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Generation, migration and function of circulating dendritic cells

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Tissue-resident dendritic cells (DCs) that migrate from peripheral sites to lymphoid organs are essential in the initiation of adaptive immune responses and for the maintenance of peripheral tolerance, and have been extensively studied. By contrast, blood-borne DCs represent a heterogeneous population, the origin, destination and function of which are still unclear. Recent studies have shown that circulating DCs capture blood-borne antigen and transport it into the extravascular space of lymphoid tissues for processing and presentation. Other findings suggest that a fraction of tissue-resident DCs might enter the blood after having acquired antigen in the periphery. Together, these studies imply that circulating DCs might modulate immune responses by translocating antigenic material from its point of origin to remote target tissues.

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Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) found in almost all peripheral tissues as well as in primary (thymus and bone marrow [BM]) and secondary (lymph nodes [LNs], Peyer's patches [PPs] and spleen) lymphoid organs (SLOs). Their function is to collect antigenic material in the periphery and to transport it to SLOs, where it is scanned by naïve T lymphocytes that continuously recirculate through these organs [1]. DCs are equipped with numerous receptors that detect signs of 'danger' in the surrounding environment. Signals associated with an ongoing infection, such as pathogen-associated molecular patterns, pro-inflammatory cytokines or byproducts of tissue damage, cause DCs to undergo phenotypic changes (maturation) that maximize their ability to induce proliferation of naïve and memory T lymphocytes [2]. By contrast, in the absence of inflammation, DCs capture and present self-antigens, but remain phenotypically in a non-activated immature state. In this context, successful T-cell receptor engagement results in abortive proliferation and anergy of reactive T cells. This mechanism is, at least in part, responsible for the establishment of peripheral tolerance [3].

The migratory properties of DCs are of fundamental importance for their function and have been extensively investigated. According to the current paradigm (Figure 1), differentiation and migration of DCs are parallel processes that follow a unidirectional path — from progenitors in the BM [4,5••] to committed precursors in the blood [6], replenishing the DC pool in the periphery. After migrating into peripheral tissues, DCs survey the environment, sample antigenic materials, and travel through afferent lymphatics to reach draining LNs where they present the collected antigens to T cells [7•]. DCs that colonize lymphoid organs directly from the blood are usually regarded as a static population of cells that form a network of SLO-resident APCs; these phagocytose soluble material from the lymph or receive antigen carried to them by migratory DCs [8]. Importantly, this paradigm has been somewhat challenged by recent studies, in which some DCs in lymphoid [9••] and non-lymphoid [10] organs were found to proliferate, suggesting that part of the DC pool might be replenished *in situ* and not (or not only) by BM-derived circulating progenitors.

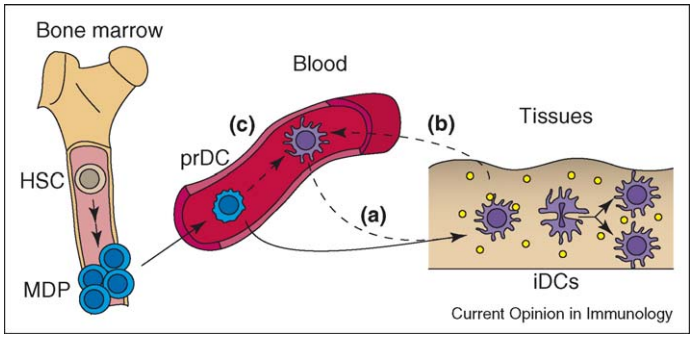
Although most DC functions appear to be confined to the draining LN, in some circumstances systemic dissemination of DCs that carry antigens administered locally has been observed [11–14]. The existence of such a route of antigen transport could have interesting implications for fundamental immune mechanisms, such as the reactivation of memory cells in organs other than peripheral LNs and the establishment of central tolerance towards tissue-specific antigens, but the exploration of DC trafficking in the circulation has been hampered by the scarcity of these cells and by their intricate subdivision into multiple phenotypic subsets.

