Murine CD8α^+ Dendritic Cell Migration

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Murine CD8α$^+$ dendritic cells (DC) are antigen-presenting cells with tolerogenic properties, including ability to prolong allograft survival. Little is known, however, regarding their migratory ability, either *in vitro* or *in vivo*. Limited work to date has yielded inconsistent findings, and potential impediments to use of this subset to promote tolerance induction are reported discrepancies regarding their ability to "home" to secondary lymphoid tissue. Despite functional differences from classic CD8α$^-$ DC, the two subsets exhibit similar phenotypes and commonly coexist in the same tissues, suggesting expression of similar homing molecules/receptors. The central hypothesis underlying these studies was that **CD8α$^+$ and CD8α$^-$ DC have equal ability to respond to migration-inducing factors, both *in vitro* and *in vivo*.** Our aim was to determine the factors and potential therapeutic targets that regulate CD8α$^+$ DC migration. Herein we have employed an *in vitro* chemotaxis assay for the determination of which, if any, CC chemokines specifically regulate the migration of CD8α$^+$ (and CD8α$^-$) murine spleen DC. We also used this assay with the addition of endothelial cell layers, to assess the adhesion molecules that facilitate DC transendothelial migration. Two chemokines (CCL19 and CCL21) elicited CD54-dependent migration of both mature DC subsets, in the presence or absence of endothelial cells, but CD8α$^+$ DC migrated in consistently fewer numbers than CD8α$^-$ DC *in vitro*. Our findings led us to investigate the importance of the *in vitro* migration-inducing chemokines *in vivo*. In these studies we compared the ability of DC subsets to migrate to T cell

areas of wild type and specific chemokine-deficient mice. Unlike our *in vitro* results, the DC subsets migrated with equal efficiency in normal recipients. After sc injection into CCL19/CC21-deficient mice, $CD8\alpha^-$ DC trafficked more efficiently than $CD8\alpha^+$ DC to draining lymphoid tissue. The necessity of specific chemokine-directed DC migration *in vivo* for alloimmune responses was borne out in transplant studies in which impaired migration of DC resulted in significant prolongation of murine cardiac allograft survival. These studies demonstrate, for the first time, potential chemokine and adhesion molecule targets for manipulation of murine DC subset migration *in vivo*.

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1. INTRODUCTION

1.1. Dendritic Cells

Dendritic cells (DC) are the most potent antigen (Ag) presenting cells (APC) of the immune system. They are essential in both innate and adaptive immune responses. As pre-DC, they can activate anti-viral and anti-tumor NK cell-mediated responses through the release of IFNα (1). Mature (m) DC can also augment NK and NK/T cell activity through the release of IL-12, IL-15, and IL-18 (2-4). Unlike other APC—B cells and macrophages (Mϕ), DC are capable of activating naïve T cells, and thus essentially are responsible for the initiation of adaptive immune responses. Though rare compared to the number of cells per tissue in which they reside, DC are found ubiquitously throughout the body, with specific concentrations of DC in the thymus and secondary lymphoid tissues. DC also are important in the regulation of autoimmunity, negatively selecting too-strongly-reactive self-recognizing T cells. Thus, though discovered just 30 years ago (5), DC are of major interest to cancer immunologists (as hopeful powerful anti-tumor immune initiators) and to transplant- and auto-immunologists and allergists (as regulators of T cell tolerance).

1.1.1. Phenotype & Function

In both the human and the mouse, DC typically are characterized by their expression of CD11c (4)[human plasmacytoid (p) DC are an exception – these DC are characterized (partly) by their lack of CD11c (6, 7)]. In the rat, DC are classified by their expression of CD11c, MHC II, and αε-integrin (OX62) (8). In non-human primates, DC are characterized by their expression of CD83, a feature they share with human DC (9). DC also express an array of other cell surface molecules not necessarily unique of their cell type, but typical to DC. They are considered to exist primarily in one of two states of maturation. Generally, immature (i) DC are $CD11c^{+}$ and

