymphocytes might play an important role in the development of subsequent renal injuries that are found in patients with lupus nephritis [39]. In addition, the beneficial effects of modulating cytokines/chemokines in lupus nephritis have been noted; it was recently shown that a novel potent fractalkine/CX3CL1 antagonist demonstrated an ability to delay the initiation and ameliorate the progression of lupus nephritis [40].

Diabetic nephropathy

Inflammatory events may be central to the pathogenesis of diabetic nephropathy. Infiltration of inflammatory cells, including macrophages and T lymphocytes, into diseased kidneys is an important histological feature that is associated with the progression of diabetic nephropathy [41, 42]. Angiotensin II-dependent up-regulation of MCP-1 has been demonstrated to play a role in the pathogenesis of glomerular and tubulointerstitial damage [43]. Therefore, activation of the renin-angiotensin system is an important determinant of the macrophage population in diabetic nephropathy, possibly by regulating certain chemokines. It is well accepted that, in addition to their blood-pressure-lowering effects, angiotensin II receptor antagonists are renoprotective in patients with type 2 diabetes mellitus with microalbuminuria [44, 45]. In addition, combination treatment with an angiotensin II receptor antagonist and an angiotensin converting enzyme inhibitor was found to be more effective in retarding the progression of non-diabetic renal diseases, in comparison with monotherapy [46]. These beneficial effects may be, at least in part, dependent on the inhibition of chemokines [43]. Interestingly, a renoprotective effect of mycophenolate mofetil has been reported, which could have derived from its well-known anti-inflammatory properties that include restriction of lymphocyte and macrophage proliferation and modulation of the expression of adhesion molecules [47]. More recently, Yozai et al. [48] reported the protective effects of methotrexate on diabetic nephropathy, which are suggested to be mediated by its anti-inflammatory actions through inhibition of NF-κB activation and consequent reduction of intercellular adhesion molecule-1 expression and immune competent infiltration. Taken together, these findings confirm that inflammatory responses are essentially involved in diabetic nephropathy and that anti-inflammatory agents might be useful and beneficial for the treatment of this disease.

Transplant nephropathy

Early nonspecific ischemic injury with leukocyte migration to the kidney has been related to subsequent immunological injuries in renal transplant rejection [49]. Rat models of transplantation have facilitated a functional study of the role of

cytokines/chemokines with leukocyte migration to the transplanted kidneys in acute and chronic renal rejection [50, 51]. In a rat model of acute renal transplant rejection, the expression of RANTES was up-regulated, at the mRNA level, by as early as 6 h, and this up-regulation was again noted at days 3–6 [52]. Increased expression of RANTES in the early hours of engraftment may be related to ischemic injury, and could, in part, induce subsequent immunological responses. In addition, lymphocytes, macrophages and their secreted products play important roles in the eventual immune-mediated rejection process. Moreover, increased production of certain cytokines (IL-8/CXCL8, IL-10, IL-15), chemokines (RANTES, MCP-1) and hepatocyte growth factor (HGF) by infiltrating immune competent cells, tubular epithelial cells, and endothelium may have a determinant role in renal transplant rejection [52, 53]. In addition, we recently reported the amelioration of acute rejection with reduced leukocyte migration and prolonged survival by combined therapy of FR167653, a specific inhibitor of p38 MAPK, and cyclosporin [54]. Especially, for chronic allograft nephropathy (CAN) should be possible to achieve an improvement in the long-term outcome of renal allografts in the clinical setting of renal transplantation. Met-RANTES not only reduced vascular and tubular damage in acute renal transplant rejection [50], but also protected renal allografts from long-term deterioration [55]. The expression of MCP-1 in acute renal transplant rejection correlated with the number of infiltrating macrophages [56], and elevated urinary MCP-1 excretion during rejection episodes, which diminished after successful treatment. In addition, FTY720 may be promising for the treatment of transplant rejection, since FTY720 prevents renal T lymphocyte infiltration after ischemia-reperfusion injury [57]. A recent study has shown for the first time a potential role of B lymphocyte chemoattractant (BLC)/B cell-attracting chemokine 1(BCA-1)/CXCL13 and its specific receptor CXCR5 in recruitment of B lymphocytes in renal allograft rejection [58]. In this report, a striking colocalization of CXCL13 expression with $CXCR5^+$ and $CD20^+$ B lymphocytes in renal transplants undergoing rejection was described. Further, Kerjaschki et al. [59] demonstrated that numerous chemokine receptor $CCR7^+$ cells within the nodular infiltrates seemed to be attracted by secondary lymphoid tissue chemokine (SLC)/CCL21, which is produced and released by lymphatic endothelial cells in a complex with podoplanin. They speculated that lymphatic neoangiogenesis not only contributes to the export of the rejection infiltrate, but is also involved in the maintenance of a potentially detrimental alloreactive immune response in renal transplants, and thus provides a novel therapeutic target.

Expansion of chemokine universe beyond lymphocyte migration to the kidney

Recent studies have demonstrated that chemokines and their receptors expand their universe beyond lymphocyte migration to the kidney in health and renal diseases.

For instances, IP-10, which was originally reported to be a chemoattractant to Th1 T lymphocytes, is a mitogenic factor for mesangial cells and possibly acts *via* the cognate receptor, CXCR3 [60]. In human mesangioproliferative glomerulonephritis, IP-10 may serve as a growth factor to promote and escalate mesangial proliferation in addition to its effect in the recruitment and activation of Th1 T lymphocytes. The constitutive glomerular expression of CCR7 and its ligand SLC by adjacent renal cells suggests the involvement of this specific chemokine/chemokine receptor interaction in regulating glomerular homeostasis and regeneration [61]. In addition, the regulation of IL-8 and MCP-1 is closely related to the urinary excretion of protein in both experimental models [26, 62] and human nephrotic syndrome; the glomerular protein leakage is possibly due to increased permeability of the glomerular capillaries. A recent report described the expression of CCR4, CCR8, CCR9, CCR10, CXCR1, CXCR3, CXCR4, and CXCR5 in cultured podocytes; the expression of CXCR1, CXCR3, and CXCR5 was also detected in podocytes of human kidney sections [63]. It is likely that the release of oxygen radicals that accompanies the activation of CCRs and CXCRs may contribute to podocyte injury and the development of proteinuria [63]. Importantly, MCP-1/CCR2 signaling has been reported to be involved in renal fibrosis with the infiltration of macrophages and T lymphocytes, which is supposed to be a strong determinant for renal fibrosis [64, 65]. These results suggest that particular chemokines participate in renal homeostasis, and in progressive renal diseases, resulting in renal fibrosis in addition to lymphocyte migration.

Concluding remarks

The current concept of lymphocyte migration to the kidney that has resulted in a better conceptual understanding of progressive renal diseases has been briefly summarized. Our understanding of the molecules involved in the pathogenesis of various renal diseases may provide new therapeutic choices, and lead to the discovery of gene-based therapeutic options. It is likely that selective intervention of chemokines, at the appropriate phase of a particular disease, may have the therapeutic potential for site- and phase-specific intervention of lymphocyte migration and the progression of renal diseases.

Acknowledgment
T.W. is a recipient of a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture in Japan.

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Leukocyte migration to pancreatic islets: a critical step in the pathogenesis of type 1 diabetes

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Introduction

Type 1 diabetes (T1D) is an autoimmune disease resulting from selective destruction of insulin-producing beta cells in the pancreas by immune cells, including macrophages, dendritic cells (DCs), T and B cells, which infiltrate the islets and mediate a local inflammatory reaction. [1, 2]. A well-characterized model to study T1D is represented by the non-obese diabetic (NOD) mouse, which spontaneously develops an autoimmune insulin-dependent diabetes mellitus form closely resembling human T1D [3–5].

Although the pathogenesis of T1D is still incompletely understood, a central role for Th1 cells is well established [6]. As Th1 cells are not constitutive components of normal islets, their recruitment to the pancreas represents an important prerequisite for the occurrence of islet inflammatory immunopathology. In the NOD model, several studies have demonstrated that the initial events leading to T cell priming occur in the pancreatic lymph nodes, where antigen is presented by DCs to autoreactive T cells [7, 8]. Thus, a critical point in the pathogenesis of T1D is the migration of pathogenic Th1 cells from pancreatic lymph nodes to the pancreas, where they can meet and contribute to destroy islet β cells [8, 9]. After the inciting priming event, newly activated effector T cells modulate their homing pattern and gain access to the pancreatic islets where their cognate antigen is expressed.

Chemokines, chemoattractant cytokines involved in leukocyte recruitment and activation are key mediators of cell trafficking, and have been implicated in the development of NOD and human T1D [10]. In this review, we examine how islet chemokine production, leading to recruitment of pathogenic cells, could play an instrumental role in the early events of T1D pathogenesis. In addition, the capacity of vitamin D receptor agonists to inhibit chemokine production by islet cells, as a mechanism preventing Th1 cell migration to the pancreas and T1D development, is discussed.

Lymphocyte Trafficking in Health and Disease, edited by Raffaele Badolato and Silvano Sozzani

Chemokine production by pancreatic islet cells

Overt T1D is preceded by leukocyte infiltration into the pancreatic islets, implying a key role for chemokines produced by islet cells in disease pathogenesis. Chemokine genes are present within the diabetes susceptibility locus *Idd4* in the NOD mouse [11], one of the about 20 loci associated with T1D development. In addition, a genetic association involving a polymorphism in CCR5 and CCR2 chemokine receptors has been described in T1D patients [12]. Interestingly, different chemokines have been found to be produced by pancreatic β cells, including CCL2 [13, 14], CCL5, CXCL9, and CXCL10 [14], suggesting a direct role of β cells in leukocyte recruitment into the pancreatic islets. Chemokines have been implicated as recruiters of pathogenic [15–17] and regulatory [18] T cells to the pancreatic islets, highlighting their important role in T1D pathogenesis.

Using real-time RT-PCR, we have shown basal ex vivo expression of several chemokine transcripts in freshly isolated NOD.SCID islets [19]. Islets were obtained from immunodeficient NOD.SCID mice to exclude the contribution of infiltrating cells, detectable in the pancreas of NOD mice from about 4 weeks of age. Transcripts encoding CXCL10 were prominently expressed, followed by CCL22, CCL21, CCL3, CCL17, and CCL22. Copious amounts of CXCL10 were secreted by islet cells in the supernatant during a 72-h culture without overt stimulation (1034 pg/ml), as well as appreciable levels of CCL2 (451 pg/ml) and CCL5 (382 pg/ml), and low but detectable levels of CCL3 (5 pg/ml). Levels of CCL17 and CCL21 were below the ELISA detection limits [19]. A comparison of chemokine production by islet cultures from NOD.SCID, NOR, and BALB/c mice revealed that NOR and BALB/c islets secreted, compared to NOD.SCID islets, about 50% lower levels of CXCL10, CCL2, and CCL5.

These results demonstrate that pancreatic islets constitutively produce chemokines, and notably relatively high levels of CXCL10, CCL2, and CCL5. Islets from the diabetes-resistant NOR mouse, characterized by negligible insulitis [20], as well as from the BALB/c mouse, secrete lower levels of these chemokines compared to NOD.SCID islets, suggesting that constitutive secretion of proinflammatory chemokines by islet cells may represent a contributing factor in the development of T1D.

Pathogenic Th1 cells express on their surface predominantly two chemokine receptors: CXCR3 and CCR5 [21]. Their role in T1D is highlighted by the observation that in CXCR3-deficient mice the onset of disease is delayed [14]. In addition, the critical role of CCR5 and CXCR3 in leukocyte trafficking to islets has been shown by targeting these receptors and inhibiting islet allograft rejection [22].

Our work has established that constitutive and inducible production by mouse islet cells of CXCL10, a ligand for CXCR3 expressed by Th1 cells, is most prominent [19]. CXCL10 has previously been shown to be produced by the NOD β cell line NIT-1 stimulated with inflammatory cytokines [14]. The highest constitutive levels of this chemokine have been observed in the NOD background, compared

with diabetes-resistant strains [19], further supporting its important role in the pathogenesis of T1D. CXCL10 is also produced by human islet cells [19], and it seems to be implicated in human T1D, as elevated serum levels have been observed in diabetes patients and in autoantibody-positive subjects at risk of developing the disease [23, 24]. The role of CXCL10 as a conductor of lymphocyte trafficking to pancreatic islets has been recently supported by data indicating that islet-specific expression of CXCL10 accelerates the autoimmune process by enhancing the migration of antigen-specific lymphocytes to their target site [25].

Islets also produce CCL2 and CCL3 [19], chemokines able to recruit $CCR1^+$ and $CCR2^+$ macrophages [26] and immature DCs [27, 28]. These findings are consistent with studies showing that macrophages and DCs predominantly populate the pancreatic islets during the early stage of insulitis [29]. Intriguingly, both macrophages and $CD11c^+$ DCs are present in pancreatic islets of NOD mice at 4 weeks of age, a time point when T and B cells are just beginning to enter the pancreas [30, 31]. CCL2 has been shown to be produced also by human islet cells, and it appears to play an important role in the monocyte recruitment to transplanted islets, as low CCL2 secretion is associated with long-lasting insulin independence and good clinical outcome of islets transplanted into T1D patients [32].

Thus, pancreatic β cells, as well as other islet cell types, produce chemokines potentially able to attract all the pathogenic cells involved in β cell death.

Toll-like receptor ligands enhance islet chemokine production

T1D is a multifactorial disease, with a combination of genetic and environmental factors contributing to β cell destruction [33]. The early events in the pathogenesis of T1D are still poorly understood, but infectious agents, in particular viruses, have been implicated in disease provocation [34]. The host defense against microbial pathogens is triggered by the recognition of conserved motifs in infectious microorganisms mediated by Toll-like receptors (TLRs), surface molecules able to recognize distinct structural components of pathogens [35]. Activation of signal transduction pathways by TLRs leads to up-regulation of different genes that operate in host defense, including costimulatory molecules, cytokines and chemokines [36].

