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Migration of dendritic cells across blood and lymphatic endothelial barriers

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Summary

Dendritic cells (DC) are professional antigen presenting cells which play a pivotal role in the activation of adaptive immunity. Tissue invasion by pathogens induces the recruitment of blood DC to the site of infection and contributes to their subsequent migration to secondary lymphoid organs. This complex process relies on the expression and regulation of receptors for chemotactic factors on the surface of migrating DC and on the activation of adhesion molecules which allow DC to properly interact with both blood and lymphatic vessels. In the absence of correct tissue localization, DC fail to promote proper immune responses. Therefore, the interaction of DC with endothelial cells represents a fundamental step for DC biology.

Keywords
Dendritic cells, chemokines, endothelial cells, adhesion molecules, signal transduction

Thromb Haemost 2006; 95: 22–8

Introduction

Dendritic cells (DC) are a heterogeneous population of potent antigen presenting cells which are recruited from the blood into peripheral tissues where they reside in an immature state and exert a sentinel function for incoming antigens (1–4). Upon microbial contact and stimulation by inflammatory cytokines, DC take up antigens and traffic via the afferent lymphatics into the T cell area of the draining lymph node to initiate immune responses (2, 5–7). Therefore, DC need to transmigrate first across endothelial cell barriers to reach peripheral tissues and then across lymphatic endothelium (8). The proper localization of DC to secondary lymphoid organs and their recruitment at sites of inflammation in response to chemotactic stimuli are critical events for optimal immune response (9–11). Migration of DC into tissues depends on a cascade of discrete events which include the induction of chemokine, the activation of chemokine receptors and the regulation of adhesion molecules (5, 6, 12, 13). DC subsets possess a distinct migratory pattern. Myeloid blood CD11c^{+} DC migrate in response to a wide array of inflammatory chemotactic agonists produced at the peripheral sites of infection and immune reaction (7, 14, 15). On the other hand, CD123^{+} plasmacytoid DC are believed to enter lymph nodes across blood high endothelial venules (16). The expression and regulation of functional chemotactic receptor for chemotactic factors and the selective usage of adhesion molecules are likely to be responsible for the different distribution of DC subsets *in vivo*.

Chemokines and chemokine receptors

Chemokines are a superfamily of small proteins which play a crucial role in immune and inflammatory reactions and in viral infection (12, 17). Based on a cysteines 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 to 10), seven CXC (CXCR1 through 7), one CX3C (CX3CR1) and one XCR (XCR1) receptors have been identified. Receptor expression is a crucial determinant of

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Received October 13, 2005
Accepted after revision December 6, 2005

Financial support:
This work was supported by AIRC (Associazione Italiana per la Ricerca sul Cancro), MIUR (Ministero dell'Istruzione Università e Ricerca), Association for International Cancer Resarch (grant no. 04–223) and by Fondazione Berlucchi. We acknowledge support from the European Commission FP6 'Network of Excellence' initiative under contract no. LSHB-CT-2004–512074 DC-THERA.

Prepublished online December 12, 2005 DOI: 10.1160/TH05–10–0674

the spectrum of action of chemokines and dictates most of the differences observed in the chemotactic response of immature versus mature DC (18). 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. In addition, there are at least three promiscuous chemokine "receptors" (D6, DARC and CCX-CKR) which do not elicit migration or conventional cellular responses (19).