characteristically express MHC I and low levels of MHC II ($CD8^+$ and $CD4^+$ T cell Ag-presenting molecules, respectively), CD40, CD80, CD86 (classic costimulatory molecules), and inducible costimulator (ICOS) ligand (L), programmed death (PD)L-1 and 2 (neo-classic costimulatory molecules), with mid to high expression of CD31 and CD54 (adhesion molecules) (Table 1). iDC are also morphologically and functionally distinct. They exhibit short, "stubby" prolongations, possess multiple cytoplasmic vesicles (some of which contain Ag-laden MHC II molecules), and have manifest nucleoli (Fig 1a, c, and e). In this state, DC function primarily as environmental samplers, taking up soluble or particulate Ags (Fig 2a) and processing them for later presentation to naïve and memory B and T cells, making iDC poor immunostimulators in mixed leukocyte reactions (MLR) (Fig 2b). mDC maintain their levels of CD11c, CD31, and CD54, but upregulate Ag-presenting and costimulatory molecules, although the level of their upregulation can depend on the degree and type of stimulation (Table 1). mDC have extended cytoplasmic prolongations [dendrites, the initial observation of which gave DC their name (5)] and lobulated nuclei (Fig 1b, d, and f), do not phagocytose particulate Ags (Fig 2a), and are potent stimulators of T cell proliferation (Fig 2b).

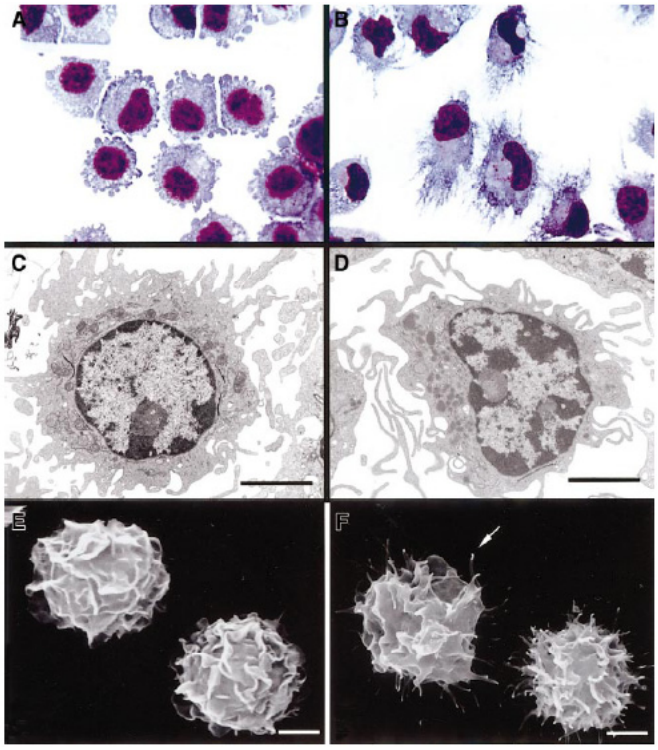


Fig 1. Morphology and ultrastructure of i(CD86$^-$) and m(CD86$^+$) murine (BM)DC.
iDC possess short, blunt prolongations (A), a round nucleus with prominent nucleoli, multiple cytoplasmic vesicles, mitochondria, few lysosomes (C), and typical "veils" (E). After maturation, CD86$^+$ DC show a typical dendritic morphology, with an eccentric, indented nucleus (B, D) and a veiled surface with delicate filamentous projections with knoblike tips (F, arrow). (A-B) May-Grünwald-Giemsa. (C-D) TEM x6000. (E-F) SEM x3500. Bar, 5 μm (10). *Copyright America Society of Hematology, used with permission.*