We have demonstrated, using real-time RT-PCR, the expression of mRNA transcripts encoding TLR1 through TLR9 in islet cells from NOD.SCID, NOR, and BALB/c mice [19]. All TLR transcripts examined were found expressed, with a relatively higher abundance of TLR2. No major differences in islet TLR expression among the different mouse strains tested were discernible. In addition, as shown in Figure 1A, all TLR transcripts were expressed, although in some cases at a relatively lower level, by NIT-1 cells, a pancreatic β cell line established from NOD mice [37]. The expression of TLR transcripts by NIT-1 cells suggests their expression also by wild-type pancreatic β cells. TLRs are also expressed on the cell surface, as indi-

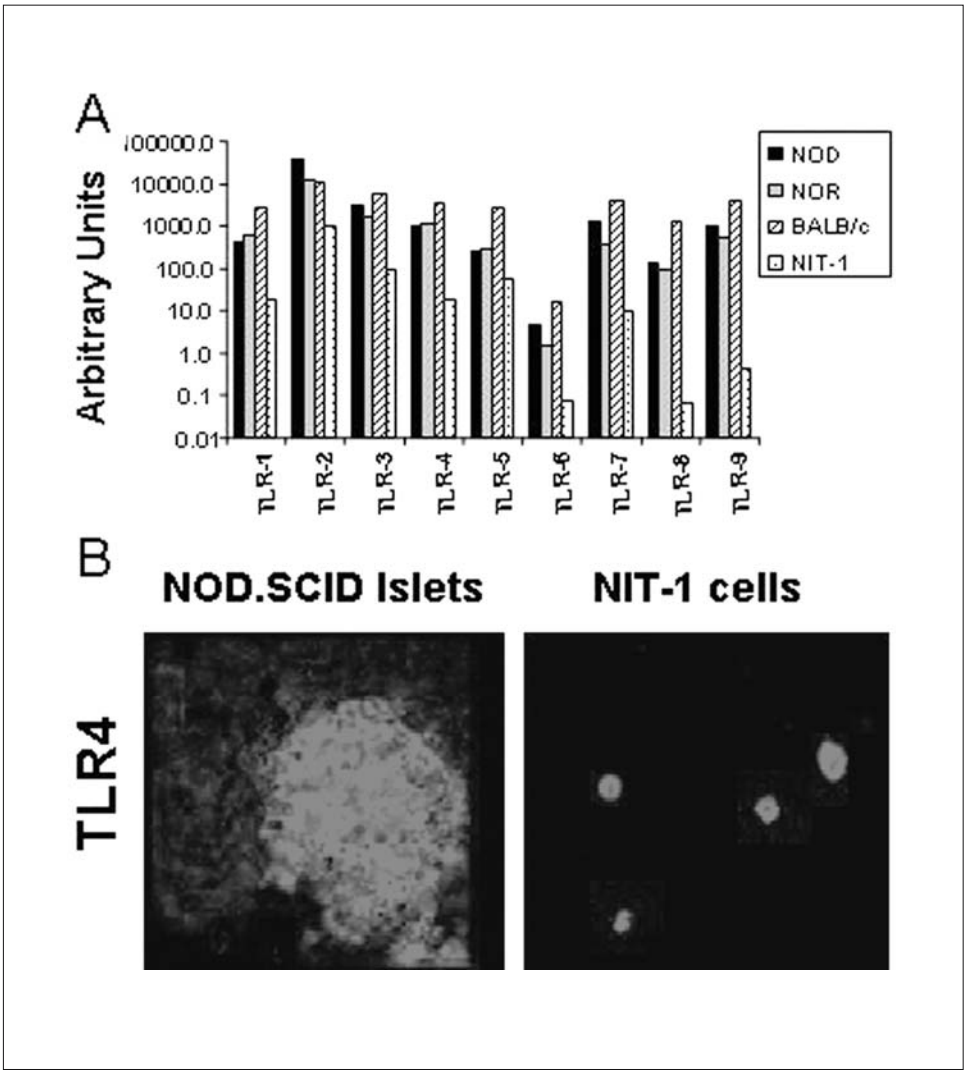

Figure 1
TLR expression in pancreatic islets and NIT-1 cells
(A) Quantification of TLR mRNA expression by real-time RT-PCR in freshly isolated NOD.SCID (filled bars), NOR (gray bars), BALB/c (stippled bars) pancreatic islets, and NIT-1 cells (open bars). The levels of mRNA are shown as arbitrary units normalized to GAPDH expression. Data are from one representative experiment out of three performed. (B) Staining for TLR4 expression by NOD.SCID islet cells and NIT-1 cells. No staining was revealed in slides incubated with isotype-matched primary antibody controls (not shown). Magnification, × 400.

cated by the strong staining of NOD.SCID islet cells and of NIT-1 cells with anti-TLR4 mAb (Fig. 1B). All TLR transcripts are also expressed by human islet cells [19]. Confocal microscopic analysis demonstrates that TLR3 and TLR4 are broadly expressed by NOD.SCID and human pancreatic islet cells, including insulin-producing β cells, as shown by co-localization of insulin and TLR expression [19]. Thus, TLRs are expressed, in addition to β cells, by other cell types in the pancreatic islets.

To determine if the TLRs expressed by islet cells are functional, we analyzed chemokine production by NOD.SCID islets in response to TLR ligands. A marked up-regulation of CXCL10, CCL5, CCL2, and CCL3 was found following islet stimulation with specific TLR ligands, such as peptidoglycan for TLR2, poly I:C for TLR3, LPS for TLR4, flagellin for TLR5, R848 for TLR7, and CpG for TLR9 [19]. Although a particularly marked enhancement of chemokine production was induced by poly I:C, all the different stimuli tested induced a significantly higher chemokine production, compared to basal levels. These results demonstrate that TLRs expressed by islet cells are functional, and suggest that different microorganisms can enhance chemokine production by islet cells, thereby potentially contributing to T1D pathogenesis. The chemokines produced, either constitutively or following TLR ligation, by islet cells are, among those tested, of proinflammatory type: CXCL10, CCL2, CCL5, and CCL3. No production of the homeostatic chemokines CCL17 and CCL19 could be detected.

The highest levels of chemokine production, among all the different TLR ligands tested, was induced by the viral RNA mimic poly I:C, a ligand for TLR3. TLR3 is a pattern recognition receptor that recognizes extracellular dsRNA released from virus-infected cells [38]. A viral component triggering the development of T1D has long been suspected, but persuasively implicated only in the case of B4 coxsackievirus, cytomegalovirus, and rubella, although at least 14 different viruses have been reported to be associated with T1D in humans and animal models [39]. Controversial results have been reported on T1D modulation by poly I:C [40, 41], but a viral component triggering the development of T1D is plausible, although viruses can also protect from T1D, at least in animal models [42, 43].

Different mechanisms have been proposed to account for virus-induced autoimmune diabetes, including molecular mimicry [44], bystander activation of autoreactive T cells [45], and direct cytopathic effects in virus-infected target cells [46]. In addition to these possible mechanisms, we would propose that the association between virus infection and T1D reflects the triggering by viral products of signal transduction *via* TLRs expressed by islet cells, in particular TLR3 and TLR9, leading to production of proinflammatory chemokines by islet cells that contribute to create the conditions for an autoimmune attack (Fig. 2). This pathogenetic mechanism, in concert with genetic susceptibility and induction of adaptive immune responses, may precipitate autoimmune diseases, as shown in the induction of autoimmune myocarditis by a combination of TLR stimulation and CD40-mediated triggering of self peptide-loaded DCs [47]. Consistent with these findings, T1D

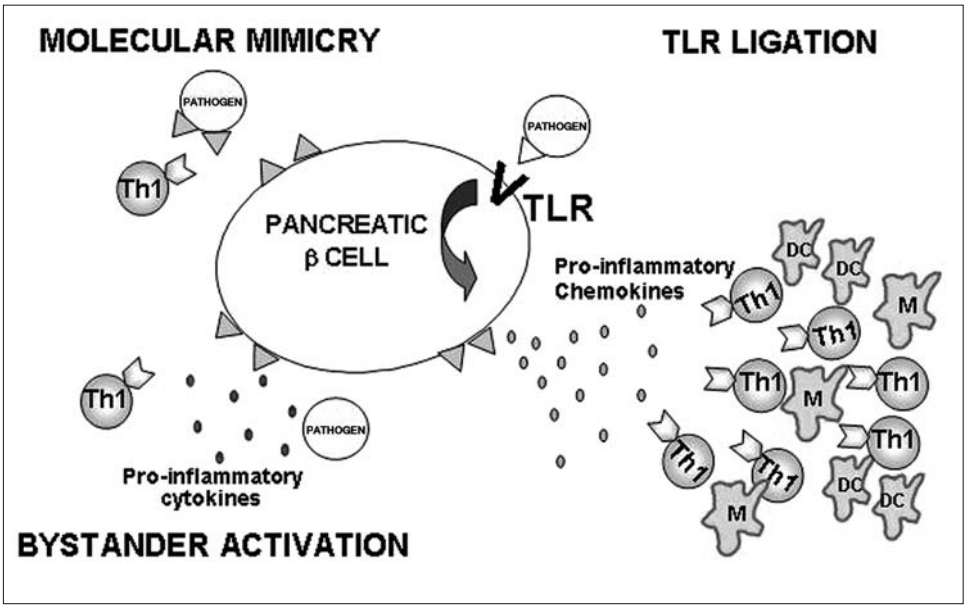

Figure 2
Pathogens provoke T1D onset: mechanistic explanations
A microbial component triggering the development of T1D has long been suspected, but the interpretation of this association has been framed only within the context of epitope mimicry or bystander activation. Islet cells constitutively produce pro-inflammatory chemokines, and up-regulation of chemokine secretion by islets, leading to leukocyte infiltration into the pancreas and to T1D development, can be also provoked by triggering TLRs expressed on the islet cell surface.

can be precipitated by a combined treatment with poly I:C and insulin B9-23 peptide [48], possibly *via* poly I:C-induced triggering of TLR3 [49]. Pancreatic β cells are known to regulate responses to viral infections, as shown by their IFN-dependent capacity to reduce permissiveness of infection and subsequent NK cell-dependent β cell death, preventing diabetes development [50]. Intriguingly, infection with lymphocytic choriomeningitis virus (LCMV) in NOD or in LCMV-RIP transgenic mice can abrogate T1D development, and this is associated with LCMV-induced expression of CXCL10 in draining pancreatic lymph node cells, creating a chemokine gradient correlated with the rapid egress of pancreas-infiltrating lymphocytes [51].

Thus, our results indicate that innate immune responses to viruses and other pathogens may contribute, *via* TLR triggering, to the pathogenesis of T1D by stimulating cells of the target organ to recruit pathogenic leukocytes. This pathogenetic

mechanism may not be restricted to islet cells, but TLR activation in target tissues could represent a situation common to many organ-specific autoimmune diseases [35], and also to systemic ones, as suggested for TLR9 in the pathogenesis of systemic lupus erythematosus [52, 53].

Modulation of islet chemokine production by vitamin D receptor agonists can affect effector and regulatory T cell recruitment in T1D pathogenesis

$1,25(OH)_2D_3$, the activated form of vitamin D, is a secosteroid hormone that has, in addition to its central function in calcium and bone metabolism, important effects on the growth and differentiation of many cell types, and pronounced immunoregulatory properties [54–56]. The biological effects of $1,25(OH)_2D_3$ are mediated by the vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors, functioning as an agonist-activated transcription factor that binds to specific DNA sequence elements in vitamin D-responsive genes, and ultimately influences the rate of RNA polymerase II-mediated gene transcription [57].

The vitamin D endocrine system is involved in a variety of biological processes able to modulate immune responses [58], and. the tolerogenic properties of VDR agonists render this class of compounds particularly interesting for the treatment of T1D [59]. $1,25(OH)_2D_3$ itself reduces the incidence of insulitis [60] and prevents T1D development [61], but only when administered to NOD mice starting from 3 weeks of age, before the onset of insulitis. $1,25(OH)_2D_3$ was found ineffective in preventing progression of diabetes in NOD mice when given from 8 weeks of age, when NOD mice present a well-established insulitis [62]. In contrast, we have identified a $1,25(OH)_2D_3$ analog 1,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor vitamin D_3 (BXL-219) that is able, as a monotherapy, to treat the ongoing type 1 diabetes in the adult NOD mouse, effectively blocking the disease course [63]. This property is likely due, at least in part, to the increased metabolic stability of this analog against the inactivating C-24 and C-26 hydroxylations, and the C-3 epimerization [64], resulting in a 100-fold more potent immunosuppressive activity compared to $1,25(OH)_2D_3$ [59]. Our results clearly show that a relatively short treatment with non-hypercalcemic doses of BXL-219 inhibits the ongoing pancreatic infiltration of Th1 cells in adult NOD mice, arresting the immunological progression of T1D and preventing its clinical onset [63].

In both islet transplantation and type 1 diabetes models, treatment with VDR agonists has a profound effect on the migration of effector T cells, preventing their entry into the pancreatic islets [63, 65]. Thus, VDR agonist-induced down-regulation of chemokine production by islet cells could represent an important mechanism of action leading to inhibition of T1D development. We have found that transcripts encoding all TLRs are expressed by mouse and human islet cells and they are functional, as demonstrated by the marked up-regulation of chemokine production fol-

lowing TLR engagement by specific agonists [19], suggesting that TLR-mediated up-regulation of proinflammatory chemokine production like CXCL10, CCL2 and CCL5 by islet cells plays an important role in the early events leading to leukocyte infiltration into the pancreatic islets.

The VDR agonist BXL-219 significantly down-regulates *in vitro* and *in vivo* proinflammatory chemokine production by islet cells, inhibiting T cell recruitment into the pancreatic islets and T1D development [19]. The inhibition of CXCL10 may be particularly relevant, consistent with the decreased recruitment of Th1 cells into sites of inflammation by treatment with an anti-CXCR3 antibody [66], and with the substantial delay of T1D development observed in CXCR3-deficient mice [14]. The inhibition of islet chemokine production by BXL-219 treatment *in vivo* persists after restimulation with TLR agonists and is associated with up-regulation of IκBα transcription, an inhibitor of nuclear factor κB (NF-κB), and with arrest of NF-κBp65 nuclear translocation [19], highlighting a novel mechanism of action exerted by VDR agonists potentially relevant for the treatment of T1D and other autoimmune diseases. These observations expand the known mechanisms of action exerted by vitamin D analogs in the treatment of T1D and other autoimmune diseases, namely arrest of DC maturation, inhibition of Th1 cell responsiveness, and enhancement of regulatory T cells [67].

Conclusions

Islet cells, including insulin-producing β cells, express all the TLRs and their triggering markedly increases the secretion of proinflammatory chemokines. Thus, initial events in T1D development leading to leukocyte infiltration into the pancreatic islets may be provoked by triggering of TLRs expressed by islet cells, which can respond to microbial signals by up-regulating the secretion of chemokines able to attract Th1 cells, macrophages, and DCs. Since these cell types are involved in the pathogenesis of T1D, the TLR-mediated up-regulation of proinflammatory chemokine production by islet cells appears to be an important element in the early steps of T1D development. TLR3 and TLR9 appear to be particularly active in leading to production of proinflammatory chemokines by islet cells that can contribute to create the conditions for an autoimmune attack. Our work has also shown that vitamin D analogs can significantly down-regulate *in vitro* and *in vivo* proinflammatory chemokine production by islet cells, inhibiting T cell recruitment into the pancreatic islets and T1D development. The inhibition of islet chemokine production *in vivo* persists after restimulation with TLR ligands and is associated with up-regulation of IκBα transcription, an inhibitor of NF-κB, and with arrest of NF-κBp65 nuclear translocation, highlighting a novel mechanism of action exerted by VDR ligands potentially relevant for the treatment of T1D and other autoimmune diseases.

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Controlling leukocyte trafficking in disease

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Introduction

In the four-step paradigm of leukocyte homing (rolling, arrest, spreading, and diapedesis), there are number of molecular events that could serve as points for intervention: selectin interactions, integrin binding, and the chemotactic factors directing diapedesis and migration. By far the best studied set of chemotactic factors are the chemokine ligands and their receptors. The chemokine receptors belong to one of the most pharmacologically exploited protein families, the G protein-coupled receptors (GPCRs), and are therefore the focus of most of the efforts in therapeutically controlling leukocyte trafficking in disease [1].

Chemokine receptor expression on hematopoietic cells displays a great deal of redundancy and inflammatory lesions often express a large set of chemokines which will then attract multiple cell types. Regardless, strategies at targeting single chemokine receptors have shown great success in animal models of disease [2–4]. To date this success has not been recapitulated in human clinical trials testing these agents. Most of these trials have been based on the concept that antagonism of a single receptor can generate a broad enough effect to elicit efficacy in disease. While it is certainly too early to close the door on this targeted receptor approach, it is worth exploring the potential for using more promiscuous targeting to control leukocyte trafficking. In addition to the evaluation of promiscuous targeting, there has been a heightened interest in harnessing the homeostatic trafficking of leukocytes for therapeutic benefit. There are a number of recent reviews that provide a detailed summary of the current activities in developing chemokine receptor small molecule antagonists, and the reader is referred there for additional information on specific receptor programs [4–8].