Here, we review our current understanding of the migration and function of circulating DCs and how they might affect immunity and tolerance.

DCs in blood and lymph

It is commonly assumed that DCs circulating by way of the blood reflect committed BM-derived precursors on their way into peripheral organs. Although such precursors have been identified in the mouse [6], several other

Figure 1



Putative origins of circulating DCs. The solid arrows represent migratory and developmental pathways that have been recapitulated experimentally, from common macrophage and DC progenitors (MDP [5••]) in the BM, to committed circulating precursors (prDC) that replenish peripheral tissues with immature DCs (iDC), some of which might also proliferate *in situ*. The dotted arrows correspond to the three migration and differentiation routes that have been postulated to explain the presence of *bona fide* DCs (purple) in the blood. **(a)** They could represent a transition step between prDCs and tissue-resident iDCs; **(b)** they could be DCs that are re-entering the bloodstream after having assumed temporary residence in peripheral tissues; or **(c)** they could be BM-derived DCs that remain in the circulation to sample blood-borne antigens for processing and presentation. HSC, hematopoietic stem cell.

subsets of DCs in various maturation states can be detected in human and mouse blood [15].

DCs in human blood

Human peripheral blood has been a common source of material for DC studies, either by isolation and *in vitro* differentiation of monocyte-derived DCs [16] or by extensive purification of the rare population of cells that already express DC markers [17]. Human circulating DCs are defined as HLA-DR$^+$lineage$^-$, where the term 'lineage' includes all cells that express markers for lymphocytes, granulocytes or monocytes, although there are differences in the choice of the lineage-specific antibodies used by different investigators. The HLA-DR$^+$lineage$^-$ population can be broadly divided in two subsets: CD11c$^-$CD123$^+$ plasmacytoid DCs (pDCs) and CD11c$^+$CD123$^-$ myeloid DCs (myDCs) [18]. Until recently, the circulating myDC population was thought to be mainly composed of DC precursors, but with the development of more discerning markers it was found that blood myDCs can be assigned to at least two different phenotypic classes, characterized by mutually exclusive expression of CD1c (also known as blood DC antigen 1 [BDCA1]) or BDCA-3 [19], with the possible addition of a third population that expresses CD16 [20]. This implies that further complexity can be found in this cell population and raises the possibility that not all blood DCs share the same history and migratory properties. For example BDCA-1$^+$ as well as BDCA-3$^+$ blood DCs have a transcriptional profile more similar to full-fledged DCs harvested from SLOs than to other circulating DC subsets [21], which suggests that they could give rise to or originate from tissue-resident DCs.

DCs in mouse blood

CD11c is usually considered a better marker for mouse DCs than it is for human DCs [22]. Although some mouse lymphocytes express low levels of CD11c [23], it is rather straightforward to exclude these cells by their expression of markers for B, T or natural killer lineage, which are not expressed on *bona fide* DCs. Circulating DC precursors that give rise to both myDCs and pDCs *in vitro* have been identified in the mouse and are characterized by little to no expression of MHC class II and low to intermediate expression of CD11c [6,24]. This leaves unaccounted for the small but distinct subset of CD11c^{hi}MHC-II$^+$ DCs in mouse blood, the number of which has been estimated to range from 1000 cells/mouse [24] to 20 000 cells/mouse [25]. The small number of these cells should not make us underestimate their potential importance, because a single DC in lymphoid organs can be scanned by more than 10 000 T cells every day [26]. It is also possible that DCs are quickly removed from the circulatory pool by efficient recruiting mechanisms; in fact, the half-life of adoptively transferred splenic DCs in the murine circulation is in the order of 2–4 hours (R Bonasio *et al.*, unpublished), therefore small numbers of circulating DCs at steady-state could still translate into a significant daily flux of cells from the blood to target organ(s). Finally, the above-mentioned studies were carried out in animals that were protected from environmental pathogens and were therefore immunologically naïve. It is not yet known whether the number and maturation state of circulating DCs might be modulated by infection and inflammation. As for humans, the precursor–product relationship of circulating *bona fide* DCs with peripheral or SLO-resident DCs has not been established.