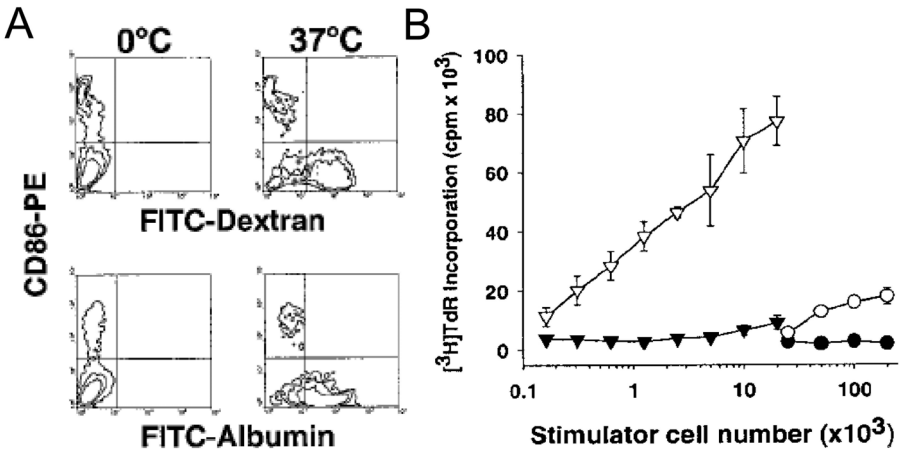


Fig 2. i and m (BM)DC function.
(A) FITC-dextran and FITC-albumin uptake by murine CD11c$^+$ bead-sorted CD86$^-$ (i) and CD86$^+$ (m) BMDC. Only iDC internalized FITC-dextran and FITC-albumin at 37°C, a phenomenon down-regulated at 0° C. Results are representative of 3 independent experiments. (B) Allostimulatory activity of γ-irradiated, FACS-sorted CD86$^-$ (i) ( ) or CD86$^+$ (m) B10 DC (▽), assessed using C3H splenic T cells as responders. B10 (H2^b) DC (d 7 of culture) were set up at graded concentrations with 2-3x10^5 responder naive C3H (H2^k) T cells, and the cultures were maintained for 72 hours. [^{3}H]TdR was added 18 hours before harvesting. The MLR stimulatory activity of freshly-isolated allogeneic (B10 [ ]) or syngeneic (C3H [ ]) bulk spleen cells is also shown. Results are expressed as mean cpm ± 1 SD and are representative of at least 3 separate experiments (10). *Copyright America Society of Hematology, used with permission.*

Table 1. Typical Dendritic Cell Markers.

cell surface molecule	function	state of maturation		subset(s)	human/mouse /primate/rat	major ligand(s)	
		iDC	mDC			molecule(s)	cell(s) expressed
CD1a[a]	non-classic MHC	++	++	LC	h	TCR	T cells
CD8α	T cell marker	++	++	$CD8\alpha^{+}$[b];	m	MHC I	all cells
CD11b (Mac-1; CR3)	AM[a]	++	++	$CD8\alpha^{-}$[c]	h,m,p,r	CD54, iC3b, fibrinogen	Mo, neutrophils, DC, FDC
CD11c	AM[a]/DC marker	+++	+++	all but human/primate $CD45R^{+}$	h,m	iC3b, fibrinogen	N/A
CD31 (PECAM-1)	AM	++	++	all	h,m,p,r	CD31	DC, B cells, T cells, Mo, EC neutrophils
CD40	costim	-/+	+++	all	h,m,p,r	CD154	activated T cells, NK cells
CD45R (B220)	signal transduction/ DC marker	++	++	pDC	h[d],m,p	unknown	N/A
CD80 (B7.1)	costim	++	+++	all	h,m,p,r	CD28/ CTLA4	naïve/activated T cells
CD86 (B7.2)	costim	+	+++	all	h,m,p,t		
CD83	Ig superfamily/ DC marker	-	++	all	h,p	(unknown)	(unknown)
CD123 (IL-3Rα)	IL-3R	++	++	$CD45R^{+}$	h,p	IL-3	activated T cells, mast cells, eosinophils
CD205 (DEC 205)	ER	++	++	$CD8\alpha^{+}$[e]; LC	m	mannosylated proteins? (unknown)	pathogens
CD207 (Langerin)[f]	induces BG/ER	++	++	LC	h,m,p,r	mannosylated proteins? (unknown)	pathogens
ICOSL	costim	-/+	+++	all[g]	h,m	ICOS	naïve T cells
PD-L1	costim	-/+	+++	all[g]	h,m	PD	naïve T cells
PD-L2	costim	-/+	+++	all[g]	h,m		
TLR4	microbial recognition	++	+++[h]	$CD8\alpha^{-}$ DC	h,m	LPS[i]	bacteria
TLR9	microbial recognition	++	+++[h]	pDC	h,m	CpG	viruses

[a]AM—adhesion molecule; BG—Birbeck granules; CD – cluster of differentiation; costim—costimulatory molecule; CpG – cytosine poly-guanine; ER – endocytic receptor; F – follicular; Gr – granulocytes; ICOS – inducible costimulatory molecule; L – ligand; LC – Langerhans cell; Mo – monocytes; Mϕ -- macrophages; PD – programmed death; PECAM – platelet and endothelial cell adhesion molecule; TLR – toll-like receptor; [a]when coupled with CD18; [b]upregulated on Langerhans cells (LC) after migration to the draining lymph node after activation; [c]low levels on $CD8\alpha^{-}$; [d]$CD11c^{-}$ DC only; [e]low levels on $CD8\alpha^{-}$; [f](11); [g]although the level of expression may vary according to tissue of isolation/subset (Lau and de Creus, unpublished observations); [h] at least when activated with LPS; [i]although LPS is the primary ligand for TLR4, other stimuli also activate this receptor (12).