Chemokines elicit their biological activities through interaction with seven transmembrane domain proteins which form a distinct group of structurally related proteins within the GTP-binding proteins-coupled receptors superfamily. The presence of a significant degree of identity among chemokine receptors (25 to 80% at the aminoacid level) suggests a common origin from a common ancestor, also testified by the presence of structural features more frequently observed in chemokine receptors than unrelated seven transmembrane domain receptors, such as an acidic N-terminal segment, a short basic third intracellular loop, the presence in most cases of one cysteine residue for each of the four extracellular domains coordinating two disulfide bridges, and some conserved sequences (a LxxLxxDLLF motif in TM2; a DRYLAIVHA motif or subtle variations of it in IL2; a NPXXY motif in TM7) of proven relevance for G protein coupling and activation (20). In particular, the major biological function of chemokine receptors, i.e. their ability to induce chemotaxis, is tightly dependent on coupling and activation of Gα_i, as indicated by the ability of pertussis toxin to completely block this function. Activated G protein subunits directly stimulate downstream signal transducers, including phospholipase C (PLC)β_2 and β_3 isoenzymes, the phosphoinositide 3-kinase (PI3K) isoenzymes, the cytosolic phospholipase A_2 (cPLA$_2$), the c-Src family tyrosine kinases, and mitogen-activated protein kinases (MAPK) (21). PLCβ activation leads to the release of diacylglycerol and inositol-1,4,5-trisphosphate, which is responsible for the induction of calcium transients. Though this is the most frequent signalling pathway investigated, leukocyte migration is not dependent on PLCβ activity and consequent calcium transients, which are required for PLA$_2$ activation and arachidonic acid release (22). On the other hand cell migration requires the $\beta\gamma$-dependent activation of PI3K, which in turn regulate the contractile apparatus through recruitment and activation of pleckstrin homology domain containing proteins, such as guanine-nucleotide-exchange factors and protein kinase B (23). PI3Ks are also directly responsible for the activation of a classical signal transduction pathway which through Shc, Grb2, Ras and Raf ends on the activation of MAPKs. The α_i subunit has also been associated with the activation of Src-like kinases, such as Fgr and Lck, and other downstream effectors such as FAK and Pyk-2. The role of MAPK activation in cell migration has been questioned, and their possible involvement in other biological functions, such as transcription regulation, has also been suggested (24).

Phosphorylation and internalization of chemokine receptors results in the transient interruption of responsiveness to chemokines, a process termed cellular desensitisation. Homologous and heterologous desensitization both rely on the activity of serine-threoninine kinases, represented by G-protein coupled receptor kinase (GRK)2 and protein kinase C (PKC), respectively. While in the case of heterologous desensitization the activation of PKC is supported by G protein activation, in the case of GRK-mediated homologous desensitization the activation pathways are G protein-independent and presently unclear. In both cases however, phosphorylation of conserved residues in the receptor COOH-terminus creates a docking site for the recruitment of arrestins, which displace the G protein, thus leading to signalling termination, and couple the receptor to adaptor proteins which support clathrin-dependent receptor internalization (25).

Chemokines and dendritic cells

Immature DC express a unique repertoire of inflammatory chemokine receptors (e.g. CCR1, CCR2, CCR5, CCR6) which are responsible for the recruitment of immature DC, or their precursors, to the inflamed tissues (13, 18). 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. 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, which can precede chemokines action (Table 1).

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) (26). The formyl peptide receptor (FPR) family includes multiple proteins, two of them FPR and FPRL2 were found to be expressed by immature DC (27). FPR is the fMLP receptor, whereas FPRL2 is activated by the WKYMVM hexapeptide and F2L, a highly conserved acetylated 21-aminoacid peptide derived from the cleavage of the N-terminus of the intracellular

Table 1: Chemotactic receptors expressed by blood dendritic cell subsets.

Receptor	myeloid DC		plasmacytoid DC	
	Expression	Function	Expression	Function
CCR1	+/-	+	+/-	-
CCR2	++	+	++	-
CCR4	+	+	+	-
CCR5	+	+	++	-
CCR6	+/-	+	+/-	-
CCR7[a]	+	+	+	+
CXCR3	+	+	++	+[b]
CXCR4	++	+	++	+
FÜR	++	+	+	-
FPRL1	++	+	ND[c]	-
FPRL2	++	+	-	-
ChemR23	+	+	++	+
PAFR	+	+	ND	-
C5a	+	+	ND	ND

[a]all the receptors are functional on immature DC, with exception of CCR7 that is expressed in a functional manner only by mature DC. [b]functional in association with CXCR4 activation by CXCL12 in classical chemotactic assays. It was also reported that CXCR3 is functional when its ligands are tested in a membrane bound form (see text). [c]ND, not done

heme-binding protein (HBP) (28, 29). DC express functional receptors for platelet activating factor (PAF), a bioactive phospholipid that derives from the activation of PLA_2 (30, 31). PAF plays a crucial role in the retention of DC into peripheral tissues and may thus be relevant in the accumulation of DC observed at pathological sites, such as in atherosclerotic plaques (31).