Inflammatory trafficking

The inflammatory chemokines are characterized by high expression within inflammatory lesions that often results in the attraction of multiple cell types. These have

Lymphocyte Trafficking in Health and Disease, edited by Raffaele Badolato and Silvano Sozzani

been the primary targets for drug development, as reduction of cellular infiltration can lead to reduction of disease in chronic autoimmune disorders, chronic airway disease, atherosclerosis and graft rejection [3, 4, 6, 7, 9].

T cells

Activated T cells shift from a homeostatic trafficking phenotype by down-regulating expression of lymphoid trafficking receptor CCR7 and up-regulating expression of the inflammatory receptors [10]. Within this receptor class, CCR5 and CXCR3 are both expressed on effector T cells of the Th1 lineage, while Th2 cells express CCR3, CCR4 and CCR8. All of these receptors, except CCR8, have extensive small molecule drug development programs evaluating antagonists.

Early efforts to identify CCR5 antagonists stemmed from the recognition that CCR5, along with CXCR4, is one of the primary coreceptors for HIV infection. There exists a human CCR5 knockout-equivalent that is protected from HIV infection, where a 32-base pair deletion of this gene results in an inactive receptor (CCR5Δ32) [11–13]. This genotype also supports a role for CCR5 in leukocyte trafficking, as renal transplant recipients that are homozygous for this allele show a distinct survival advantage over the heterozygotes or wild-type recipients [14]. As activated monocytes up-regulate expression of CCR5, antagonism of this receptor would be expected to also show effects on monocyte adhesion and migration.

Pfizer, Schering Plough, Ono Pharmaceuticals, Takeda, Merck and AnorMED are among the companies with ongoing programs developing non-peptide antagonists for CCR5. While most companies are evaluating HIV infection as the primary indication for the CCR5 antagonists, Merck has presented preclinical data that a CCR5 antagonist can prolong graft survival in *cynomolgus* monkey heart allografts. Antagonism of CCR5 with one of their development compounds inhibited the influx of $CCR5^+$, $CD68^+$, $CXCR3^+$ and $CD3^+$ leukocytes to the allografts (228th ACS meeting in Philadelphia, PA, 2004). As the numerous CCR5-based HIV clinical trials advance, more of these compounds should find their way into testing for therapies related to leukocyte trafficking.

The other primary HIV coreceptor, CXCR4 is also the target of a number of clinical trials. While CXCR4 antagonists are being evaluated for stem cell mobilization and anticancer activities, there is less activity in evaluating CXCR4 antagonism for blocking lymphocyte homing, as the receptor and its ligand CXCL12 are broadly expressed both in the hematopoietic system and in other tissues [15].

A number of development programs exist that target CXCR3 in Th1-mediated diseases (psoriasis) and CCR3 in Th2-mediated diseases (allergic rhinitis and asthma). A CXCR3 antagonist (T-487) developed by Tularik/Amgen demonstrates nanomolar potency in binding, and is able to inhibit lymphocyte migration to CXCL9, CXCL10 and CXCL11. However, as reported at the Chemotactic

Cytokine Gordon Conference in 2004, the compound did not meet efficacy endpoints when tested in Phase II clinical trials as a therapy for psoriasis.

Monocytes

Monocyte homing can be driven through activation of multiple receptors: CCR1, CCR2, CCR5 and CX3CR1. CCR1 is expressed on many cell types, including neutrophils, T cells and NK cells, but much of the efficacy in these models has been attributed to its expression on monocytes that are differentiating to the macrophage lineage [16]. The most potent CCR1 antagonist reported is BX471, under development by Berlex Biosciences. This compound is able to displace the binding of multiple CCR1 ligands (CCL3, CCL5 and CCL7) with high affinity, and can inhibit the functional activation of CCR1 as demonstrated in calcium-release assays, increase in extracellular acidification rate, inhibition of CD11b up-regulation and monocyte adhesion and migration [17, 18]. Insight into the physiological and pathophysiological roles of CCR1 has been gained through the use of this CCR1 antagonists in animal models of multiple sclerosis and organ transplant rejection [17–19]. For example, in a rat experimental autoimmune encephalitis (EAE) model of multiple sclerosis, BX471 decreased the clinical score in a dose-dependent manner [17]. BX471 was evaluated in a phase II trial for multiple sclerosis, but did not meet efficacy endpoints (ECTRIMS 2005). In addition to BX471, there are other CCR1 antagonists under development, including clinical compounds by Millenium and Pfizer [5].

The CCR2 receptor and its ligands CCL2, CCL7, CCL8, CCL13 have been shown to be involved in the trafficking of monocytes. Subsequent analyses with knockout mice have shown a role for CCR2 and CCL2 in the pathogenesis of animal models of atherogenesis and multiple sclerosis [20–23]. Due to the strong correlation with numerous diseases, a number of companies are developing non-peptide antagonists. These programs are not as advanced as those for some of the other receptors discussed above, with Merck, ChemoCentryx, Johnson and Johnson, Pfizer, and Incyte all in the discovery phase [5].

Homeostatic trafficking

Efforts at targeting the chemokine receptors involved in homeostatic trafficking have lagged those of targeting the inflammatory receptors. Our understanding of the key chemokine players has resulted from detailed analysis of the microenvironment of the secondary lymphoid organs, as discussed elsewhere in this book. The homeostatic chemokines and their receptors may not show altered expression in the disease state, but antagonizing their trafficking activity can nonetheless have significant

impact on the state of the immune system in the organism. Because of the precise control of homeostatic homing, in comparison to the "cytokine (chemokine) storm" in diseased tissue, there might be the ability to more precisely affect this axis of leukocyte trafficking with specific receptor antagonists.

Dendritic cells and T cells – CCR7

Naïve T cells and activated, mature dendritic cells (DCs) use a common mechanism to reach the secondary lymph nodes. Here they come into contact, allowing the activation of the T cells by the antigen-loaded DCs. The CCR7 ligands expressed by high endothelial venules (HEVs), secondary lymphoid organ chemokine (CCL21) and EBV-induced molecule-1 ligand chemokine (CCL19), elicit entry of the leukocytes through activation of the CCR7 receptor. Much of this knowledge was elucidated through the use of a mouse strain that contains an autosomal recessive paucity of lymph node T cell (*plt*) mutation [24]. These mice lack the secondary lymphoid HEV expression of CCL19 and CCL21, and have deficiencies in trafficking of lymphocytes to the secondary lymph nodes.

When fully allogeneic islets were transplanted into streptozotocin-treated *plt* mice, they showed permanent survival of islets engrafted under the kidney capsule, whereas controls rejected islet allografts in 12 days ($p < 0.001$) [25]. These mice had normal allogeneic T cell responses, but deficient migration of donor DCs to draining lymph nodes. These results compare favorably with the animal studies blocking the inflammatory chemokine receptors CCR1 [18] and knockout models of CCR1, CXCR3 and CX3CR1 [26–28]. CCR7 antagonists are all at early stages of development. Compounds have been reported in patent literature with binding inhibition potencies of < 10 mM [29] and 3 nM by Pharmacopeia [30].

Researchers at Schering Plough are also exploring the potential of activating CCR7 in adjuvant therapy. When CCL21 was expressed in a C26 colon carcinoma tumor line, there was a reduced tumorigenicity of these cells that was correlated with an increased number of tumor infiltrating DCs [31]. Although there are no agonists under development, both Chiron and Schering Corporation have reported using CCL21 as a means of enhancing the immune component of a DC response to elicit an enhanced anticancer response [32, 33].

B cells – CXCR5

CXCR5, expressed on mature circulating B cells, is activated by the chemokine CXCL13. This receptor-ligand axis is a critical component in the homeostatic trafficking of B cells, as both CXCR5 and CXCL13-deficient mice lack B cell follicles in the spleen and Peyer's patches [34, 35]. While they do develop secondary lym-

phoid organelles that are deficient in B cell zones, these mice are able to generate a full spectrum of immunoglobulin responses, including antibody maturation [36].

A common feature of autoimmune disease is the formation of B cell clusters in ectopic lymphoid structures: rheumatoid arthritis, acute renal transplant rejection, Sjögren's syndrome, autoimmune thyroid disorders, and multiple sclerosis [37–39]. These follicle-like structures contain germinal centers that are thought to participate in the generation of autoreactive antibodies associated with pathogenesis. Given the lack of phenotype seen with the knockout mouse, CXCR5 might be an interesting target that could selectively affect the development of these disease-specific lymphoid structures, while leaving the normal secondary lymphoid functions competent.

In addition to the expression on mature B cells, CXCR5 is also expressed on a subset of memory T cells [10], and there are data tying CXCR5 to both T and B cell migration to diseased tissue, in this case acting like an inflammatory chemokine. CXCL13 is significantly up-regulated in actively demyelinating and chronic inactive lesions of multiple sclerosis patients [40]. Demonstrating that CXCL13 is involved in the pathology, neutralizing antibodies against CXCL13 or CXCL13$^{-/-}$ mice are able to reduce disease progression and abrogate relapse in EAE, presumably through a reduced infiltration of T cells in this model (Bagaeva, 6th World Congress on Inflammation). Despite these interesting initial findings, there are no reports of CXCR5-targeted therapies in development, although Bayer has a published patent claiming methods related to screening for antagonists of CXCR5 [41].

FTY-720

The immunomodulatory drug FTY-720 is a prodrug that when phosphorylated *in vivo* acts as an agonist on a subset of the members within the GPCR family of sphigosine-1-phospate (S1P) receptors. Systemic administration of FTY-720 results in a rapid lymphopenia that is lymphocyte selective, leaving monocytes and NK cells unaffected. This is the result of sequestration of naïve and activated CD4 and CD8 T cells and B cells in lymph nodes and Peyer's Patches [42, 43]. S1P gradients are essential for the egress of lymphocytes from the lymph nodes, and part of the activity of FTY-720 may be attributed to its ability to cause internalization of S1PR, in essence acting as a non-competitive agonist.

While much of the activity of FTY-720 is directly attributable to blocking the SIP1R-induced migration [44], it is clear that there is also a component of the homeostatic chemokine system that is downstream of SIPR. Activation of S1PR was first reported to sensitize the CCR7 receptor to signaling by its ligands (CCL19 and CCL21) [45]. Additional chemokine receptors have since been implicated in FTY-720's affect on lymph node homing, including CCR2 and CXCR4 [45]. However, the exact mechanism of how FTY-720 and the chemokines are acting in concert to affect egress and recirculation of lymphocytes is not clear.

Through these actions of lymphocyte sequestration, FTY-720 is able to prolong human and murine allograft survival, without affecting T cell activation and proliferation [46]. This mechanism of action is unique among immunosuppressive agents, and could lead to an improved safety profile for chronic dosing. This compound is being developed by Novartis AG and is in phase 3 clinical trials for transplant rejection [47]. There are also ongoing clinical trials for multiple sclerosis (Phase II) and Crohn's disease (Phase I). It may be that FTY-720, by moving upstream of the chemokine receptor itself, is able to generate a broad activity profile that overcomes some of the problems identified in clinical trials with antagonists on individual chemokine receptors. In the next section we discuss this concept of promiscuous targeting in more detail.

With the entry of chemokine receptor small molecule antagonists into clinical trials, the hopes were high that these magic bullets would provide validation of the concept that specific inhibition of leukocyte trafficking could show therapeutic potential in humans. As previously mentioned, in the case of CCR1 and CXCR3, these selective target antagonists have failed to show efficacy when transitioned from animal models to disease in humans.

Promiscuous targeting

New complexities in the chemokine systems continue to emerge. As research discovers new receptor-ligand pairs that are involved in leukocyte trafficking (RDC1-CXCR4), or finding new uses for the usual suspects (CCR2 lymph node homing) [45]. Often, this complexity can be understood with meticulous studies. In the case of the homeostatic chemokines, individual chemokine gradients are set up within the lymphoid systems that precisely arrange the cellular partners involved in the required cellular dance (elsewhere in this book) [15].

In the chemokine system that regulates inflammatory trafficking, it is less clear if the system involves a degree of complexity that we have yet to understand, exhibits functional redundancy, or some combination of the two. Knockout studies of murine atherosclerosis suggest functional redundancy in monocyte attraction, as many different chemokine receptor knockouts (CCR2, CXCR1, CX3CR1) give partial protection against diet-induced atherosclerosis [3]. However, it may be that we simply do not understand the specific non-redundant contribution of each of these to the pathology. Most importantly, as demonstrated by the growing number of failures of single-receptor antagonists in clinical trials, there are challenges in trying to control the system of leukocyte trafficking by targeting a single receptor. Approaches are under evaluation that attempt to circumvent this problem, although they mostly utilize systems that are quite different from the non-peptide antagonist approach.

Antibody and modified ligand based approaches

Often proof-of-concept research utilizes biological agents such as antibodies and modified chemokines, rather than small molecule chemical compounds, to impact the leukocyte trafficking system. Neutralizing antibodies against a broad range of chemokines and chemokine receptors have been generated and tested in animal models such as transplant rejection, rheumatoid arthritis and multiple sclerosis (EAE), to name a few [27, 48].

The large number of studies demonstrating efficacy of neutralizing antibodies in animal models continues to support this as a valid development approach. Human Genome Sciences and Progenics have both humanized anti-CCR5 antibodies in Phase I trials for inhibition of HIV infection. Cambridge Antibody Technology Group is testing an anti-CCR3 antibody in Phase II trials in efforts to block leukocyte trafficking in allergic rhinitis, allergy, and asthma. Another example currently in the clinic is an anti-CCR2 antibody developed by Millennium (MLN-1202). This is in Phase II trials evaluating efficacy in rheumatoid arthritis and other inflammatory diseases including multiple sclerosis, chronic obstructive pulmonary disorder and atherosclerosis.

There is certainly much hope that one or more of these trials will result in a marketed therapy. However, as with the non-peptide antagonists described above, the anti-receptor antibodies are by nature highly specific and do not generate promiscuous targeting. In contrast, anti-chemokine antibodies could affect the activity of many receptors. For example, CCL5 neutralizing antibodies have demonstrated efficacy in models of disease, presumably by blocking CCL5 activation of CCR1, CCR5 and possibly CCR3 [49, 50]. However, few anti-chemokine antibodies are under clinical development. Abgenix has tested ABX-IL8, a humanized anti-CXCL8 antibody in Phase II clinical trials for chronic obstructive pulmonary disorder, psoriasis and rheumatoid arthritis [51]. Although the therapy was well tolerated, none of these studies demonstrated sufficient efficacy and there are currently no development activities for this antibody.

Yet another approach is to take the natural promiscuity of some of the chemokines and, by modifying them, use this to block multiple receptors. For example modifications of CCL5 (RANTES) can affect the activity of CCR1, CCR3 and CCR5. AOP-RANTES, consisting of a chemical addition of aminooxypentane to the N terminus of CCL5/RANTES, binds to CCR5 with subnanomolar affinity [52]. It was initially investigated for its anti-HIV fusion activity, but has since shown efficacy and reduced cellular infiltration in rat experimental glomerulonephritis and an OVA-sensitized murine model of human asthma [53]. However, AOP-RANTES is not acting purely as a multi-receptor antagonist, as it can cause internalization and has full agonist activity on human CCR5, with partial activity on CCR3 and CCR1 [54]. Despite this, it is apparently able to disrupt the endogenous chemokine-regulated trafficking. Gryphon Technology was last reported to be

investigating chemically synthesized AOP-RANTES as a therapeutic against HIV infection.