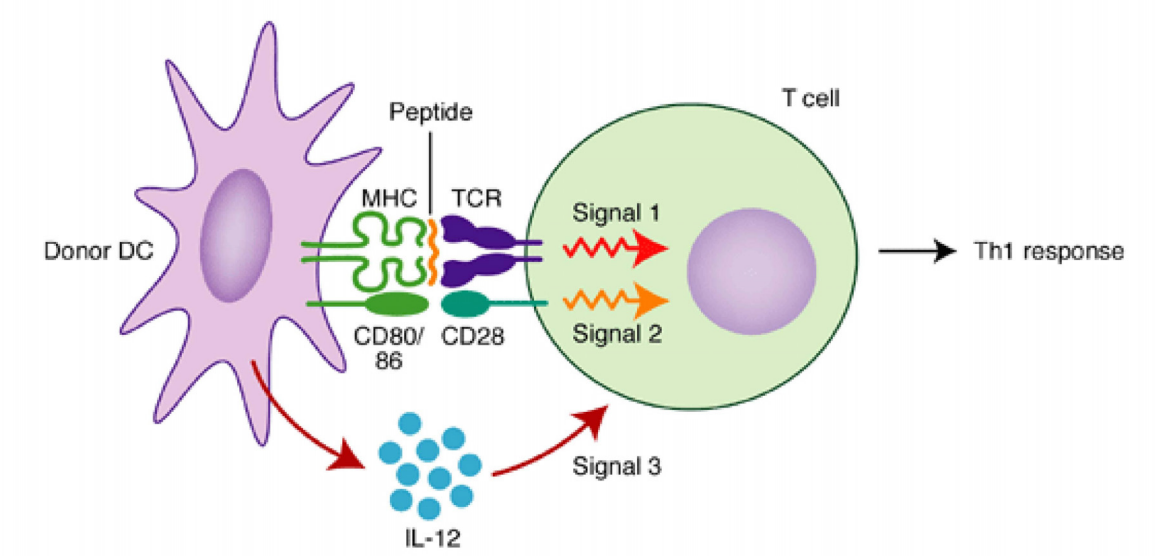
In response to the release of inflammatory – "danger" – signals [e.g., tumor necrosis factor (TNF)α, cytosine poly-guanine (CpG), and lipopolysaccharide (LPS), -- all of which are also used to mature DC *in vitro*] from injured tissues/pathogens, local iDC become activated. Activated DC begin to lose their ability to take up Ag, upregulate Ag-presenting and costimulatory molecules, and downregulate receptors for inducible chemokines and upregulate receptors for constitutive chemokines (further discussed in Section 1.2). Mature/semi-mature DC[1] then traffic to the nearest draining (D) LN, spleen, or intestinal Peyer's patch. Once in secondary lymphoid tissues, mDC enter (primarily) T cell areas for activation of naïve and memory T cells [while some DC also traffic to B cells areas for aid in the differentiation of B cells to memory and plasma cells (4, 13-15)].

Not all DC subsets (discussed further in Section 1.1.2) are capable of responding to the same stimuli (16). Toll-like receptors [TLR; so-called because of their conserved homology to toll receptors, first described in *Drosophila* (12)] are expressed specifically on various cell types and respond to specific microbial and autologous signals. TLR are restricted both by subset as well as state of maturation on DC. While TLR 1, 2, 4, and 5 are expressed at high levels on iDC, all are downregulated upon maturation (17). TLR3, on the other hand, is expressed only on mDC (18). Human blood-isolated, plasmacytoid DC (pDC), identified by their expression of CD123 and lack of CD11c (6, 7) express TLR7 and 9, and produce IFNα in response to binding of unmethylated CpG bacterial DNA fragments to TLR9 (19-23). "Myeloid" DC ($CD11c^{+}CD123^{-}$) reportedly express TLR1-5 (12, 16, 17, 20). Murine spleen-isolated DC show a slight different

[1] Though "veiled cells" have been identified in lymphatic vessels (13-15), little is known about the order of the expression of cell surface molecules on and state of maturation of activated DC during their transition from the periphery to secondary lymphoid tissues.

expression pattern for TLR. Interestingly, while TLR3 is expressed only on mDC in humans, it is selectively expressed on $CD8\alpha^+$ murine iDC and TLR5 and 7 are absent (24). Further, while the murine spleen pDC do express TLR7 and 9, $CD8\alpha^-$ DC also express TLR7, a TLR exclusive to pDC in humans. However, this divergence from human DC may be more tissue-related than species-related, as murine bone marrow-derived DC express a similar TLR pattern to human blood-derived (25).