Recent work has also shown that DC may have a pivotal function in the induction of autoimmunity (32). Histidyl-(HisRS) and asparaginyl-(AsnRS) tRNA synthases, two cytoplasmic proteins involved in protein synthesis which function as autoantigens in myositis, were shown to induce the migration of immature DC through the interaction with CCR5 (33). 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 (34). Therefore, self-antigens may promote autoimmunity also through the recruitment of antigen presenting cells at sites of tissue injury.

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 (35–37). DC activation is associated with the acquisition of a mature phenotype consisting in an up-regulation of co-stimulatory 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 which are expressed at the luminal side of high endothelial cells and in the T cell rich areas of secondary lymphoid organs, such as tonsils, spleen and lymph nodes (36, 38, 39). The crucial role of CCR7 and its ligands is documented *in vivo* in mice deficient for these proteins (10, 11). CCR7 expression by DC is also required for the entry of DC into lymphatic vessels at peripheral sites both in steady state and inflammatory conditions (40, 41). 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 (41). The relevance of chemotactic factors in DC migration *in vivo* has been clearly documented in mice lacking the gamma isoform of phosphoinositide-3 kinase (PI3Kγ) (9). PI3Kγ is located downstream seven-transmembrane chemotactic receptors and plays a non-redundant role in cell migration in response to chemotactic agonists (23). 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 PI3K$\gamma^{-/-}$ mice and most importantly, this defect was associated with a defective ability of PI3K$\gamma^{-/-}$ mice to generate a specific immune response (9).

Table 2: Lymphatic endothelial cell and blood vascular endothelial cell preferential gene expression. (Adapted from Hirakawa et al. Am J Pathol 2003; 162: 575-86 and Saharinen et al. Trends Imm 2004; 25: 387-95.)

	Blood EC	Lymphatic EC
Adhesion and transmembrane molecules	Integrin α5	Integrin α9
	Integrin β5	Integrin α1
	ICAM-1, ICAM-2	Macrophage mannose receptor 1
	N-cadherin	LYVE-1
	Selectin P, selectin E	Podoplanin
	CD44	
Cytokines, chemokines, growth factors and their receptors	IL-8, IL-6	IL-7
	CCL2	SDF-1
	CXCR4, CCRL2	SLC
	IL-4 receptor	CCL20
	Axl	CCL5
	NRP1	Angiopoietin-2
	VEGF-C, PIGF	VEGFR-3
Cytoskeletal proteins	Vinculin	Desmoplakin I and II
	Claudin 7	Plakoglobin
	Actin, α2	α-actinin-2 associated LIM protein
	Profilin 2	
	β-catenin	
ECM molecules	Collagens 8A1, 6A1, 1A2	Matrix Gla protein
	Laminin, γ2, α5	Reelin
	Versican	TIMP-3
	Proteoglycan	
	MMP-1, MMP-14	
	uPA, PAI-1	
	Cathepsin C	

Interaction of DC with blood endothelium

Migration is a multistep process which involves the adhesion of DC with endothelial cells and the interaction with physical obstacles, such as basement membranes and collagen meshwork. Circulating DC first need to tether to endothelial cells through the interaction of E-and P-selectins with their respective ligands (42). Firm adhesion between DC and endothelial cells is dependent on the engagement of chemotactic receptors and subsequent integrin activation on DC (43–45). *In vitro*, DC express CD31, the β2 integrins LFA-1, Mac-1 and p150,95, the β1 integrins VLA-4 and VLA-5 which mediate their binding to both resting and activated endothelial cells (EC) and to EC-derived extracellular matrix (ECM) (44) (Table 2). Transmigration of DC across an EC monolayer, unlike adhesion, involves the engagement of CD31. Activation of endothelial cells by oxLDL, TNF-α, or hypoxia strongly increases DC adhesion and transmigration (44, 46). Interestingly, EC apoptosis also markedly enhances DC adhesion (47). *In vivo*, mice defective in β2 integrin functions (48) and α6 integrin (49) showed a reduced ability in the migration of cutaneous DC to the draining lymph nodes. An accumulation of DC was reported in atherosclerotic areas (50) and in vascular regions prone to develop atherosclerosis (51, 52). Furthermore, modulation of the endothelial nitric oxide synthase (NOS) is involved in DC-EC interaction (53). Release of nitric oxide by activated endothelial cells inhibits DC adhesion and transmigration, whereas inhibition of nitric oxide synthesis increases DC-endothelial cell interaction (46). These evidences provide

new insight into DC-endothelial cell interaction, which plays an emerging role in inflammation and atherogenesis.