Together, these observations are consistent with at least three non-exclusive mechanisms to explain the presence of phenotypically distinct subsets of circulating DCs, as depicted in Figure 1: (a) CD11c^{hi} blood DCs might be the immediate BM-derived precursor of peripheral tissue and

SLO DCs (however, see update); (b) fully differentiated peripheral DCs might be able to reenter the circulation to maintain their phenotypic identity; or (c) blood-borne DC precursors might be able to differentiate into phenotypically distinct DCs without leaving the circulation.

DCs in efferent lymph

It has long been thought that once peripheral DCs reach a draining LN they do not leave again, that is they do not enter the efferent lymph, which is channeled back to the blood by way of the thoracic or the right lymphatic ducts. However, cells that have phenotypic characteristics ascribed to DCs have been detected in low numbers (1 in 5000–10 000 cells) in the efferent lymph of rats [27,28] and sheep [7•,29]. Our own experiments on efferent lymph collected from a mouse thoracic duct fistula found a discrete $CD11c^+$ population that was negative for T, B and natural killer cell markers [30••]. This population represented 0.01% of the total cellular flux in the thoracic duct. Given a total leukocyte flux of $\sim 7 \times 10^6$ cells/hour [31], we estimated that ~20 000 DCs reach the systemic circulation every day. The scarcity of $CD11c^+$ cells in mouse lymph and the technical difficulties in collecting it have, to date, prevented an extensive phenotypic and functional characterization of these cells.

Techniques for tracking and imaging DCs *in vivo*

How do $CD11c^{hi}MHC\text{-}II^+$ (i.e. fully differentiated) DCs reach the blood? What are their trafficking properties? Which molecules guide their migration? What immunological consequences are elicited by these cells? To address these questions a number of experimental approaches have been developed for the study of DC trafficking: some are aimed at tagging endogenous DCs, usually in a specific tissue, to follow their movement in time; others rely on the *ex vivo* labeling of bulk numbers of DCs, followed by adoptive transfer to recipient animals.

Labeling of endogenous DCs

A frequently used method to label endogenous DCs takes advantage of their phagocytic capacity by delivering molecular labels to tissues in which DCs are relatively frequent (e.g. the skin) and studying their uptake and transport. For example, the small organic fluorochrome fluorescein isothiocyanate (FITC) can be applied epicutaneously and is picked up by Langerhans cells (LCs) and dermal DCs, which then migrate to the LN; here, they can be detected by flow cytometry or microscopy [32]. Alternatively, fluorescent beads can be injected and phagocytic cells that have incorporated the beads can then be tracked; this approach preferentially targets monocyte-derived DCs [33]. DCs that populate the airways have also been tagged by intranasal administration of carboxyfluorescein succinimidyl ester (CFSE) [14,34]. For functional studies, labeling of peripheral DCs has been coupled to antigen delivery by administration of antigens conjugated to fluorochromes [35] or fused to fluorescent proteins [36].

These approaches share the advantage of minimizing the *ex vivo* manipulation of DCs, providing a physiological setting in which to study their migration. However, they carry the risk that the precise location(s) at which cells are labeled can be difficult to control. For example, small molecules and proteinaceous antigens are known to reach local LNs by way of the lymph, and might be acquired by resident DCs [36]; moreover, as antigenic material is handed over from migratory to LN-resident DCs [34], the fluorescent label can also be transferred. Thus, whereas these strategies can faithfully track the fate of exogenous antigen, one must be careful when drawing conclusions about the migratory history of the labeled DCs. [8]. Another potential drawback is that the labeling intensity decreases with time, because normal metabolism and protein turnover dilute or destroy the fluorescent molecules.