Naïve T cells need three signals to become activated: (i) TCR/MHC ligation, (ii) CD28/CD80/CD86 costimulation, and (iii) secretion of T helper cell (Th)-inducing cytokines by DC, for example IL-12 (Fig 3). In the absence of signal one, naïve and memory T cells ignore DC, and thus do not become activated. In the absence of signal two and/or signal three, naïve T cells can become anergic or apoptotic. Memory T cells do not need all three signals and therefore can be activated by non-DC APC [such as B cells, Mϕ, and, in rare instances, EC, none of which have the capacity to deliver signals two and three (26-28)].



The interaction between dendritic cells (DCs) and T cells involves three signals

Fig 3. DC are the only APC capable of delivering the three signals necessary for activation of naïve T cells.

mDC present peptides to naïve T cells via MHC/TCR interactions (Signal 1). The interaction of CD80/86 on mDC with CD28 on the T cell results in the delivery of Signal 2. Ligation of CD40 on DC with CD40L on the semi-activated T cell (for example, not shown here), in turn further activates the DC, resulting in secretion of IL-12 by the DC, inducing a Th1 phenotype in the formerly naïve T cell (Signal 3). (29) *Reprinted with the permission of Cambridge University Press.*

DC (principally $CD8\alpha^+$ DC) regulate central tolerance through negative selection of autoreactive T cells in the thymus. DC also are thought to regulate peripheral tolerance in the absence of inflammation through steady state trafficking from the periphery to DLN bearing self and "innocuous" non-self Ag, likely from engulfed apoptotic bodies (30-32). Interactions of ($CD11c^+CD8\alpha^+$) DC bearing Ag with naïve T cells in the absence of danger have been reported to induce anergy (32), deletion (33), or both (34), or regulatory T cells (T reg) (30, 35, 36).

1.1.2. Origin

DC, like all leukocytes, arise from hematopoietic stem cells (HSC). The elusive DC-specific precursor that gives rise to DC only, however, is yet to be identified. DC can arise from progenitor cells as plastic as a BM precursor (pre)-DC (10) (which can give rise to a variety of cells of the myeloid lineage in the appropriate cytokine environment), or as differentiated as reverse-transmigrating monocytes (which differentiate into macrophages if they remain in the subendothelial collagen) (37). Nevertheless, DC can be generated from pre-DC in *in vitro* culture systems or isolated directly from tissues. Human DC studies typically utilize DC derived from blood-derived $CD34^+$ progenitor cells (38) or $CD14^+$ monocytes (39, 40), or iDC emigrated from skin explants (Langerhans cells; LC) (38, 41). Non-human primate studies utilize blood- or lymph node-derived DC (42, 43). Rat DC can be generated from BM and secondary lymphoid tissues such as the gut associated lymph (GALT), Peyer's patches, lamina propria, and the mesenteric lymph nodes (LN) (8). DC in murine studies commonly are generated from the bone marrow (BM). However, mouse DC have been reported to be directly isolated for study from the skin (4, 38), Peyer's patches (44, 45), spleen (46-48), kidney (49, 50), liver (51-54), and blood (55). Derivation of murine DC subsets also has been reported for a variety of precursors, as discussed further below.

Precursor cell populations require a variety of cytokines to differentiate into DC depending on the species and tissue of origin. Human $CD34^+$ hematopoietic precursor cell (HPC) cultures give rise to $CD1a^+CD14^-$ and $CD1a^-CD14^+$ cells which differentiate into LC [skin immature (i) DC which express Langerin (CD207) and E-cadherin and possess Birbeck granules (11)] and dermal (which lack these markers and express Factor XIIIa and CD2, CD9, and CD68) DC, respectively, after addition of granulocyte/macrophage-colony stimulating factor (GM-CSF) and tumor

necrosis factor (TNF) (38). DC are generated from human and non-human primate blood mononuclear cells cultured in the presence of GM-CSF and IL-4 (9, 39, 40). Addition of transforming growth factor (TGF)β to GM-CSF and interleukin (IL)-4-cultured monocytes also yields LC (56). Interestingly, it has been shown that G-CSF, which reportedly selectively mobilized the pDC population in human blood (57), recently has been shown to augment pDC numbers in peripheral blood through downregulation of CD62L (and consequent prevention of pDC exodus into lymphatics), not through inducement of proliferation of blood pDC progenitors (58). After red blood cell lysis and complement depletion of other precursors (B, NK, red blood, and T cells and granulocytes), murine BM cells cultured in media supplemented with GM-CSF and/or IL-4 differentiate into iDC between days 4-6. Although GM-CSF alone is sufficient to produce DC, addition of IL-4 to BMDC cell cultures generally results in a more mature DC phenotype at an earlier stage than in cultures supplemented with GM-CSF alone (~6-7 d vs ~8-9, respectively) (59).