JAM-A (junctional adhesion molecule 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 (54, 55). JAM-A is expressed by endothelial cells, platelets and leukocytes and localizes in close proximity to tight junction components. Other members of the JAM family (JAM-B and JAM-C) have been identified, but they have a more restricted distribution (56). JAM-A comprises an extracellular domain, a transmembrane segment and a cytoplasmic tail. The extracellular domain forms parallel dimers (57) and binds several ligands such as JAM-A itself (58, 59), the leukocyte integrin αLβ2 (60) and the reovirus protein s-1 (61). 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 αLβ2 (60).

We have recently described that DC express JAM-A and that this expression has a biological relevance both *in vitro* and *in vivo* (62). DC generated from JAM-A-defective mice showed a selective increase in random migration and transmigration across lymphatics. Conversely, migratory capacity in response to chemotactic agents was not affected, indicating that only random motility in DC is influenced by JAM-A. No difference in DC random migration across blood endothelial cells was observed. One possible explanation for the different migration of DC across lymphatic and blood vessels may be ascribed to the fact that lymphatics present weak intercellular junctions with a specific molecular organization, compared to blood vessels (63, 64). JAM-A$^{-/-}$ mice showed increased localization of skin DC to lymph nodes and an exaggerated response in a contact hypersensitivity model, which was directly related to an increased migration of DC (62). Therefore, JAM-A possesses a non-redundant role in the regulation of DC trafficking and function.

Interaction of DC with lymphatic endothelium

The migration pathways which lead DC from periphery to secondary lymphoid organs through the lymphatics, are still poorly understood and may involve multiple signals (8). As mentioned above, CCR7 expression of maturing DC is required for their efficient entering into lymphatic vessels (41). In addition, a recent study proposed that CCR8 and its cognate ligand CCL1 are involved in the emigration of mouse DC from the skin (65). A role for CCR4/CCL22 has been also described in the formation of T cell-DC cluster in both inflamed skin and lymph nodes (66).

Studies on the biology of lymphatic endothelium have been limited by the complexity of *in vitro* culturing of these cells. Both human and mouse lymphatic endothelial cells were isolated and characterized in short term cultures, but unfortunately cell growth was limited to a few *in vitro* passages (67, 68). We have recently reported the characterization of a mouse endothelial lymphatic cell line (MELC) (69, 70). MELC express the lymphatic endothelial markers VEGFR-3/Flt-4 and podoplanin and the chemokine decoy receptor D6; moreover they express endothelial markers and adhesion molecules relevant for the physiological circulation of leukocytes from tissues to secondary lymphoid organs through the lymphatics, such as CD34, ICAM-1, VCAM and JAM-A, but not CD31, VE-cadherin and E-selectin (Table 2). Upon stimulation with TNF-α they upregulate the expression of adhesion molecules such as ICAM-1 and VCAM and produce increased amounts of IL-6 and CCL2.

D6 is a chemokine scavenger receptor strategically located on endothelial cells lining afferent lymphatics (71) and has been suggested to have a role in limiting and preventing excessive transfer of inflammatory chemokines to lymph nodes (72). By recognizing CCL22, D6 expressed on lymphatic endothelial cells may regulate the traffic of CCR4-expressing cells, such as DC during migration to lymph nodes via afferent lymphatics (72). In the cornea, a close interaction between DC and the eye lymphatic axis was reported (73). Under steady state conditions, cornea resident DC express VEGFR-3 and the expression of this receptor, and its ligand VEGF-C is upregulated during inflammatory reactions (73). These findings suggest a potential link between lymphangiogenesis and immunity. The induction of lymphatic vessels into the cornea might facilitate the delivery of antigen presenting cells to draining lymph nodes which can contribute to immunogenic inflammatory reactions, such as rejection of corneal grafts (74). The model proposed by Hamrah et al. (73) suggests that the increased cytokine secretion during inflammation leads to increased expression of VEGFR-3 and VEGF-C; the signalling through VEGFR-3 can then lead either to lymphangiogenesis or DC recruitment. VEGF-C could also promote the molecular interactions of DC with lymphatic endothelial cells, inducing the entry of DC into lymphatic vessels, similarly to the promotion of tumor metastatic spread via lymphatics (75, 76).