Researchers at Serono are pursuing a novel strategy to affect chemokine signaling whereby mutation of the essential heparin-binding site of CCL5 eliminates the ability of the protein to form higher-order oligomers [55, 56]. This protein is still a functional agonist at chemokine receptors, but by forming monomers with endogenous CCL5, it inhibits the formation of higher-order oligomers. Therefore, this mutant form [(44AANA47)-RANTES] acts as a dominant-negative inhibitor for endogenous CCL5. The modified CCL5 can inhibit infiltration of inflammatory cells to the peritoneal cavity and bronchoalveolar air spaces. Further studies demonstrated an ability of this compound, when dosed intraperitoneally at 10 μg, to reduce the severity of disease in a mouse model of chronic autoimmune encephalomyelitis. After 14 and 21 days of treatment with the 10 μg dose, the area under curve measurement of clinical score was significantly lower. Serono is investigating this technology for the potential treatment of multiple sclerosis and has a compound (AS-839562) that entered preclinical studies in early 2004.

Protein based therapies – viral mimetics

Although a relatively new approach in the arena of chemokine antagonist drug development, the use of viral proteins as a starting point for compound development may prove to be a rich area. Large DNA viruses (poxviruses and herpesviruses) have generated a veritable toolbox of proteins with the primary purpose of subverting the efforts of the immune system. Many of these proteins have the ability to act as promiscuous effectors of the chemokine system, not a surprise as they have gone through 'optimization by evolution'. There are three main classes of chemokine-interacting proteins. The first two are chemokine and receptor mimics that the virus has copied from the host genome and modified. The last class consists of secreted proteins that bind to chemokines but share no homology with the natural chemokines or their receptors. These may be the most interesting for drug development as they represent proteins with no known mammalian homolog. The viral receptor proteins are not discussed here. As they are multi-span membrane proteins, they are less applicable for use as therapeutic agents. There are a number of excellent reviews that provide a thorough overview of this interesting class of proteins [57].

Viral chemokine mimics can exist as either agonists or antagonists. The agonists might induce the migration and attraction of specific lymphoid subsets that are targets for viral replication, while the chemokine antagonists might interfere with antiviral immune responses. Human herpesvirus 8 (HHV-8) produces the chemokine receptor antagonist vMIP-II. Glaxo reported in 1997 that vMIP-II blocked the primary coreceptors used by HIV to gain entry into cells (CCR5, CCR3, CXCR4 and

the viral receptor US28). This vMIP-II antagonist activity has subsequently been extended to also include CCR1, -2, -4, -10, CXCR3, and CX3CR1 [58–62]. Demonstrating the utility of this broad spectrum antagonist *in vivo*, vMIP-II has shown efficacy in rat models of spinal cord contusion and glomerulonephritis [63, 64]. While there is no development reported for the clinical use of vMIP-II as a therapeutic, there is research using this protein to assist in developing antagonistic agents for example, against CX3CR1 and CXCR4 [65, 66].

The murine herpesvirus 68 (MHV-68) secretes the 44-kDa M3 protein that can bind to both human and murine chemokines of all classes, despite the fact that it has no homology to human chemokines or receptors [67, 68]. The chemokine binding by this viral protein has high affinity (K_d in the nanomolar range), and can inhibit binding to the receptor as well as the subsequent calcium signaling [68]. M3 has the ability to block binding of chemokines to glycosaminoglycans as well, a property unique among the viral proteins. Possibly through this mechanism, M3 can inhibit *in vitro* migration of CCR7-transfected cells to the homeostatic chemokines CCL19 and CCL21. Further, transgenic mice co-expressing CCL21 and M3 in the pancreas show a reduced recruitment of lymphocytes compared to the same mice without M3 expression [69]. The M3 protein was previously under investigating by Xenova for the potential treatment of inflammation and cancer (inhibition of angiogenesis). In 2002, the protein was undergoing preclinical studies for the potential treatment of autoimmune and inflammatory diseases. However, Xenova has since been purchased, and there has no been further development reported.

CBP1 is a poxvirus (Parapoxvirus) secreted protein that also has the ability to bind a wide range of chemokines. Viron is in preclinical development investigating compounds based on CBP1 that have similar broad activities and show efficacy in a number of animal models of inflammatory-based disease. In a presentation at the 18th International Congress of the Transplantation Society Meeting (2000), the company reported that CBP1 could prevent chronic rejection in rat renal allografts. In these models, CBP1 in conjunction with cyclosporin could significantly reduce graft rejection.

Small molecule CKR antagonists

Approaches that utilize protein or peptide-based therapies will face challenges in drug development as they can not be administered orally and there is the potential for a humoral response against the administered agent. Therefore, the ideal promiscuous antagonist would be a non-peptide, orally available, small molecule. There is a wealth of literature demonstrating that small molecule drugs can bind to multiple GPCRs. Usually this is seen as an off target effect and improving specificity for the target GPCR is part of the drug development process. However, if this receptor promiscuity could be suitably harnessed in the design of dual inhibitors of GPCRs,

it could be a very useful approach in a number of situations. For example, dual CXCR4 and CCR5 inhibitors would be very useful therapeutically as fusion inhibitors to prevent HIV infection. Although the idea is quite attractive, the question remains whether the design of dual GPCR inhibitors is possible. There are examples where efforts to design such dual inhibitors have met with success.

Given the current progress in the field of GPCR antagonist design, it is clear that the design of dual receptor antagonists is possible if the binding pockets of the receptors share some common determinants. A number of homology models of chemokine receptors have been described. Of particular interest are the highly related receptors CCR1 and CCR3, which share around 59% sequence identity [70, 71]. A 2-(benzothiazolylthio) acetamide compound from Takeda binds to both receptors with high affinity displacing CCL3 with an IC_{50} of 450 nM for CCR1, and CCL11 with an IC_{50} of 33 nM for CCR3 [72]. Similarly, the antagonist UCB 35625 is a potent antagonist for both receptors inhibiting chemotaxis by CCL3 in CCR1 with an IC_{50} of 9.6 nM and CCL11 in CCR3 with an IC_{50} of 93.7 nM [73].

The receptors CCR2 and CCR5 may also have a binding pocket amenable to the generation of dual receptor antagonists. Many of the non-peptide CCR5 antagonists have reported some activity at CCR2. For example, Takeda's TAK-779 has only a 15-fold specificity inhibition at CCR5 compared to CCR2 (IC_{50} of 1.4 and 27 nM, respectively). This is despite no activity at CCR1, CCR3, or CCR4. Novartis is exploiting this common binding pocket, and is developing compounds to antagonize activity at both CCR2 and CCR5 to inhibit monocyte trafficking (Pacifichem 2005).

If the intent is to design promiscuous antagonists with maximum therapeutic benefit, these will need to be designed to either: overcome the potential redundancy of chemokine receptors on the same cell type (e.g., CCR1, CCR2 or CCR5 for monocyte trafficking), or to target multiple receptors on multiple cell types involved in the pathogenesis of a disease (e.g., CCR2 for monocytes and CCR5 or CXCR3 for T cells in inflammatory diseases) [74]. The design of such dual-receptor antagonists will provide a challenge to medicinal chemists as these are often structurally unrelated receptors.

Conclusion

The pharmaceutical industry has made amazing progress in developing candidate drugs against the chemokine receptors, bringing agents into clinical trials only a decade after the cloning of the first target (e.g., CCR1 and BX471). These efforts utilizing this strategy continue to expand with an increasing number of chemokine receptor small molecule antagonists entering the clinic each year. Although none have yet made the leap to marketed drug, there is hope that this will be seen soon, especially in the therapeutic area of HIV. It may be that new strategies will be required, however, to see optimum efficacy in preventing leukocyte trafficking.

Many of these strategies, such as the viral proteins, are only now making their way into clinical trials. The additional efforts that biologists and medicinal chemists are now placing on promiscuous small molecule antagonists should only increase the potential for market therapeutics that control human disease by controlling leukocyte trafficking.

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Inherited disorders of lymphocyte trafficking

Leukocyte adhesion deficiency

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Introduction

Leukocyte trafficking from bloodstream to tissue is important for the continuous surveillance for foreign antigens, as well as for rapid leukocyte accumulation at sites of inflammatory response or tissue injury. Leukocyte emigration to sites of inflammation is a dynamic process, involving multiple steps in an adhesion cascade. These steps must be precisely orchestrated to ensure a rapid response with only minimal damage to healthy tissue [1, 2]. Leukocyte interaction with vascular endothelial cells is a pivotal event in the inflammatory response and is mediated by several families of adhesion molecules.

Several years after reporting the structure of the leukocyte integrin molecule, a genetic defect in the subunit of the molecule (ITGB2) was discovered. This syndrome, now called leukocyte adhesion deficiency (LAD) I (OMIM 116920), has been described in more than 300 children, and is characterized by delayed separation of the umbilical cord, recurrent soft tissue infections, chronic periodontitis, marked leukocytosis, and a high mortality rate at early age (Tab. 1). Currently, the only definite therapy is bone marrow transplantation. *In vivo* and *in vitro* studies have shown a marked defect in neutrophil motility. Ten years later, in 1992, a second defect, LAD II (OMIM 266265), was discovered in two children, and was found to be due to defect in the synthesis of selectin ligands. Later on several more cases were reported. While several adhesive functions of LAD II leukocytes are markedly impaired *in vitro*, the clinical course with respect to infectious complications is a milder one than for LAD I. However, LAD II patients present other abnormal features, such as growth and mental retardation, which are related to the primary defect in fucose metabolism, which are not observed in LAD I. The primary defect is mutation in the specific fucose transporter to the Golgi apparatus (FUCT1).

Recently, a third rare LAD syndrome has been described. Patients with LAD III suffer from severe recurrent infections, similar to LAD I, and a severe bleeding tendency. Although integrin structure is intact, a defect in integrin activation is the primary abnormality in LAD III.

Lymphocyte Trafficking in Health and Disease, edited by Raffaele Badolato and Silvano Sozzani

Table 1 - Leukocyte adhesion deficiency syndromes

	LAD I	LAD II	LAD III
Clinical manifestation			
Recurrent severe infections	+++	+	+++
Neutrophilia			
basal	++	+++	++
with infection	+++	+++	+++
Periodontitis	++	++	?
Skin infection	++	+	++
Delayed separation of the umbilical cord	+++	–	+++
Developmental abnormalities	–	+++	–
Bleeding tendency	–	–	+++
Laboratory findings			
CD18 expression	↓↓↓ or absent	NL	NL
SLeX expression	NL	Absent	NL
Neutrophil motility	↓↓↓	↓↓	↓↓
Neutrophil rolling	NL	↓↓↓	NL
Neutrophil adherence	↓↓↓	↓	↓↓↓
Primary genetic defect	+(ITGB2)	+(FUCT1)	?

LAD I

Clinical and laboratory findings

LAD-I is an autosomal recessive disorder caused by mutations in the common chain (CD18) of the β_2 integrin family. The prominent clinical feature of these patients is recurrent bacterial infections, primarily localized to skin and mucosal surfaces. Sites of infection often progressively enlarge, and they may lead to systemic spread of the bacteria. Infections are usually apparent from birth onward, and a common presenting infection is omphalitis with delayed separation of the umbilical cord. The most frequently encountered bacteria are *Staphylococcus aureus* and gram-negative enteric organisms, but fungal infections are also common. The absence of pus formation at the sites of infection is one of the hallmarks of LAD I. Severe gingivitis and periodontitis are major features among all patients who survive infancy. Impaired healing of traumatic or surgical wounds is also characteristic of this syndrome [3].

The recurrent infections observed in affected patients result from a profound impairment of leukocyte mobilization into extravascular site of inflammation. Skin windows yield few, if any, leukocytes, and biopsies of infected tissues demonstrate inflammation totally devoid of neutrophils. These findings are particularly striking considering that marked peripheral blood leukocytosis (5–20 times normal values) is consistently observed during infections. In contrast to their difficulties in defense against bacterial and fungal microorganisms, LAD I patients do not exhibit a marked increase in susceptibility to viral infections [4].

The severity of clinical infectious complications among patients with LAD I appears to be directly related to the degree of CD18 deficiency. Two phenotypes, designated severe deficiency and moderate deficiency, have been defined [5]. Patients with less than 1% of the normal surface expression exhibited a severe form of disease with earlier, more frequent, and more serious episodes of infection, often leading to death in infancy, whereas patients with some surface expression of CD18 (2.5–10%) manifested a moderate to mild phenotype with fewer serious infectious episodes and survival into adulthood.

The defective migration of neutrophils from patients with LAD I was observed in studies *in vivo* as well as *in vitro*. Neutrophils failed to mobilize to skin sites in the *in vivo* Rebuck skin-window test. *In vitro* studies demonstrated a marked defect in random migration as well as chemotaxis to various chemoattractant substances. Adhesion and transmigration through endothelial cells were found to be severely impaired [6]. With the use of an intravital microscopy assay, it was found that fluorescein-labeled neutrophils from an LAD I boy rolled normally on inflamed rabbit venules, suggesting that they were capable of initiating adhesive interactions with inflamed endothelial cells [7]. However, these cells failed to perform activation-dependent, β_2 integrin-mediated adhesion steps and did not stick or emigrate when challenged with a chemotactic stimulus.

Patients with LAD I exhibit neutrophilia in the absence of overt infection with marked granulocytosis with neutrophils in peripheral blood reaching levels of up to 100 000/μl during acute infections.

Integrin are also important in the optimal T cell response and indeed a wide variety of *in vitro* abnormalities of lymphocyte function have also been described in LAD I, but their *in vivo* relevance remains unclear [8].

Early on, several lines of evidence supported an autosomal recessive pattern of inheritance. Equal numbers of male and female patients were described, and family studies showed heterozygous male and female carriers who expressed 50% of the normal amount of the β_2-integrin molecules on their neutrophils. Furthermore, a frequent history of consanguineous marriages strongly supported the concept that LAD I is inherited as an autosomal trait [9]. Early studies showed that patients with this disorder were uniformly deficient in the expression of all three leukocyte integrins (Mac-1, LFA-1, p150,95), suggesting that the primary defect was in the common β_2-subunit, which is encoded by a gene located at the tip of the long arm of

chromosome 21q22.3. To date, none of the reported affected individuals has demonstrated a selective deficiency of a single β_2-integrin heterodimer.

Subsequently, several LAD I variants were reported in which there was a defect in β_2-integrin adhesive functions, despite normal surface expression of CD18. A child with classical LAD I features with normal surface expression of CD18 was reported [10], in whom a mutation in CD18 was found to lead to a non-functional molecule. Another child with a moderate form of LAD I was found to have novel point mutation in CD18, leading to the expression of dysfunctional β_2 integrin [11].

The molecular basis for CD18 deficiency varies [12]. In some cases, it is due to the lack or diminished expression of CD18 mRNA. In other cases, there is expression of mRNA or protein precursors of aberrant size with both larger and smaller CD18 subunits. Analysis at the gene level has revealed a degree of heterogeneity, which reflects this diversity. A number of point mutations have been reported, some of which lead to the biosynthesis of defective proteins with single amino acid substitutions, while others lead to splicing defects, resulting in the production of truncated and unstable proteins [13].

Notably, a high percentage of CD18 mutations identified in LAD I is contained in the extracellular domain of the CD18 (on exon 9), which is a highly conserved regions. Domains within this segment are presumably required for association and biosynthesis of precursors, and may represent critical contact sites between the α-subunit and β-subunit precursors.