It also has been reported that "lymphoid-related" DC [putative tolerogenic CD8α^+-expressing mouse DC (60)] and pDC [interferon (IFN)α-secreting mouse, human, and non-human primate DC (6, 7, 42, 61, 62, 63 Coates, 2003)] both requiring addition of IL-3, and regulatory DC (putative T reg-inducing DC) requiring TNFα and IL-10 (64) (all discussed in Section 1.1.4) can be generated from BM cell cultures, but the complex cytokine cocktails [i.e., IL-1β, TNFα, IL-7, stem cell factor, IL-3, FL, and CD40L for CD8α^+ DC (60)] necessary for the differentiation have led most investigators to isolate these DC directly from tissue.

The belief that murine CD8α$^+$ DC were of lymphoid lineage has lead to several investigations regarding the origin of these DC. Though the subject of debate, CD8α$^+$ and CD8α$^-$ DC reportedly can arise from either committed lymphoid or myeloid progenitors [CLP or CMP, respectively, as discussed further in Section 1.3 (65, 66)]. The hematopoietic growth factor fms-like tyrosine kinase 3 ligand (Flt3L; FL) also has been found to be useful in the generation of human and mouse DC both *in vitro* and *in vivo*, and it has been particularly useful in facilitating the growth of mouse pDC *in vitro* (25, 54). Rat DC can be generated under culture conditions similar to those for mouse BMDC (67). DC arise from *in vitro* culture systems from anywhere between 4-10 days, depending on precursor cell populations and culture conditions.

As discussed in Section 1.1.1, early *in vitro*-generated DC are immature in phenotype, morphology, and function. *In vitro*-generated DC continue to differentiate into mature (m) DC with extended culture or with the addition of inflammatory stimuli, such as TNFα, LPS, CpG, and/or CD40L, as evidenced by changes in their phenotype, morphology, and function, and concordant with the nuclear translocation of nuclear factor (NF)- κβ [the blocking of which impedes DC differentiation and maturation (68, 69)]. Freshly-isolated tissue DC are fully-differentiated DC which resemble early iDC from *in vitro*-cell cultures, although due to their rarity, mice typically are pre-treated with a hematopoietic growth factor(s) such as FL (47, 70, 71), GM-CSF (72), or the combination of both before harvesting (62, 73). Many investigators administer FL intraperitoneally (ip) into mice for 10 days before sacrifice, as FL reportedly expands both CD8α$^+$ and CD8α$^-$ DC subsets, while GM-CSF selectively expands the CD8α$^-$ DC population (73). FL dramatically increases DC in the BM, GALT, liver, spleen, LN, lung, peritoneal cavity, thymus, and peripheral blood, in addition to stem cells and various leukocyte

populations (74). Aside from their increased number, FL-DC are otherwise reportedly identical to DC from non-growth factor treated mice (48, 75). Unlike *in vitro*-generated DC, however, tissue-isolated DC (from both FL- and untreated animals) mature after overnight culture (16-18 h), without the addition of exogenous factors. GM-CSF, though not necessary for maturation, is typically added to overnight cultures of tissue-isolated DC to aid in the maintenance of cell viability (52).

1.1.3. DC Subsets

Another complication in determining the origin of DC is the existence of multiple DC subsets. In mouse secondary lymphoid tissue, and some non-lymphoid tissues [such as the kidney (50) and liver (53, 54, 76, 77)], at least three major DC subsets have been defined: $CD11c^{+}CD8\alpha^{-}CD11b^{h}$ (immature "myeloid"), $CD11c^{+}CD8\alpha^{+}CD11b^{lo}$ (immature "lymphoid"), and $CD11c^{+}B220^{+}CD11b^{+}CD8\alpha^{+/-}$ (immature "plasmacytoid") (54, 61, 62, 78). In the human and non-human primate, there are at least two: $CD11c^{+}CD123^{-}$ (immature "myeloid") and $CD11c^{-}CD123^{+}$ (immature/pre-"plasmacytoid") (6, 7, 42). These subsets are not only phenotypically distinct, but they can be classified functionally as well.