To overcome this hurdle and to achieve more reliable labeling of endogenous DCs, genetic approaches are desirable. Making use of transgenes driven by the CD11c promoter, two groups have obtained permanent [37] or conditional [38] expression of green fluorescent protein (GFP) in DCs *in vivo*. However, this approach is somewhat limited by the specificity of CD11c expression, which is not entirely exclusive to DCs. In addition, $CD11c^+$ cells in all tissues of these transgenic mice express GFP simultaneously, making the technique unsuitable for tracking isolated subpopulations, unless adoptive transfer strategies are employed (see below).

A particularly elegant genetic approach was developed by Garg *et al.*, [39] who took advantage of a transgenic mouse strain that carried a *lox*P-flanked neomycin cassette followed by the bacterial *lacZ* gene [40]. Gene-gun vaccination of these animals with gold particles carrying a construct that expressed the Cre recombinase resulted in efficient deletion of the intervening cassette and permanent expression of the β-gal reporter by migrating DCs; these could be detected in the draining LN up to two weeks after vaccination [39]. A possible caveat associated with this technique is that the presence of unmethylated CpG motifs in bacterial plasmid DNA is known to activate skin DCs [41], which could alter the migration of the tagged cells.

Adoptive transfer techniques

Although elegant and biologically sound, the experiments discussed above can be limited by their inefficient sensitivity: because DCs are scarce in many tissues and labeling efficiency is presumably low, it can be difficult to document unequivocally subtle migratory events, such as the emigration of DCs into efferent lymph or the

trafficking of circulating CD11c^{hi} DCs. Moreover, experiments that aim to interfere with endogenous DC migration (e.g. by blocking specific molecules with neutralizing antibodies) could inadvertently alter also the migration and distribution of surrounding non-DCs. In these cases, adoptive transfer of *ex vivo* manipulated DCs is often advantageous. In order to obtain a sufficient number of cells to carry out these experiments in mice, it is necessary to apply strategies that boost the number of DCs available for adoptive transfer. Several protocols for the expansion or culture of primary DCs or DC-like cell lines have been developed (Box 1).

Once a suitable population of DCs has been obtained, the cells can be either marked with organic fluorochromes or radioactive isotopes [42] or tracked after adoptive transfer into recipients by virtue of their expression of a distinct congenic marker [30••] or fluorescent protein, such as GFP [43]. In these homing experiments, the molecules required for recruitment to different organs can be individually tested by treating DCs *ex vivo* with neutralizing antibodies or by using gene-targeted animals as donors or recipients. In addition, adoptive transfer of purified and labeled DCs has made intravital observation of the intravascular behavior of these cells possible and, consequently, has identified the role of several traffic molecules in the intravascular adhesion cascade (see below).

Box 1 Techniques for generating bulk numbers of DCs.

Cell lines

D1: Myeloid DC line generated by serial passaging of loosely adherent splenocytes from bulk cultures of C57BL/6 mice. Propagated *in vitro* by culture in medium that contains supernatant from GM-CSF transfected 3T3 fibroblasts, and can be activated by exposure to LPS [63].

XS52: Murine epidermal DC line that was generated from newborn BALB/c skin by long-term culture in GM-CSF and keratinocyte-conditioned medium. This resembles freshly isolated mouse LCs [64].

DC2.4: Immortal cell clone, generated by serial transduction of C57BL/6 BM with viruses that express GM-CSF to increase the number of proliferating DCs, and with *myc* and *raf* to confer immortality. DC2.4 cells are highly phagocytic, express homogeneously the DC marker DEC-205, and are efficient APCs [65].

Primary cell cultures

Human monocytes: Non-proliferating CD14$^+$ peripheral blood monocytes were amongst the first sources of human DCs. *In vitro* culture with GM-CSF and IL-4 induces a homogenous population of APCs that can be activated by exposure to TNF-α or CD40L [16]. Adding TGF-β to the cytokine cocktail promotes a phenotype skewed towards LCs [66].

Human cord blood: CD34$^+$ progenitors, enriched in cord blood, can be induced to differentiate into DCs *in vitro* by culture in the presence of GM-CSF and TNF-α [67].