Thus, LAD I can be caused by a number of distinct mutational events, all resulting in the failure to produce a functional leukocyte β_2-subunit. While in most cases a point mutation, small insertions, or deletions in the CD18 (ITGB2) gene have been reported, an infant with LAD I and gross abnormality in chromosome 21, representing a deletion of q22.1-3, was described [14].

Diagnosis and treatment

In any infant male or female with recurrent soft tissue infection and a very high leukocyte count, the diagnosis of LAD I should be considered. The diagnosis is even more suggestive if a history of delayed separation of the umbilical cord is present. To confirm the diagnosis, absence of the α- and β-subunits of the β_2-integrin complex must be demonstrated. This can be accomplished with the use of the appropriate CD11 and CD18 monoclonal antibodies and flow cytometry. Sequence analysis to define the exact molecular defect in the β_2-subunit is a further option.

As leukocytes express CD18 on their surface at 20 weeks of gestation, cordocentesis performed at this age can establish a prenatal diagnosis [15]. In families in whom the exact molecular defect has been previously identified, an earlier prenatal diagnosis is possible by chorionic biopsy and mutation analysis.

Patients with the moderate LAD I phenotype usually respond to conservative therapy and the prompt use of antibiotics during acute infectious episodes. Prophylactic antibiotics may reduce the risk of infections.

Although granulocyte transfusions may be live saving, their use is limited because of difficulties in supply of daily donors and immune reactions to the allogeneic leukocytes.

At present, the only corrective treatment that should be offered to all cases with the severe phenotype is bone marrow transplantation [16]. The absence of host LFA-1 may be advantageous in these transplants because graft rejection appears to be in part dependent upon the CD18 complex.

The introduction of a normal β_2-subunit gene (ITGB2) into hematopoietic stem cells has the potential to cure children with LAD I [17]. Retroviral-mediated transduction of the CD18 gene was shown to reconstitute a functional CD11a/CD18 in lymphoblastoid cell lines derived from patients with LAD I [18]. With the future development of gene therapy techniques that allow efficient gene transfer without severe side effects, into true hematopoietic stem cells, severely deficient LAD I patients will be ideal candidates for this procedure.

LAD II

Clinical and laboratory findings

LAD II syndrome (OMIM266265) results from a general defect in fucose metabolism, causing the absence of SLeX and other fucosylated ligands for the selectins. LAD II was first described in two unrelated Arabs with consanguineous parents [19]. This is an extremely rare condition with only six patients reported [20].

Affected children were born after uneventful pregnancies with normal height and weight. No delay in the separation of the umbilical cord was observed. They have severe mental retardation, short stature, a distinctive facial appearance, and the rare Bombay (hh) blood phenotype. From early life they have suffered from recurrent episodes of bacterial infections, mainly pneumonia, periodontitis, otitis media, and localized cellulitis. Several mild to moderate skin infections without obvious pus have also been observed [21]. The infections have not been life-threatening events and are usually treated in the outpatient clinic. Interestingly, after the age of 3 years, the frequency of infections has decreased and the children no longer need prophylactic antibiotics. At older age their main infectious problem is severe periodontitis, as is also observed in patients with LAD I [22]. During times of infections the neutrophil count increases up to 150 000/μl.

Overall, the infections in LAD II appear to be comparable to the moderate rather than the severe phenotype of LAD I. It is possible that the ability of LAD II neutrophils to adhere and transmigrate *via* β_2-integrin under conditions of reduced

shear forces [7] may permit some neutrophils to emigrate at sites of severe inflammation where flow may be impaired, thereby allowing some level of neutrophil defense against bacterial infections.

Indeed, while the neutrophils of the patients and their parents exhibit normal levels of the integrin subunits, LAD II neutrophils were found to be deficient in expression of the SLeX antigen [19]. In contrast to LAD I neutrophils, which fail to flatten and spread on phorbol ester-treated glass coverslips, the majority of LAD II neutrophils flattened and extensively spread with pseudopod formation [23]. The poor migration toward chemoattractants in an under-agarose assay and the defective homotypic neutrophil adhesion could not be readily explained by the biochemical deficiency of SLeX, and suggested a more global defect in cell activation or adhesion. Nevertheless, LAD II neutrophils could be activated normally with upregulation of various adhesion molecules [23].

Neutrophils isolated from peripheral blood of one LAD II patient failed to bind to purified platelet-derived P-selectin and recombinant E-selectin *in vitro* [23]. No adhesion of LAD II neutrophils was observed when the endothelial cells were activated with IL-1β or TNF-α, both potent inducers of E-selectin, whereas normal neutrophils bound avidly. On the other hand, normal adhesion to endothelial cells was observed after activation with PMA, an activating stimulus for β_2-integrins.

Rolling, the first step in neutrophil recruitment to site of inflammation is mediated primarily by the binding of the selectins to their fucosylated glycoconjugate ligands. Using intravital microscopy, the *in vivo* behavior of fluorescein-labeled neutrophils from a normal donor and from LAD I and LAD II patients was investigated during their passage through inflamed microcirculation of rabbit mesentery [7]. The rolling fraction of normal donor neutrophils in this assay was around 30%, and LAD I neutrophils behaved similarly. In contrast, LAD II neutrophils rolled poorly (only 5%) and failed to emigrate.

To examine *in vivo* chemotaxis, the response of patient neutrophils to cutaneous inflammation was assessed by both skin chamber and skin window techniques. Neutrophil emigration was markedly diminished in both tests, the values being approximately 1.5% and 6% of normal in the skin-window and skin chamber tests, respectively [24]. Notably, neutrophils from a patient with LAD I, studied concurrently, showed the same magnitude of defect in these assays.

Since the first two LAD II patients identified were the offspring of first-degree relatives, and since the parents were clinically unaffected, autosomal recessive inheritance was assumed. The basic abnormality in LAD II is a general defect in fucosylation of macromolecules.

In addition to the Bombay phenotype (absence of the H antigen), the cells of LAD II patients were also found to be Lewis a-and b-negative and the patients were non-secretors. The three blood phenotypes (Bombay, Lewis a and b) have in common a lack of fucosylation of glycoconjugates. These facts suggested that the primary defect in LAD II must instead be a general defect in fucose production.

After the observation that the defect in the Arab patients may be localized in the *de novo* GDP-1-fucose biosynthesis pathway [25], the two enzymes involved with this pathway, GMD and FX protein, were measured and were found to be normal with no mutation in cDNA isolated from LAD II patients. Another child, from a Turkish origin, was also described with LAD II in whom decreased GDP-1-fucose transport into the Golgi vesicles was detected [26]. These studies were performed also in the Arab patients, and indeed the same general defect in fucose transporter to the Golgi vesicles was found. Still, marked kinetic differences were observed between the Turkish and the Arab patients [27]. This may explain the different response to fucose supplementation in the Turkish and the Arab children (see below). Using the complementation cloning technique, the human gene encoding the fucose transporter was found to be located on chromosome 11 [28]. The Turkish child was found to be homozygous for a mutation at amino acid 147 in which arginine is changed to cysteine, while the two Arab patients examined were found to have a mutation in amino acid 308 in which threonine is changed to arginine [28]. Both mutations are located in highly conserved transmembrane domains through evolution. LAD II is thus one of the group of congenital disorders of glycosylation (CDG), and is classified as CDG-IIc [29]. Although only four mutations have been described so far, some genotype-phenotype correlation can be observed [30].

From the biochemical aspect, once the primary defect was found, several studies were done to clarify the defect. As growth and mental retardation are prominent features in LAD II, and Notch protein, which is important in normal development, contains fucose, Sturla et al. [29] looked at the fucosylation process in LAD II. Fractionation and analysis of the different classes of glycans indicated that the decrease in fucose incorporation is not generalized, and is mainly confined to terminal fucosylation of N-linked oligosaccharides. In contrast, the total levels of protein O-fucosylation, including that observed in Notch protein, were unaffected [29]. Indeed, it was recently observed that the O-fucosylation process take place in the endoplasmic compartment and not in the Golgi apparatus [31]. Thus, it is still unclear what leads to the severe developmental delay observed in LAD II.

Diagnosis and treatment

LAD II is a very rare syndrome, described so far only in six children. As the clinical phenotype is very striking, the diagnosis can be made based on the presence of recurrent, albeit mild infections, marked leukocytosis, and the Bombay blood group, in association with mental and growth retardation.

An analysis of peripheral blood leukocytes by flow cytometry using a CD15s monoclonal antibody should be performed to determine SLeX expression. To confirm the diagnosis sequence analysis of the gene encoding the GDP-fucose transporter should be performed.

Prenatal diagnosis was made in a female fetus from one of the two affected, unrelated families. This fetus was found to have the Bombay blood phenotype and was aborted. Now that the gene involved in LAD II has been cloned, prenatal diagnosis can be done earlier using chorionic villus samples for DNA analysis.

Each of the patients described so far with LAD II suffered from several episodes of infections, which responded well to antibiotics. No serious consequences were observed, and prophylactic antibiotic is not needed. The patients' main chronic problem has been periodontitis, a condition that is especially difficult to treat in children with severe mental retardation [22]. The oldest LAD II patient is now 17 years and has a severe psychomotor retardation with only mild infectious problems.

Because of the proposed defect in fucose production, supplemental administration of fucose to the patients has been suggested. Indeed fucose supplementation caused a dramatic improvement in the condition of the Turkish child [32]. A marked decrease in leukocyte count with improved neutrophil adhesion was noted. Unfortunately, while using exactly the same protocol, no improvement in laboratory data or clinical features were seen in two Arab children [33]. This difference may be due to the fact that the genetic defect in the Turkish child leads to a decreased affinity of the transporter for fucose, and thus an increase in the cytosolic concentration of fucose would be expected to overcome, at least in part, the defect in fucose transport.

LAD III

Recently, a rare autosomal recessive LAD syndrome that is distinct from LAD I has been reported [34]. Although leukocyte integrin expression and intrinsic adhesive activities to endothelial integrin ligands were normal, *in situ* activation of all major leukocyte integrins, including LFA-1, Mac-1 and VLA-4, by endothelial-displayed chemokines or chemoattractants was severely impaired in patient-derived lymphocytes and neutrophils. Although LAD leukocyte rolling on endothelial surfaces was normal, they failed to arrest on endothelial integrin ligands in response to endothelial-displayed chemokines. G protein-coupled receptor (GPCR) signaling on these cells appeared to be normal and the ability of leukocyte to migrate towards a chemotactic gradient was not impaired. The key defect in this syndrome was attributed to a genetic loss of integrin activation by rapid chemoattractant-stimulated GPCR signals [34]. This novel LAD shows significant similarities to three previous cases commonly referred to as LAD I variants [35–37]. All four cases had similar clinical symptoms, characterized by severe recurrent infections, bleeding tendency and marked leukocytosis. In all cases tested, integrin expression and structure were intact with a defect in integrin activation by physiological inside-out stimuli. As these events cannot take place in LAD I leukocytes, it was proposed to designate this

group of integrin activation disorders as LAD III [38]. The term LAD I variant, which has been ascribed to these unique syndromes, is inaccurate because these syndromes do not evolve from structural defects in leukocyte or platelet integrins.

In all of the LAD III cases, defects in GPCR-mediated integrin activation are also accompanied by variable defect in non-GPCR-mediated inside-out activation of leukocyte integrins. A growing body of evidence implicates the Ras related GTPase, Rap-1, as a key regulator of integrin activation by these and other inside-out stimuli [39]. Rap-1 was also implicate in the activation of platelet and megakaryocyte GpIIbIIIa, which is defective in LAD III. It is thus highly attractive that one or more of the new LAD III cases involve either a direct or indirect defect in Rap-1 activation of leukocyte and platelet integrins. Although lymphocytes from two cases expressed normal level of Rap-1, in one case studied an aberrant activation pattern was observed [40].

However, the ubiquitous expression of Rap-1 in most tissues and its highly diverse functions in non-hematopoietic cells [41], make it unlikely that Rap-1 is structurally mutated in any of the new LAD III cases. It is thus possible that a hematopoietic-specific effector of Rap-1 activity, or a specific hematopoietic adaptor that links this GTPase to cytoskeletal integrin partners regulating both inside-out and outside-in integrin activation processes, is functionally deleted in these and related LAD III cases.

These patients need prophylactic antibiotics as well as repeated blood transfusion. The only curative treatment is bone marrow transplantation, which should be performed as early as possible.

E-selectin deficiency

Another potentially inherited defect in the selectin system was described in a child with moderate neutropenia and severe recurrent infections [42]. There was markedly reduced expression of E-selectin on blood vessels of inflamed tissue with increased levels of circulating soluble E-selectin, suggesting increased cleavage of surface E-selectin. Notably, the E-selectin gene sequence was normal.

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Wiskott-Aldrich syndrome as a model of cytoskeleton defects

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Introduction

Wiskott-Aldrich syndrome (WAS) is an X-linked immunodeficiency with an estimated incidence of 2–4 per million live births [1, 2] that results from mutations in the WAS gene. The gene product, Wiskott-Aldrich Syndrome protein (WASp) is exclusively expressed in non-erythroid hematopoietic cells [3–5] where it has an important role in cytoskeletal regulation and thus WASp deficiency is linked to functional defects in most blood lineages [5, 6]. Here we discuss the pathogenesis of WAS-associated immune disease at the molecular and cellular levels as a model to highlight the importance of the actin cytoskeleton for leukocyte function and generation of normal immune responses.

WASp is a key cytoskeletal regulator

WASp is a key cytoskeletal regulator, transmitting and integrating actin-regulating signals essential for multiple cell functions including antigen uptake, cell migration and immune cell-cell interactions. Other WASp family homologues are more widely expressed than WASp itself and in vertebrates these include neural WASp (N-WASp) and three homologues of WASp family Verprolin-homologous protein (WAVE), also called suppressor of G protein-coupled cyclic-AMP receptor cAR (SCAR) [7, 8]. All family members are composed of modular domains identified by sequence homology and binding interactions that serve to integrate signals for regulating WASp activity and subcellular localization (Fig. 1). WASp shares closest sequence homology with N-WASp, with common domains in these proteins having similar binding partners and functions. All WASp family proteins contain a characteristic C-terminal tripartite VCA domain (verprolin homology, central, acidic) capable of activating the actin-related protein (Arp)2/3 complex to initiate formation of new actin filaments (Fig. 2) [9]. A detailed discussion of WASp domain interactions is outside the scope of this chapter but has been reviewed elsewhere [10, 11].

Lymphocyte Trafficking in Health and Disease, edited by Raffaele Badolato and Silvano Sozzani

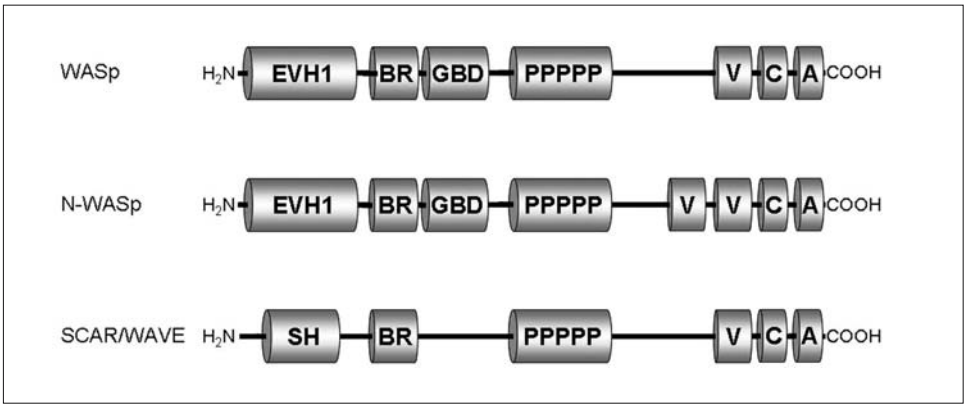

Figure 1
Domain structure of WASp family proteins
Schematic diagram representing the WASp family proteins. The WASp family proteins are organized into domains. The C terminus, which is critical for regulation of actin polymerization, is highly conserved between members, but the N terminus is divergent. Members of the SCAR family do not contain an EVH1 domain, but are homologous with each other at the N terminus. The function of the N terminus is unclear, but may involve localization of the proteins to membranes (EVH1, Ena Vasp homology domain; BR, basic region; GBD, GTP-ase binding domain; V, verprolin homology domain; C, central domain; A, acidic domain; SH, SCAR homology domain; NH2, N terminus; COOH, C terminus).