1.1.3.1. Mouse DC Subsets

$CD11c^{+}CD8\alpha^{-}CD11b^{hi}$ DC (denoted henceforth by their common nomenclature, $CD8\alpha^{-}$ DC) have been previously referred to as "classic" myeloid DC and generally describe any mouse DC from any tissue/culture condition that does not express/result in CD8α or B220 expression (Figs 4 and 10 and data not shown). The best-studied of murine DC subsets, these DC are considered to reside in the marginal zone of secondary lymphoid tissues (31, 79) and generally are considered to be the immunogenic mouse DC subset (32, 47, 80-84), at least in the mature state

(10, 47). In addition to their high expression of CD11b and the absence of CD8α on their surface, they also are frequently characterized by their low to non-existent expression of DEC-205 (CD205) (Table 1). Early experiments with the $CD11c^{+}CD8\alpha^{-}$ DC subset suggested they were the Th1-inducing DC subset (75, 85), but almost immediately it was shown they can induce Th2 cells as well (86).

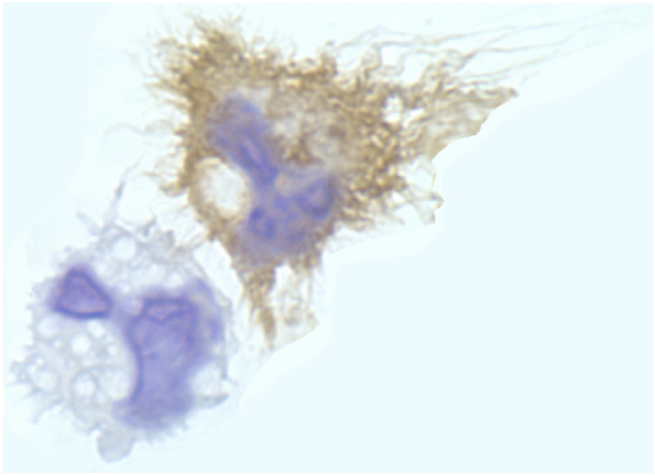


Fig 4. Despite some phenotypic differences, $CD8\alpha^{-}$ and $CD8\alpha^{+}$ DC are similar in morphology.
Metrizamide-enriched, splenic mDC were cytospun and then stained for expression of CD8α with biotinylated anti-CD8α and ABC/alkaline-phosphatase (brown). Nuclei were stained with Giemsa (blue). Both mDC subsets exhibit similar dendritic extensions and lobulated nuclei (Amanda Schell, Summer 2001, unpublished data).

Formerly known as lymphoid or "lymphoid-related" DC, $CD11c^{+}CD8\alpha^{+}CD11b^{lo}$ ($CD8\alpha^{+}$ DC) DC were first discovered in the thymus (78), spleen, and LN (46), but now are known to reside in the Peyer's patches (44, 45), the kidney (50), and the liver (51, 52, 54) as well. Morphologically similar to $CD8\alpha^{-}$ DC (Fig 4), $CD8\alpha^{+}$ DC express high levels of CD205 and low levels of CD11b (Table 1). Despite their presence in numerous tissues, even optimized isolation procedures require large numbers of animals to procure enough DC for study. Therefore, FL, which dramatically increases both $CD8\alpha^{+}$ and $CD8\alpha^{-}$ DC numbers [as compared to GM-CSF, which selectively augments the expansion of $CD8\alpha^{-}$ DC (72)], is frequently administered to the animals for 10 days prior to tissue harvest (42, 48, 50, 52, 71, 74).

Early reports of these DC suggested that their expression of surface FasL (CD178) induced activation induced cell death in $CD4^+$ T cells (84) and that they indirectly suppressed $CD8^+$ T cell proliferation through suppression of IL-2 production (83) (both in an allo-MLR). However, neither these T cell suppressive properties nor the expression of FasL have been repeated by other groups. Nevertheless, O'Connell *et al* (47) found that $CD8\alpha^+$ DC, in both the immature and mature states, significantly prolonged mouse heart allograft survival, while only $CD8\alpha^-$ iDC could delay graft rejection -- $CD8\alpha^-$ mDC accelerate the rejection process. Further, another group has shown that populations of $CD8\alpha^-$ DC containing only 3% $CD8\alpha^+$ DC are impaired in their ability to instigate an immunogenic response against a tumorigenic self-peptide due to $CD8\alpha^+$ DC secretion of indoleamine 2,3-dioxygenase (IDO), the first enzyme of the tryptophan degradation pathway. Interestingly, this ability of $CD8\alpha^+$ DC to suppress $CD8\alpha^-$ DC immunogenicity can be itself abrogated by addition of IL-12 to cultures (82). However, further addition of IFNγ blocks the ability of IL-12 to overcome the $CD8\alpha^+$ DC-induced suppression (87). Additionally, the thymic DC population is nearly 95% $CD8\alpha^+$ (78), delegating this DC subset to what is likely an entirely tolerogenic role in this primary lymphoid organ. Therefore, despite confusion with early reports, these DC still remain of interest in the fields of transplantation and tumor immunology.