Mouse bone marrow: Mouse bone marrow can be cultured *in vitro* with GM-CSF [68] or Flt3L [69] for about one week to give rise to DCs that over time spontaneously acquire an increasingly mature phenotype. Whereas culture in GM-CSF only promotes outgrowth of myDCs, Flt3L additionally generates pDCs [70].

***In vivo* expansion**

Flt3L: Daily injections of recombinant Flt3L (a growth factor for DCs) dramatically increases the number of all subsets of DCs in every organ in mice [71] and in men [72].

B16–Flt3L: Implantation of a melanoma line (B16) genetically engineered to secrete a soluble form of Flt3L results in massive *in vivo* expansion of all DC subsets [73]. Ten days after inoculation, the spleens of treated mice contain up to 50×10^6 CD11c$^+$ DCs. This protocol is much cheaper and less labor-intensive than the daily injection of recombinant cytokine.

Tissue tropism and immunological function of circulating DCs

Homing experiments provide the least biased approach to study DC trafficking. Knowledge of the tissue tropism of *ex vivo* purified, adoptively transferred DCs and of the molecular interactions involved in their migration is of paramount importance for cell-based vaccination strategies that are now being explored for treatment of a broad range of neoplastic and infectious diseases [44].

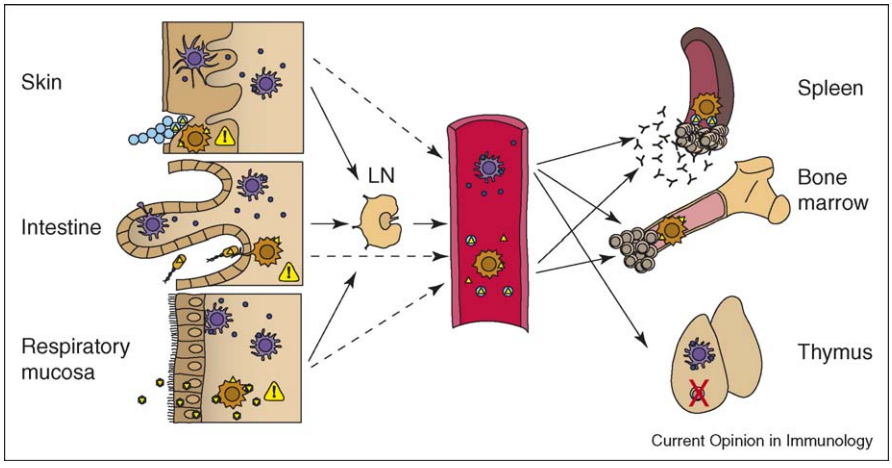
Blood-borne leukocyte trafficking is governed by a sequence of molecularly distinct adhesion and signaling events known as the multi-step adhesion cascade [45,46]. This entails a tethering and slow rolling step followed by a chemoattractant-mediated signal that triggers integrin activation, which in turn causes the arrest (sticking) of the rolling leukocyte onto the vessel wall. Specific recruitment of leukocyte subsets to different tissues is achieved by discrete combinations of adhesion molecules and chemoattractants presented within the microvasculature of each organ; only circulating leukocytes that express the corresponding counter-receptors have a chance to be recruited. The molecules responsible for organ-specific recruitment of circulating DCs have been uncovered in several settings, and we will review them and their putative immunological consequences in the following section.

DC homing to the spleen and other SLOs

Shortly after intravenous administration, mouse DCs are mainly found in spleen, liver and lung [30••,47], but they are poorly retained in the latter. DCs migrating to the spleen are likely to engage in productive interactions with the many resident lymphocytes. For example, Balazs *et al.* [48] have shown that a circulating DC subset is responsible for capturing blood-borne bacteria and stimulating a T cell-independent B cell response in the spleen, possibly by recycling intact three-dimensional antigens through their phagocytic compartment and back to the cell surface [49•] (Figure 2).