Regulation of WASp activity

Regulation by Cdc42

Studies investigating the activation of WASp-mediated actin polymerization have demonstrated a highly complex regulation involving several separate but co-operative pathways. Cytosolic WASp adopts an auto-inhibited configuration in which the C-terminal VCA region is bound to a proximal GTPase-binding domain (GBD) [12] (Fig. 2). Binding of the Rho-GTPase Cdc42, to the WASp GBD, via a complex formed with Toca-1 (transducer of Cdc42-dependent actin assembly), induces a conformational change that exposes Arp2/3 and actin-binding sites, allowing actin polymerization to proceed [12, 13]. Cdc42-GBD binding was recently shown to be preceded by electrostatic interaction of Cdc42 with the adjacent WASp basic region (BR) [14], a step not shared by other Rho family proteins but required for optimal Cdc42 affinity and unfolding of auto-inhibited WASp. Other signaling molecules synergize with Cdc42 for optimal WASp activation. These includes the Src family kinase Lck which interacts with WASp through its SH2-SH3 module [15], and the

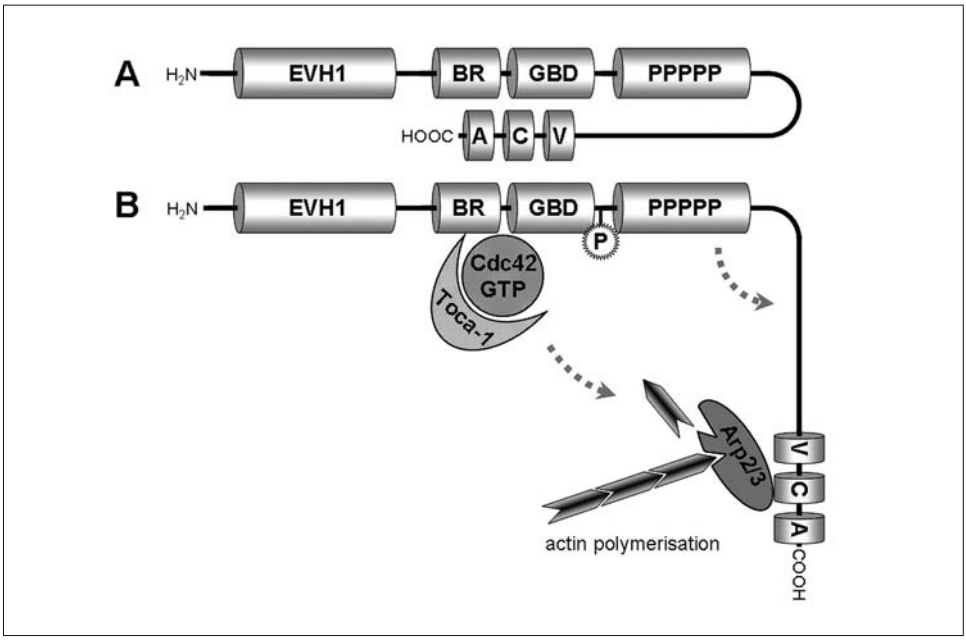

Figure 2
Regulation of WASp activity
Schematic representations of WASp. (A) Cytosolic WASp exists in an auto-inhibited conformation in which the VCA region is bound to the proximal GBD. (B) WASp activation by the Cdc42/Toca-1 complex or by phosphorylation disrupts auto-inhibition, exposing Arp2/3 and actin binding sites.

phosphoinositide phosphatidyl 4,5-bisphosphate (PIP_2) [16], which may either directly interact with WASp or exert its effects upstream of Cdc42 [17].

Regulation by phosphorylation

Phosphorylation provides a separate important mechanism for WASp activation and occurs in response to a variety of physiological stimuli including T cell receptor (TCR) stimulation, mast cell IgE receptor stimulation and collagen receptor stimulation of platelets [18–21]. Reported as a target for Btk and Src family kinases, Y291 was identified as a specific WASp residue, which when phosphorylated leads to destabilization of WASp's auto-inhibited configuration. This sensitizes WASp to Cdc42 activation, enables further activation by SH2 domain-containing

proteins, and enhances actin polymerization both *in vitro* and *in vivo* [15, 22–24]. *In vivo*, Y291 phosphorylation occurs independently of Cdc42 binding and is required to initiate WASp effector functions after TCR stimulation [18], indicating that distinct routes of activation may direct specific WASp responses. Separately, serine phosphorylation at sites in the VCA domain by casein kinase 2 enhances WASp activity *in vitro* by increasing the affinity of the VCA domain for the Arp2/3 complex [25].

Regulation by WIP

An important binding partner for the Ena-Vasp homology 1 (EVH1) domain is WASp-interacting protein (WIP) [26, 27], an ubiquitous actin regulator that binds monomeric actin, stabilizes actin filaments and promotes filopodia and ruffle formation [26, 28, 29]. The majority of inactive cytosolic WASp appears to be complexed with WIP, and WASp levels are substantially reduced in WIP-deficient lymphocytes, suggesting that WIP protects WASp from proteolysis [30, 31]. Other effects of WIP on WASP-mediated actin polymerization are complex, however, as it appears that interaction of WIP with WASp/N-WASp retards Arp2/3-mediated actin polymerization, possibly through stabilization of the auto-inhibited conformation [29, 30]. On the other hand, WIP that is released during WASp activation may enhance Arp2/3-mediated actin polymerization, and WIP activity itself can regulate cytoskeletal rearrangement in a partially redundant fashion with WASp [31]. The importance of WASp-WIP interactions for cell function is highlighted by the fact that a significant proportion of WAS gene mutations result in expression of WASp with amino acid substitutions in the EVH1 domain that would be predicted to interrupt binding of WIP [32].

Regulation by other mechanisms

Other less well-defined molecular interactions also regulate the efficiency of WASp-mediated actin polymerization *in vitro*. For example, the polyproline (P), BR and EVH1 domains are all required for optimal actin polymerization by both WASp and N-WASp [33–35], although the exact mechanisms remain unclear.

Localizing WASp activity

For normal cell functions, spatial as well as quantitative regulation of actin polymerization is essential. WASp localizes to areas of new actin polymerization, such as phagocytic cups, specialized adhesion plaques in macrophages and dendritic cells

(DC) and during assembly of the immunological synapses in T cells and NK cells [36–39]. To date, the mechanisms spatially regulating WASp activity to discreet intracellular compartments are largely unknown. WASp-WIP interaction is one possible mechanism as WIP recruits WASp for immune synapse formation in T cells after TCR stimulation via a Zap70-CrkL-WIP-WASp complex [30]. Phosphoinositides such as PIP_2 could also play a role, but although binding to the BR domain of N-WASp has been reported [40, 41], direct interaction of PIP_2 with WASp remains to be demonstrated. In addition SH3 domain-containing proteins, such as the endocytic proteins syndapin and intersectin, may facilitate localization to membrane sites via polyproline domain interactions [42, 43].

Defective WASp function leads to clinical disease

Classical WAS is characterized by the triad of immunodeficiency, microthrombocytopenia and eczema, but in fact only the bleeding diathesis is uniformly present and disease severity is highly variable [44]. As might be expected by the pattern of WASp expression, defective function has been described in most immune cell lineages, leading to a combined cellular and humoral defect, which results in a susceptibility to severe and life-threatening bacterial, viral and fungal infections [44, 45]. Abnormal immunological laboratory parameters can include generalized lymphopenia, abnormal T cell proliferation (especially in response to CD3 stimulation) and aberrant immunoglobulin responses to protein and, more particularly, to polysaccharide antigens, including isohemagglutinins [44, 46, 47]. Immune dysfunction additionally manifests as autoimmunity, which was seen in 40% and 72% of WAS patients in two independent studies [44, 47]. A wide range of autoimmune diseases can occur even as early as infancy, with autoimmune cytopenias, arthritis and vasculitis being the most common complications. Poorly understood at a cellular level, WAS-related autoimmunity can be difficult to manage and is a poor prognostic indicator [45, 47]. Hematopoietic malignancies are an additional serious complication of WAS that may result from defective immune surveillance, although, as with autoimmunity, the pathogenic mechanisms are presently unclear [45, 48, 49].

Without treatment, the prognosis for severely affected WAS patients is poor, with bleeding and severe infections constituting the major causes of morbidity and mortality in infancy and early childhood. At present, the only curative therapy for classical WAS is bone marrow or hematopoietic stem cell transplantation, but it is likely that corrective gene therapy will be available in the near future [50, 51]. In contrast, X-linked thrombocytopenia (XLT), which results from mutations in the same gene, has a milder phenotype generally restricted to microthrombocytopenia, and consequently is associated with a much-improved prognosis [45, 52–54].

The clinical variability of the XLT/WAS spectrum is in part due to genotype, but is also influenced by other factors that may include pathogen exposure and sec-

ondary disease modifying genes. Approximately 300 unique mutations have been reported throughout the WAS gene, with a predominance of missense mutations located in the first four exons (WASpbase: http://homepage.mac.com/kohsukeimai/wasp/WASPbase.html, and [45, 55]). Five specific mutations, three associated with XLT and two with WAS, occur with high frequency and have been termed 'mutational hotspots' (Fig. 3) [55]. The effect of the mutation on protein expression strongly influences clinical phenotype, as mutations resulting in complete absence of WASp are associated with severe disease while persistence of reduced amounts of WASp correlate with a milder phenotype [45, 55, 56]. This likely results from preservation of partial WASp function including WASp-Arp2/3-mediated actin polymerization in a subset of patients. Once proteotype has been determined for any given mutation, there is a strong genotype/proteotype association that is likely to be clinically useful for prognostication and directing patient management [56]. Intriguingly, mutations which give rise to constitutively active WASp result in X-linked neutropenia, a completely different disease characterized by congenital neutropenia and severe bacterial infections [57, 57a], highlighting the importance of WASp-mediated cytoskeletal regulation for normal cell function. Studies of the effects of WASp deficiency *in vivo* have been assisted by the generation of two separate WASp-deficient mouse models, both of which provide good mimics for the hematopoietic features of human WAS [58, 59], although neither express significant autoimmune or malignant disease.

Importance of the actin cytoskeleton for generation of normal immune responses

Effective host protection against invading pathogens requires continual immune surveillance by tissue-resident leukocytes and rapid recruitment of blood-borne immune cells. The actin cytoskeleton is crucial for both of these events at multiple points. At the inflammatory site, phagocytosis of particulate antigens and uptake of soluble antigens by endocytosis/pinocytosis require cytoskeletal reorganization for protrusion or retraction of the plasma membrane [60, 61]. Capture of foreign antigen initiates DC migration to draining lymphoid tissue via afferent lymphatics and homing to lymphoid T cell areas for antigen presentation [62–64]. Dynamic cytoskeletal rearrangement is mandatory for cell motility, cell-cell interactions and for formation of an immunological synapse during T cell priming [60]. Cell motility is also essential for homing of blood-borne effector cells into inflamed tissue, which occurs via a sequence of events that is often referred to as the multistep paradigm of leukocyte emigration [65]. During this process, cells tether, roll and firmly adhere to the vascular endothelial surface before crawling through a junction between adjacent endothelial cells (diapedesis) to enter tissue and migrate to inflam-

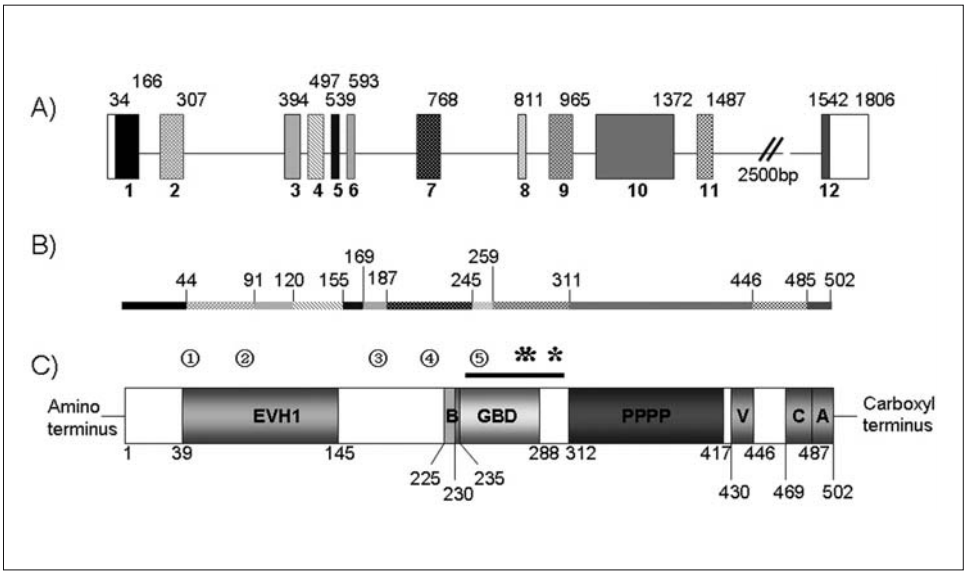

Figure 3

WASp genomic organization and protein structure

(A) Schematic diagram of WASp genomic structure. The 12 exons are shaded and numbered. Uncoded regions are shown as unshaded blocks. Numbers above the blocks indicate base pairs. (B) Line diagram representing the WASp protein. Shaded regions correspond with encoding exons and numbers indicate amino acids. (C) Schematic diagram representing the WASp protein. The EVH1, basic (B), GBD (minimal high affinity Cdc42-binding site is shown), polyproline (PPPP) and VCA domains/regions are shown. Amino acids are numbered below. The black line above the GBD indicates the VCA binding region (amino acids 242–310). Black circled numbers denote mutational hotspots [55] as follows: 1, T45M; 2, R86S/G/C/H/L; 3, IVS6 (+5g>a); 4, R211X and 5, IVS8 (+1g>a/c/t). Mutations resulting in constitutive activation are represented by black asterisks: these are (from left to right) L270P [57], L272P [57a] and I294T [57a]. With permission adapted from [48].

matory sites [65]. Both the rigidity to sustain tethering and rolling forces and the high flexibility required for physical migration are provided by dynamic regulation of the cell's actin cytoskeletal machinery.

WASp-mediated cytoskeletal rearrangements are essential for many immune response events. The following sections detail the consequences of WASp deficiency on the immune function of myeloid and lymphoid lineages and discuss how these specific defects are likely to be important for disruption of normal immunity.

The effects of WASp deficiency on individual immune cell lineages

Monocytes, macrophages and DC

Blood-borne monocytes respond to inflammatory stimuli by exiting the circulation to populate inflamed tissues, where they differentiate into macrophages or DC [66–69]. WASp-deficient monocytes demonstrate defective polarization and migration in response to inflammatory chemokines [70, 71]. Therefore, although stimulated up-regulation of integrin expression is normal, diapedesis and subsequent translocation through tissues are likely to be defective *in vivo*.