Selective recruitment of dendritic cell subsets

Blood DC includes two main subsets, myeloid and plasmacytoid DC. These two cell subsets differ for the expression of some membrane molecules and for the ability to release cytokines. Myeloid DC produce large amounts of IL-12 whereas, plasmacytoid DC secrete high levels of type I interferon (16, 77).

The expression of chemokine receptors on blood myeloid DC and plasmacytoid DCs is, in general, fairly similar (78). Both subsets express relatively high levels of CC chemokine receptor CCR2 and CXCR4. In contrast, CCR1, CCR3, CCR4, CCR6, CXCR1, CXCR2, and CXCR5 are very weakly, or not expressed, on both circulating myeloid DC and plasmacytoid DC. Conversely, CCR5 and CXCR3 expression is clearly divergent in the two subsets, being low on blood myeloid DC, but high on plasmacytoid DC (78, 79). In contrast with the overall similar pattern of chemokine receptor expression, circulating myeloid DC and plasmacytoid 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 (78) or in transmigration assays across an endothelial cell monolayer (80). In classical chemotaxis assays, the ligands of CXCR3, namely CXCL9, CXCL10 and CXCL11, are inactive in inducing plasmacytoid DC migration but can promote plasmacytoid DC migration in response to CXCL12 (81, 82).

DC subsets also differ for their ability to interact with endothelial cells *in vitro*. Myeloid DC were shown to vigorously

migrate across endothelium in the absence of any chemotactic stimuli, whereas spontaneous migration of plasmacytoid DC was limited (80). On the contrary, the interaction with an endothelial cell monolayer greatly favored transmigration of plasmacytoid DC in response to CXCL1 and CCL5 (80), ChemR23 (see below) (15) and in response to CXCR3 ligands (79).

Plasmacytoid DC are normally absent from peripheral tissues and they are believed to migrate constitutively from the blood into lymph nodes through high endothelial venules (83–85). This migration is mediated by L-selectin and is increased by an E-selectin-dependent mechanism, when lymph nodes are exposed to inflammatory conditions (85–87). Accordingly, plasmacytoid DC express high levels of CD62 ligand and PSGL1, the counter ligands of P- and E-selectins (79, 83). Recruitment of plasmacytoid DC to non-lymphoid tissues is observed in some pathological conditions, such as autoimmune diseases (i.e. lupus erythematosus disease, psoriasis and rheumatoid arthritis) (77, 88, 89), allergic diseases (i.e. contact dermatitis and in nasal mucosa polips) (90) and in tumors (91–93). However, the mechanisms leading to the recruitment of plasmacytoid DC to inflammatory sites remain unresolved. Recently, chemerin, a new chemotactic factor was proposed as a key signal for the recruitment of plasmacytoid DC into pathological tissues (15). Chemerin is a novel chemotactic protein identified as the natural ligand of ChemR23, a previously orphan G protein-coupled receptor expressed by immature dendritic cells and macrophages (94). Chemerin was purified from ovarian cancer ascites and found to correspond to the product of the Tig-2 gene. 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 (95) or released by leukocytes convert prochemerin into a full agonist of ChemR23 by proteolytic removal of the last six amino acids (96). ChemR23 is expressed by blood plasmacytoid DC, and chemerin was found active in inducing their transmigration across an endothelial cell monolayer. *In vivo* ChemR23 was expressed by plasmacytoid DC localized in reactive lymph nodes and in skin lesions of lupus erythematosus patients. Of note, chemerin was selectively expressed by high endothelial venules 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 plasmacytoid DC to lymph nodes and to pathological tissues (15).

Concluding remarks

DC are professional antigen presenting cells. To accomplish their biological function, 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 include the interaction with both blood and lymphatic endothelium and the response to chemotactic signals. In the past few years, many chemokines were 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.

Abbreviations

DC, dendritic cells; TM, transmembrane segment; PLC, phosholipase C ; PI3K, phosphoinositide 3-kinase; $cPLA_2$, cytosolic phospholipase A_2; GRK, G-protein coupled receptor kinase; FPR, formyl peptide receptor; PAF, platelet activating factor; IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; EC, endothelial cells; ECM, extracellular matrix.

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