Circulating DCs are, for the most part, excluded from other SLOs [30••], although a small population of cells can gain entry to LNs and PPs by a pertussis toxin-sensitive pathway. These cells could be the precursors that have been described to access lymphoid organs directly from the blood to give rise to SLO-resident

Figure 2



Routes of antigen transport by circulating DCs. Peripheral DCs continuously sample autologous (blue circles) and exogenous (yellow triangles) antigenic material. The presence of danger signals associated with infection (yellow exclamation signs) induce DC maturation and full immunogenicity (orange cells), whereas immature DCs remain in a tolerogenic state (purple cells). DCs leave the tissues and reach the blood, possibly, but not necessarily, by way of draining LNs and efferent lymph; in addition, circulating DCs might also capture blood-borne pathogens and antigens. From the blood, DCs disseminate to different lymphoid and non-lymphoid organs where they present antigen to resident lymphocytes; this has different consequences that depend on the organ of origin, the maturation status of the DC, and the developmental stage of the lymphocyte. For example, in this illustration, splenic B cells are alerted to the presence of a blood-borne pathogen and are induced to differentiate into antibody-producing cells; BM-resident T_{CM} are activated and expanded; and self-reactive thymocytes might be removed from the repertoire by thymus-homing DCs that carry self-antigen (see text for more details).

DCs [6,50]. Interestingly, splenic DCs utilize integrin $\alpha_4\beta_7$ to adhere to high endothelial venules in PPs, and chronic administration of antibodies against MAdCAM-1 — the endothelial ligand for $\alpha_4\beta_7$ — causes a dramatic reduction of DC numbers in PPs (R Bonasio *et al.*, unpublished). This suggests that circulating DC precursors populate PPs (which lack afferent lymph vessels) by way of the blood, and could provide a molecular mechanism for the observed redistribution of skin-derived DCs to PPs after exposure to certain stimuli [11,12].

Bone marrow

In addition to its hematopoietic role, the BM also functions as a hub for recirculating leukocytes and as a site for antigen presentation and lymphocyte activation [51–53]. In particular, central memory T cells (T_{CM}) have a dedicated niche in the BM, where they are actively recruited [54] and undergo homeostatic proliferation [55]. In addition, memory T cells in the BM mount stronger effector responses than the corresponding population in the blood [53], which suggests that the BM functions as a repository for highly immunoprotective memory cells.

We have recently shown that, regardless of their maturation state, DCs injected intravenously home to and are retained within the BM, where they can establish long-lasting antigen-driven interactions with resident T_{CM}, inducing their activation and proliferation [30••]. Using intravital microscopy we have established that DCs slowly roll along the BM endothelium by interacting with P- and E-selectin, whereas firm adhesion is partially dependent on DC-expressed integrin $\alpha_4\beta_1$ binding to vascular-cell adhesion molecule 1 (VCAM-1). Considering that T_{CM} are recruited to the BM by a similar cascade [54], the transport of recall antigen by (re)circulating DCs might help to boost secondary immune responses (Figure 2).

Thymus

A small but functionally relevant number of DCs migrates by way of the blood to the thymus [56] by a conventional three-step cascade, which requires P-selectin for rolling, followed by chemoattractant-mediated activation of $\alpha_4\beta_1$, causing firm adhesion on VCAM-1 (R Bonasio *et al.*, unpublished). After migration to the thymic medulla, blood-borne DCs join the ranks of intrathymically generated DCs and medullary epithelial cells and, in an adoptive transfer setting, are able to present antigens to developing thymocytes, thus eliminating potentially auto-reactive cells by clonal deletion (R Bonasio *et al.*, unpublished). Although the physiological role of this migratory pathway in the induction of tolerance remains to be clarified, recirculation of tissue-derived DCs by way of the blood helps to explain how the immune system might establish central tolerance towards antigens that are expressed in peripheral organs but not in the thymus (Figure 2). However, we need to point out that, in at least one experimental setup, ovalbumin expressed in the pancreas did not induce clonal

deletion of ovalbumin-reactive thymocytes [57]. By contrast, Huseby *et al.* [58] reported evidence for central tolerance towards an epitope of myelin basic protein not expressed in the thymus, and proved that BM-derived APCs were responsible for the phenomenon. This might signify that recirculation of peripherally-derived DCs is restricted by the organ(s) of origin or by the developmental phase of the organism, and suggests that experiments directly aimed at this question need to be carried out before the matter is settled.