Similar defects of actin rearrangement are found in WASp-deficient macrophages and immature DC, resulting in aberrant polarization and defective formation of lamellipodial protrusions at the cell's leading edge [36, 70, 72]. Additionally, there is a striking lack of dynamic actin-containing adhesion structures called podosomes [73, 74], which normally are assembled by cells of the monocytic lineage when in contact with substratum [36, 75, 76]. Detailed analysis of podosomes using confocal laser scanning microscopy has demonstrated that they comprise a core of F-actin and actin-associated proteins (such as WASp) surrounded by a ring of integrins and integrin-associated proteins (Fig. 4) [74]. The main function of podosomes appears to be adhesion to substratum, and they have been implicated to be important for cell migration as they localize close to the leading edge of migrating cells [74]. Podosome formation is severely impaired in macrophages and DC of both WAS patients (Fig. 4) and $WASp^{-/-}$ mice [36, 73, 77, 78], and failure to normally recruit β_2 integrins to sites of podosome assembly results in reduced adhesion to ICAM-1 [77] with possible implications for transendothelial migration.

Together, these cytoskeletal defects significantly impair migratory responses. For example in macrophages of WAS patients, chemotaxis (directional movement towards a chemoattractant) is significantly impaired [78, 79]. DC translocation *in vitro* is also severely abnormal in both human and murine WASp deficiency [36, 72, 80, 81]. In addition, DC migration from the skin to secondary lymphoid tissue and homing to splenic T cell areas is defective in WASp-deficient mice [80, 81], suggesting that DC migratory defects significantly contribute to failure of T cell responses in WAS patients. Curiously, no motility defects have yet been described for mature human DC (which do not normally polarize or assemble podosomes in contact with substratum [48]), and this may reflect their major role as presenters rather than transporters of antigen.

Identical morphological and migratory defects can be induced in normal DC by down-regulation of WASp using lentiviral vector-mediated RNA interference [82], indicating that WASp is directly responsible for the cytoskeletal defects observed in WASp-deficient myeloid cells. Importantly, reconstitution of WASp by gene replacement restores podosome formation in macrophages and DC of WAS patients [36, 78] and $WASp^{-/-}$ mice [83], and normal chemotaxis in WAS macrophages [78],

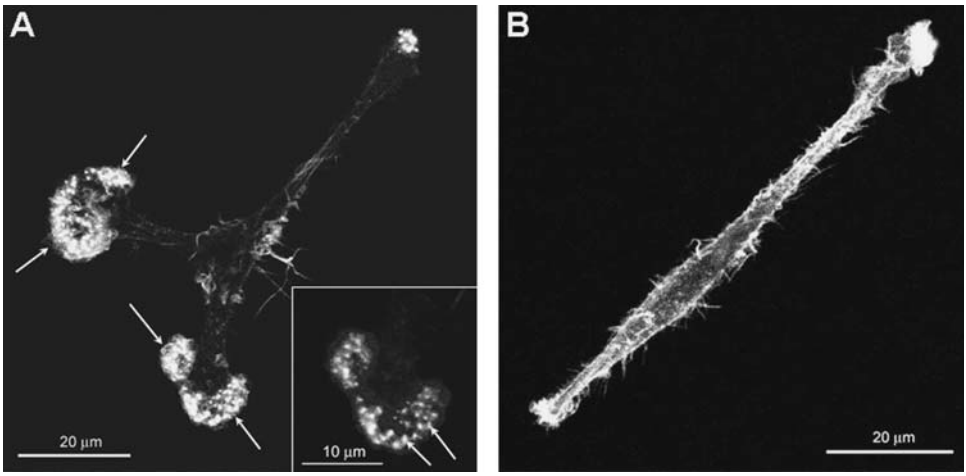

Figure 4
Immunofluorescence staining of podosomes in human DC
(A) Podosome formation directly behind the leading edge in a DC of a healthy subject. The inset shows more detail at higher magnification. (B) DC of a WAS patient fail to form podosomes or distinct leading edges. Arrows indicate podosomes. Bars represents 20 µm, and 10 µm in inset.

demonstrating that myeloid functional defects could be corrected by gene therapy in the future.

Phagocytosis for particulate antigen uptake and removal of apoptotic cells is a separate function of monocytes, macrophages and DC that requires dynamic cytoskeletal rearrangement. While uptake of soluble antigen by pinocytosis or endocytosis, is not affected by WASp deficiency [58, 84, 85], FcgR-mediated phagocytosis is impaired in macrophages of WAS patients and $WASp^{-/-}$ mice [37, 86]. Similarly, phagocytosis (but not binding) of apoptotic cells by $WASp^{-/-}$ murine macrophages is defective [86]. These observed defects result from a failure of phagocytic cup formation, which normally requires WASp recruitment for new actin polymerization [37, 86], and specifically lead to reduced DC-induced T cell responses to particulate antigens *in vivo* [85].

Granulocytes

Despite the importance of granulocytes for innate immunity, $WASp^{-/-}$ neutrophils have, to date, received relatively little attention. Surprisingly, granulocyte-mediated phagocytosis has not been investigated in WAS patients, although this function is

defective in WASp$^{-/-}$ murine neutrophils [59]. Similarly, neutrophil migratory responses are impaired in WASp$^{-/-}$ mice both *in vitro* and *in vivo* [81], but human data are conflicting. While granulocytes isolated from patients with WAS [79] demonstrate normal chemotaxis, there appears to be an inhibitory factor in WAS patient serum which may impact *in vivo* neutrophil function by unknown mechanisms [87]. Additionally, migration of normal human neutrophils, but not integrin activation or adhesion, is inhibited by injection of the SCAR VCA domain and to a lesser extent the WASp VCA domain [37]. These proteins exert a dominant negative effect through competitive binding to Arp2/3 and actin, and therefore these results suggest a role for WASp family proteins, if not WASp itself, in human neutrophil migration. Clarification of the role of WASp and redundancy between WASp family proteins for human neutrophil function is clearly an area that requires future study.

T lymphocytes

The role of WASp in TCR signaling and immune synapse assembly has been a major focus of recent research. Formation of the immune synapse (comprising TCR, costimulatory molecules and adhesion molecules) at the DC-T cell contact site is required for optimal T cell activation, and its assembly normally involves clustering of lipid rafts, actin polymerization and recruitment of signaling molecules including talin, ZAP-70, PKC-θ and WASp [30, 88, 89]. In the absence of WASp, TCR-mediated lipid raft clustering, actin polymerization and immune synapse assembly are all impaired at the T cell-antigen-presenting cell contact site with resultant defects of TCR signaling and TCR-mediated proliferation [38, 46, 58, 59, 90–92].

Other cytoskeletal defects in WAS-deficient T cells include abnormal morphology, reduced expression of microvilli [46, 93] and reduced migration both *in vitro* and *in vivo* [31, 81]. Resultant defects of homing and cell-cell interactions may partly account for the poorly developed T cell areas observed in both WAS patients and WASp-deficient mice [94, 95]. Additionally, impaired migration could contribute to the reduced number of T cells in the blood of WAS patients [87, 96] and WASp-deficient mice [58, 59], although WASp also appears to be required for normal T cell development and survival [48]. As has been demonstrated for other cell types, gene correction by viral transduction of murine WASp$^{-/-}$ hematopoietic stem cells or human WAS T cells corrects many T cell defects *in vitro*, including WASp expression, actin polymerization, TCR stimulated proliferation and IL-2 expression [83, 97–101].

B lymphocytes

Patients with WAS demonstrate humoral defects including reduced numbers of circulating B lymphocytes and impaired specific antibody responses, particularly to

Table 1 - WASp deficiency influences cellular function at multiple levels

Cell type	Cytoskeletal rearrangements	Migration	Cellular function
Monocytes	↓ polarization	↓	Normal oxidative burst
Macrophages	↓ podosomes formation, ↓ polarization	↓	↓ phagocytosis
Dendritic cells	↓ podosomes formation, ↓ polarization	↓	↓ phagocytosis, normal maturation
Granulocytes	Not documented	↓	↓ phagocytosis
T cells	↓ microvilli on surface, ↓ immune synapse formation	↓	↓ TCR activation, ↓ IL-2 expression
B cells	↓ membrane protrusions, ↓ homotypic clustering	↓	Disrupted spleen architecture, normal isotype switching

For references see text

polysaccharide antigens [44, 58, 96, 102]. It is not clear to what extent these observations result from primary B cell dysfunction or from failure of appropriate T cell help or changes in secondary lymphoid tissue architecture. There is good evidence for a contributing primary defect, as both human and murine WASp-deficient B cells demonstrate impaired actin polymerization [103, 104], which can be corrected by restoration of WASp expression via retrovirus-mediated gene transfer [105]. In addition, defective cytoskeletal regulation results in aberrations of morphology including polarization, spreading, microvilli expression and homotypic clustering [104, 106]. Furthermore, B cell migration is abnormal in both WAS patients and WASp-deficient mice [38]. Inefficient homing may explain the paucity of postgerminal (marginal zone) $CD27^+$ B cells [102], and poor follicle formation found in the spleen of WAS patients [94, 95] and $WASp^{-/-}$ mice [59, 80, 106, 107]. Reduced numbers of marginal zone B cells may also provide an explanation for impairment of T-independent antibody responses found in WAS patients.

As for other B cell functions, studies are incomplete. Uptake, processing and presentation of soluble antigen is apparently normal [85], but phagocytosis and presentation of particulate antigen remains to be investigated. In contrast with TCR studies, evidence of a requirement for WASp in downstream signaling from the B cell receptor (BCR) is contradictory. $WASp^{-/-}$ B cells proliferate normally in response to mitogenic stimuli [58, 59] and BCR activation, although not to anti-IgM-mediated BCR activation [58, 59, 108].

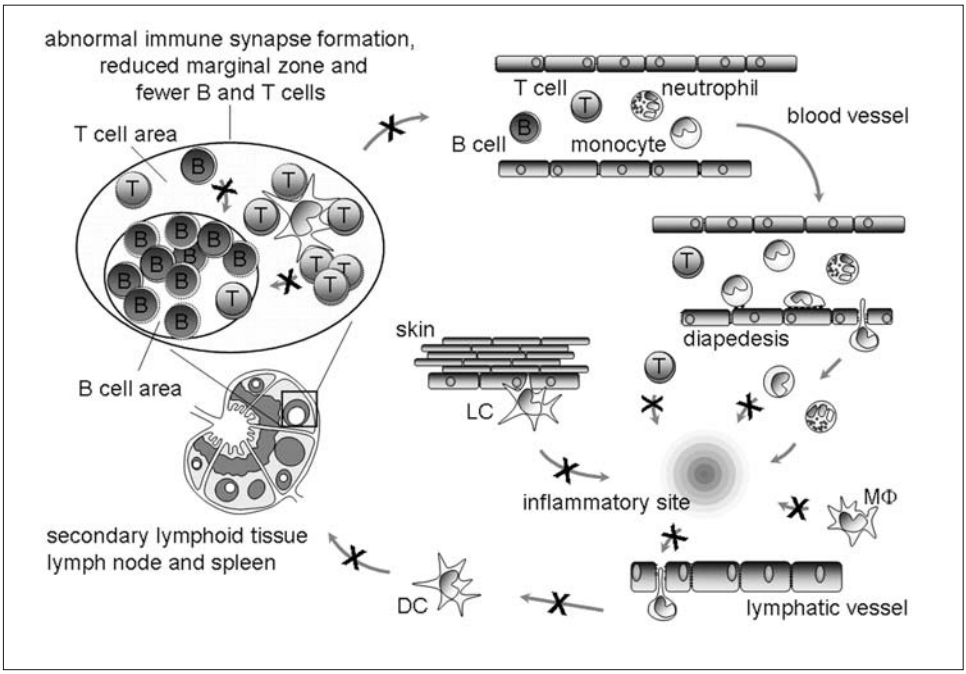

Figure 5
Migratory defects in multiple immune cell lineages in WAS
Schematic diagram representing immune cell trafficking. Multiple cell lineages including monocytes, macrophages, DC and B and T cells have been shown to exhibit migratory defects in WAS resulting in dysregulated immune cell traficking and impaired immunity. Arrows represent routes of trafficking and black crosses indicate those that have been reported to be abnormal in WASp deficiency. For more detail see text.

The impact of leukocyte homing defects in WAS

While WASp deficiency may exert its influence on innate and adaptive immunity at multiple levels (see also Tab. 1), it is likely that a reduced migratory potential of immune cells significantly contributes to the immunodeficiency seen in both WAS patients and in WASp$^{-/-}$ mice (Fig. 5). Kinetic or quantitative defects of cell recruitment to inflammatory sites, as have been demonstrated for WASp-deficient myeloid cells [70–72, 78–81, 84], would be predicted to impair efficiency of pathogen clearance [107] and timely antigen presentation. Additionally, aberrant DC and lymphocyte homing [31, 72, 80, 81, 84, 106] may in part explain aberrations of splenic

architecture and adaptive immunity as seen in both WAS patients [95, 96] and $WASp^{-/-}$ mice [106, 107]. For example, the chemokine CXCL13 is thought to be crucial for attracting B cells to the follicles [109], and migration of B lymphocytes to CXCL13 and CXCL12 is reduced in WAS [106]. Similarly CCL19 and CCL21 direct the homing of T cells to the T cell area of lymphoid tissue [110, 111], and reduced migration of T lymphocytes to CCL19 may thus be responsible for abnormal T cell areas in the spleen. Furthermore, DC migration to CCL19 and CCL21, believed to direct DC to T cell areas of lymphoid tissue [63, 111–113], is impaired in WASp-deficient murine DC [80, 81], providing a further possible mechanism to explain abnormal lymphoid architecture and defective activation of antigen-specific lymphocytes in WAS.

Defective leukocyte homing may also significantly contribute to the pathogenesis of WASp-mediated autoimmunity. In particular, defective homeostatic trafficking of DC, which normally takes place in the absence of inflammation and tolerizes T cells to self-antigen [114–116], may be important. However, other cytoskeleton dysfunctions, such as impaired clearance of apoptotic cells or aberrant TCR signaling could also play a role, and rigorous investigation is required, which will be important not only for clarifying pathogenic mechanisms in WAS but also for understanding the basis of human autoimmune disease.