Skin and sites of inflammation

Adoptively transferred DCs undergo adhesive interactions with the endothelium of the skin, mainly through P- and E-selectin [59]. Importantly, the recruitment of DCs to the skin is more effective in the event of inflammation [59,60] and is restricted to cells that have an immature phenotype [60], which are most apt at phagocytosis. These results demonstrate that circulating immature DCs retain the ability to migrate into peripheral tissues, suggesting that at least part of the DCs found in blood might in fact represent a circulating pool of APCs available for immediate recruitment to sites of inflammation, where their antigen sampling and processing function is most needed.

Evidence for DC recirculation

If tissue-derived DCs can indeed return to the circulation after sampling peripheral antigens, it is even more important to understand the migration and function of such blood-borne DCs. The suggestion that this might happen comes mainly from circumstantial evidence. First, $CD11c^+$ cells appear in thoracic duct lymph at steady state, and their number increases when particular stimuli are used; the only possible origins of these cells are the downstream LNs and/or the tissue area drained by them. Second, molecular labels or antigens that had been deposited in the periphery, including intracutaneously [11,12], intranasally [14] and orally [13], could be retrieved in association with DCs in the spleen or in other organs that were not directly connected to the administration site by anatomic conduits other than the vascular system. Third, DCs emigrate from cardiac allografts and disseminate by way of the blood to the spleen in mice [61] and in rats [62], thus demonstrating that, at least in these non-physiological circumstances, a direct path of reverse migration from tissue to blood is open to these cells. Fourth, in our own experiments, splenic $CD11c^+$ DCs deposited in high numbers in the footpad could be detected 1–2 days later in spleen and BM, again arguing for a blood-borne route of recirculation [30••]. The efficiency of this last process in our investigation was rather low: we only recovered one DC in spleen and BM for every 10 000 cells that had been injected in the footpad. However, this might reflect limitations of our experimental setup, in particular the intrinsic difficulty in detecting rare cell populations by flow cytometry.

Conclusions

Although the physiological significance of recirculating DCs remains elusive, many independent observations provide mounting evidence for their existence. Whatever their origin may be, it is clear that circulating DCs represent a heterogeneous population that contains, amongst others, cells competent in: phagocytosis of antigenic material (sometimes directly from the blood [48]); efficient processing and presentation of the acquired antigens; and migration to different lymphoid and non-lymphoid organs to communicate with resident lymphocytes.

The immunological implications of such a migratory route are potentially far-reaching. Although sometimes immune responses need to be locally confined, in many circumstances it will be advantageous to alert all available lymphocytes in the organism to the presence of their cognate antigen, either because a rapid recall response is required from the largest possible lymphocyte pool (including cells that reside in tissues not reached by the lymph) or because developing T cells can only be informed about their auto-reactivity if the antigen they recognize is present in the thymus. Given these considerations, we propose that DCs, beyond their role as local sentinels of the immune system, have also evolved the ability to carry their antigenic cargo not only to the draining LNs but also to wherever else it might be needed.

Update

In a recent study, Naik *et al.* [74•] described a population of mouse DC precursors that can give rise to splenic $CD11c^+MHC\text{-}II^+$ DCs in 5 days. These immediate precursors represent 0.05% of mononuclear cells in the spleen, whereas their frequency among blood leukocytes, judging from *in vivo* differentiation assays, is ~50-fold lower. This suggests that immediate DC precursors are exceedingly rare in the circulation (~0.001%) and probably constitute only a minor fraction of the cells expressing DC-specific markers that are found in the blood.

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