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From CXCR4 mutations to WHIM syndrome

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Biology of CXCR4-CXCL12 interaction

Although the role of the CXCR4-CXCL12 has been extensively investigated for the hematopoietic compartment, animals with genetic deletions of CXCR4 or of its ligand CXCL12 have multiple defects in heart, brain and vessel development [1–3]. CXCR4 is expressed on cell surface of a variety of immature and terminally differentiated cells including adult and embryonic stem cells. Primordial germ cells, skeletal muscle satellite progenitor cells, neural stem cells, retinal progenitor cells and fetal liver-derived hematopoietic stem cells express CXCR4 and are functionally responsive to CXCL12, suggesting that CXCR4 and CXCL12 exert a general role during organogenesis. Early studies of CXCL12 knockout mice have revealed that this chemokine is secreted at early stages during embryogenesis by bone marrow stroma to induce the colonization of bone marrow by hematopoietic stem cells which are derived from liver [1–3]. In $CXCR4^{-/-}$ embryonic marrow, myelopoiesis and B cell development are severely impaired, whereas erythropoiesis is not affected [4]. CXCL12 is expressed during ontogeny by bone marrow stromal cells to induce proper homing of circulating hematopoietic progenitor stem cell (HPSCs) to the site of hematopoiesis [5]. This precise role of CXCL12 in homing and retention of stem cells in the bone marrow is still maintained in adult life, when the secretion of the chemokine in the hematopoietic environment is essential to prevent the egress of progenitor cells into peripheral blood. In contrast, reduced CXCL12 expression or increased proteolysis of the chemokine by the action of neutrophil elastase decrease adhesion of hematopoietic cells expressing CXCR4 to the stromal microenvironment of bone, which subsequently leads to their release to the bloodstream [6]. The role of CXCL12/CXCR4 axis in homeostasis of bone marrow is reinforced by the observation that injection of pertussis toxin, the specific inhibitor of G protein signaling, leads to long-lasting leukocytosis, and abolishes functional response of mobilized cells to its ligand [7]. The recent discovery of CXCR4 antagonists and their use to induce HPSCs mobilization and to increase the mobilizing effect to granulocyte colony-stimulating factor (G-CSF) have highlighted the importance of the

Lymphocyte Trafficking in Health and Disease, edited by Raffaele Badolato and Silvano Sozzani

critical balance between CXCL12 expression by stromal cells and CXCR4 levels for normal hematopoiesis [8]. Subcutaneous administration of the CXCR4 antagonist AMD3100 to healthy volunteers or to multiple myeloma patients have shown that not only HPSCs, but also neutrophils and lymphocytes rapidly increase in the blood following treatment [9–12].

Genetics and physiopathology of WHIM syndrome

Analysis of numerous pedigrees has shown that the disease is usually inherited as an autosomal dominant trait in the majority of pedigrees, but an autosomal recessive inheritance can be also observed [13, 14]. Hernandez et al. [13] have shown by positional analysis that heterozygous mutations of the chemokine receptor gene CXCR4 account for all cases of WHIM syndrome with autosomal dominant inheritance. In contrast, CXCR4 mutations could not be detected in a minority of WHIM patients and in a single family characterized by an autosomal recessive inheritance pattern [13, 15].

All the mutations of CXCR4 that have been identified in WHIM patients affect the intracellular tail of the chemokine receptor and cause a loss of the last 10–19 amino-acid residues [13–15]. Analysis of neutrophil and T cell chemotaxis in WHIM patients has shown that truncation of CXCR4 intracellular tail confers increased cell responsiveness to the CXCR4 ligand CXCL12 (the previous term for CXCL12 was stromal derived factor 1, SDF-1) [14, 15]. Specifically, T lymphocytes of WHIM patients display a striking increase of chemotaxis, endothelial adhesion and protracted actin polymerization in response to CXCL12 [14, 15]. Upon ligand-activation, CXCR4, like the other seven-transmembrane-spanning receptors (7MSR), induces dissociation of G proteins into the active subunits $G\alpha$ and $G\beta\gamma$ that signal by activating phospholipases and ion channels, thereby leading to phosphoinositide hydrolysis and increased intracellular calcium concentration. Study of intracellular signaling in cell lines engineered to express a WHIM-associated form of CXCR4 (that lacks the last 19 amino acid residues) have shown that the mutant receptor displays a more efficient activation of G proteins and increased calcium flux in comparison to the wild-type receptor [15, 16]. At the basis of the long-lasting response of cells expressing truncating mutations of CXCR4 to the ligand, there is decreased internalization following stimulation of cells with CXCL12/SDF-1, and a faster recovery of the receptor to the cell surface when the ligand is removed from cultures [16].

Exposure of leukocytes to chemokines leads to agonist-stimulated desensitization that is partially dependent on the removal of chemokine receptors from the cell surface by internalization. In the case of CXCR4, lack of C-terminal tail prevents normal receptor internalization, thereby leading to continuous activation (Fig. 1). Study of CXCR4 signaling has revealed that after CXCL12 binding, the chemokine

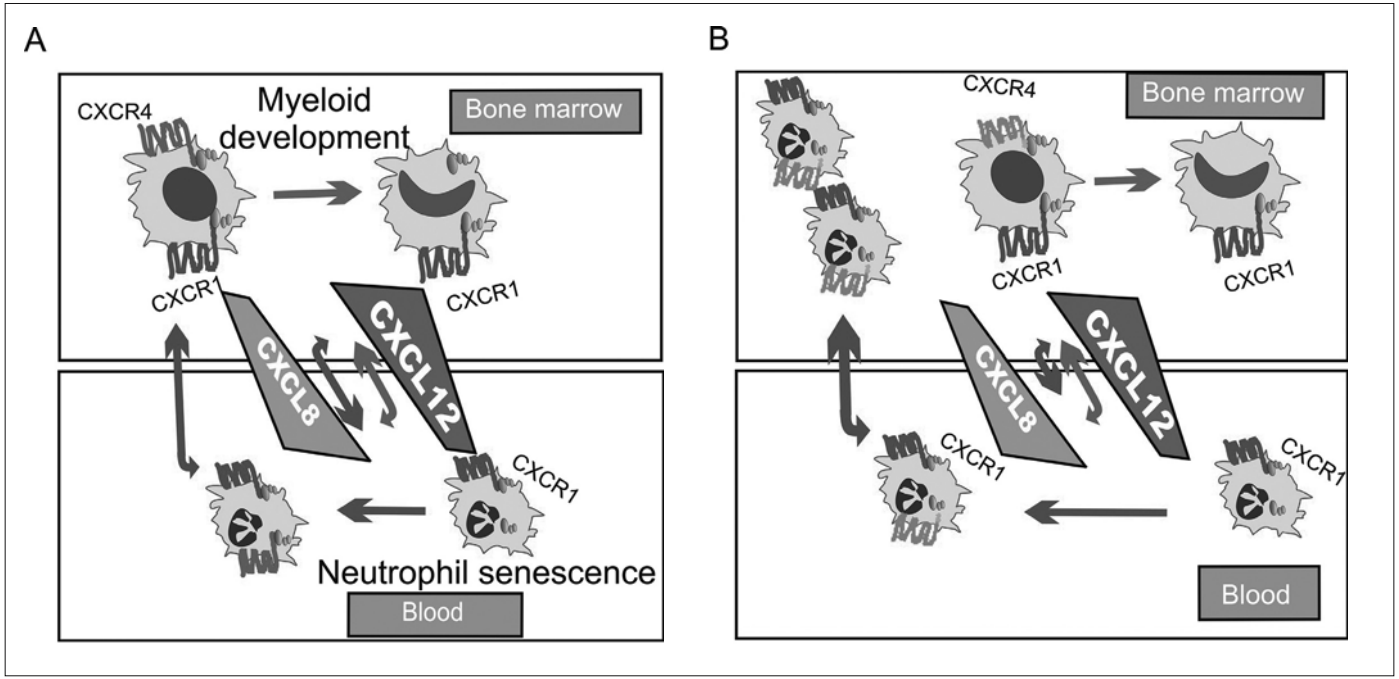

Figure 1

Leukocyte trafficking between bone marrow, lymphoid organs and blood

(A) In normal subjects, neutrophils, which express low levels of CXCR4 and are unresponsive to CXCL12, recirculate between bone marrow and blood. These cells, which express CXCR1 and CXCR2 are recruited to inflammatory sites in response to CXCL8 signals (B) in WHIM patients, senescent neutrophils which express CXCR4 at high levels and are highly responsive to CXCL12, are retained in bone marrow thereby leading to myelokathexis, which constitutes the typical manifestation of the disease. Nonetheless, elevated blood concentrations of pro-inflammatory chemokines, such as CXCL8, may mobilize these neutrophils from bone marrow to the blood circulation, thereby overcoming the condition of neutropenia during infections.

receptor is rapidly phosphorylated at the serine and threonine residues that are situated at the C-terminal tail [17]. CXCR4 phosphorylation is mediated by serine/threonine kinases that are recruited to the receptor tail to start the signaling cascade leading to receptor uncoupling from its cognate G proteins, and thereby to endocytosis. Cytoplasmic tail deletion mutants of CXCR4 resembling mutations of WHIM syndrome are less sensitive to chemokine-mediated endocytosis in response to CXCL12, and are refractory to receptor phosphorylation after CXCL12 activation [17–20]. By analogy with other G protein-coupled receptors, CXCR4 desensitization, induced by CXCL12, is mediated by G protein-coupled receptor kinases (GRK) that phosphorylate the C-terminal tail, induce recruitment of beta-arrestin and subsequent endocytosis [21]. It is likely that loss of the distal portion of CXCR4 may profoundly affect the mechanism of homologous desensitization, which is strictly dependent on the ligand-mediated internalization of the receptor. However, the analysis of beta-arrestin binding to CXCR4 mutants has shown that lack of the intracellular tail does not abolish the interaction of beta-arrestin to truncated-CXCR4, suggesting that other mechanisms are responsible for the impairment of CXCR internalization in WHIM patients [22]. Coexistence on cell surface of truncated and wild-type forms of CXCR4 may also affect internalization of the receptor. In fact, experiments of co-expression of wild-type and truncated CXCR4 demonstrate that the presence of the mutated receptor on the cell surface prevents the internalization of the wild-type receptor, probably acting by a trans-dominant mechanism [15]. This is in accordance with the autosomal dominant inheritance of WHIM syndrome and with a model of CXCR4 multimerization, in which C-terminal tail mutant receptors interact with the wild-type form of CXCR4, preventing its endocytosis [23]. The recent identification of CXCR7, formerly designated as orphan receptor RDC1, as an alternative receptor for CXCL12 suggests that its expression is mostly restricted to T lymphocytes and that this novel chemokine receptor may affect the biological response of this cell type to CXCL12 and probably influence the pathogenesis of WHIM syndrome [24].

Immune features of WHIM syndrome

In the vast majority of WHIM patients, a simultaneous reduction of neutrophils and lymphocytes is commonly observed, but neutropenia is usually more severe. It is likely that increased responsiveness of WHIM leukocytes to CXCL12 may favor their sequestration in the bone marrow, where the chemokine is expressed at the highest levels [6] (Fig. 1). On the basis of this model, circulating neutrophils, which are newly released from bone marrow and express CXCR4 at low levels on the cell surface, are refractory to CXCL12 chemotactic activity. Senescent neutrophils, which display high CXCR4 and increased sensitivity to CXCL12 migrate to the bone marrow where they can be removed from circulation by tissue macrophages

[25, 26]. In WHIM patients, the enhanced response of mature neutrophils to CXCL12 may favor their migration from blood to the bone marrow compartment, thereby accounting for myelokathexis and subsequent neutropenia. Moreover, in the context of bone marrow, CXCR4 ligation by CXCL12 may induce expression of both TNF-related apoptosis-inducing ligand (TRAIL) and of TRAIL receptors on senescent neutrophils, thereby increasing their sensitivity to apoptotic stimuli [27]. This is in accordance with the increase in the number of apoptotic neutrophils observed in the bone marrow of WHIM patients [28].

This hypothetical picture can be rapidly reverted during infections or after G-CSF treatment of WHIM patients. Under these conditions, circulating neutrophil numbers increase due to an acute release of cells from the bone marrow [29, 30]. This residual capacity of bone marrow to release neutrophils under inflammatory circumstances is reflected in the mild neutropenia and the favorable outcome of infections that has been reported for most WHIM patients.

Although the absolute number of lymphocytes is decreased, analysis of lymphocyte subpopulations may not reveal any obvious abnormality, because T cell percentages, including CD4 and CD8 subsets, are within the normal range [14, 30–32]. In addition, functional studies of T cell activation in response to mitogens and delayed-type hypersensivity to antigens do not usually reveal any obvious defect of cell-mediated immunity [32–34]. However, a careful analysis of naïve/memory subsets of T cells in WHIM patients usually demonstrates a prevalence of effector/memory T cells, whereas naïve T cells are markedly reduced [14]. These abnormalities of T cell subsets are associated with normal or even increased thymic output as measured by determination of T cell receptor excision circles (TRECs) levels, suggesting that thymic function is probably normal in WHIM patients [14]. Indeed, WHIM patients show neither susceptibility to common opportunistic viral infections nor require special precautions for live viral vaccines, suggesting that the formation of warts that is observed in some WHIM patients may arise from a specific mechanism of selective susceptibility to human papillomavirus (HPV) infection. Nonetheless, two cases of B cell lymphoma, following Epstein-Barr virus (EBV) infection, have been reported, suggesting that EBV infections may also represent a potential threat for WHIM patients [35, 36].

Analysis of the B cell compartment has shown that immunoglobulin levels are subjected to frequent changes and, especially in adult patients, may be normal [30, 32, 34]. The study of antibody production has shown a normal increase of antigen-specific immunoglobulins in children who were immunized with tetanus toxoid; however, this response was followed by dramatic decrease of antibody titers in the subsequent months, suggesting that memory B cells and plasma cells are unable to maintain a normal antibody secretion [14, 30, 37]. This change in antibody levels is reflected in the reduced circulating fraction of memory B cells ($CD19^+/CD27^+$ cells), as compared to naïve B cells, suggesting that B cell homeostasis is also altered in WHIM patients [14]. While CXCR4 is required for the generation of the early prog-

enitors of B cells and for their retention in the bone marrow [4, 38], it becomes more important for the migration of B cell subsets to the respective niches where CXCL12 and other homeostatic chemokines are expressed [38, 39]. Although CXCR4 is expressed in B cells at many phases of their development in lymph nodes and spleen, their chemotactic response to the ligand CXCL12 is tightly regulated at all these stages. In fact, high expression of CXCR4 on memory B cells and plasma cells leaving the lymph node allows them to recirculate among lymphoid organs and bone marrow where resident plasma cells are responsible for maintaining long lasting immunoglobulin production [40]. In WHIM patients, enhanced response to CXCL12 of memory B cells and plasma cells may prevent their release from bone marrow and their correct positioning in lymph nodes, resulting in deregulated B cell homeostasis.

Clinical features and treatment of WHIM syndrome

In 1964, Zuelzer [31] and Krill et al. [41] independently reported a case of a 10-year-old female who presented neutropenia associated to myelokathexis; since then, at least 30 cases have been identified on the basis of clinical and/or genetic features [13, 42, 43]. Analysis of the reported cases has shown broader variability in clinical manifestations than originally suspected, and suggested multiple genetic causes. While the majority of WHIM patients present hypogammaglobulinemia and neutropenia, favoring the development of recurrent infections and warts from infancy, in a minority of these patients, neutropenia is often moderate (PMN < 1000 cells/μl) and immunoglobulins are normal or slightly reduced. Warts are usually not observed before 3 years of age but can rapidly increase in numbers and extension in the following years. The lesions, which affect mainly the extremities and less often the genitalia, are caused by common genotypes of HPV, and are usually refractory to both medical and surgical treatments [32, 37]. WHIM patients who have multiple disseminated warts located at the genitalia, may develop cervical and vulval premalignant lesions, which require surgical ablation [29]. A few WHIM patients have developed EBV-related lymphoma and Kaposi's sarcoma [35, 36, 42], but it is unclear whether these tumors are caused by defective surveillance of immune system, or by intrinsic susceptibility of lymphocytes to neoplastic transformation.

The recent identification of the pathophysiology of WHIM syndrome has encouraged the search for novel treatment strategies targeted at the mechanism of the disease which could improve its prognosis significantly. At this time, the treatment of the disease is essentially based on use of G-CSF and/or immunoglobulin infusions in patients with neutropenia or hypogammaglobulinemia, respectively. However, the use of AMD3100 or of other CXCR4 antagonists, which are already in clinical trial, might constitute a specific treatment of the homeostasis defect observed in WHIM patients.

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