$CD11c^+B220^+CD11b^+CD8\alpha^{+/-}$, or pDC, are the most recently discovered mouse DC subset (61-63) that appears to be good a candidate for the murine equivalent of human pDC (6, 7). pDC, despite one study implicating $CD8\alpha^+$ DC (88), are reportedly the major IFNα-secreting DC subset. Although nearly any cell type can produce IFNα in response to viral exposure, pDC produce a much higher concentration of IFNα at a much lower exposure of virus (6, 23, 42, 45,

62, 89). pDC have been isolated from murine spleen (62), liver (54), blood (55), and even the thymus (90), and due to the rarity of pDC, these tissues are typically harvested from mice which have been pre-treated with FL (or FL + GM-CSF) to increase their numbers (54, 62). Freshly-isolated pDC are fragile in culture and require IL-3 and CD40L (rather than GM-CSF) for survival. Despite the fact that IFNα is a Th1 cytokine (generally considered immunogenic), pDC appear to be replacing $CD8\alpha^+$ DC as the putative endogenous tolerogenic DC subset (42, 91). This theory is supported by the suggestion that pDC are capable of inducing T reg cells (61, 92), possible powerful tools for implementation of transplant tolerance and autoimmune regulation. Even more recently described (64, 93), the *in vitro*-generated "regulatory" DC may also be important candidates.

Despite the correlation between these differences in phenotype and function, some investigators have suggested that molecules such as CD8α are markers of activation and not of lineage/subset demarcation (94). For example, LC, while not typically thought of as being/belonging to a particular subset, are especially interesting in regard to their expression of CD8α, CD11b, and CD205. As mentioned, $CD11b^{hi}$ cells characteristically express low (if any) levels of CD205 and CD8α. LC, however, express CD205 and have been shown to upregulate CD8α after phagocytosis of FITC particles taken up from painted skin and traffic to the DLN (95, 96). Further, it has been shown that a fraction of $CD8\alpha^-$ DC express CD8α and CD205 after presentation of parvovirus virus-like particles to $CD8^+$ T cells (97), and that splenic marginal zone $CD8\alpha^-$ DC upregulate $CD8\alpha^+$ expression after uptake of iv-administered allogeneic apoptotic bodies (31). Recently, it even has been shown that monocytes/Mϕ (cell lines) can

differentiate into CD8α-expressing DC *in vitro* and *in vivo* (98), further blurring the distinction between distinct lymphoid/myeloid origins for $CD8\alpha^+$/$CD8\alpha^-$ DC subsets.

Reconstitution studies to elucidate the mouse precursor cell for DC subset lineages from Ken Shortman's group have consistently suggested that $CD8\alpha^+$ and $CD8\alpha^-$ DC arise from distinct progenitors [i.e., CLP for $CD8\alpha^+$ DC vs a CMP for $CD8\alpha^-$ DC (99, 100)], while studies primarily from Ardavin's group revealed that both $CD8\alpha^+$ DC and $CD8\alpha^-$ DC can be generated from either progenitor cell type (101, 102). These latter studies have helped to shed some light on the confusing knockout (KO) mouse studies, in which both $CD8\alpha^+$ DC and LC are sometimes generated and sometimes not, in mice with depleted lymphoid or myeloid lineage cells (Table 2). As they are such essential regulators of the immune response, it is plausible to consider that DC can be derived from such a variety of progenitors as a part of the myriad of backup mechanisms built into the immune system to ensure their availability at any stage or site of an immune response.

Table 2. Essential data on DC differentiation in KO mice

Knockout mice	CD8⁻ DCs Spleen	CD8⁺ DCs Spleen	CD8⁺ DCs Thymus	LCs Skin	Comments
Ikaros DN[a]	NO	NO	NO	Yes	Marked deficiency in lymphoid development, normal myeloid development
Notch1[b]	Yes	Yes	Yes	Yes	Marked deficiency in T-cell development, normal B-cell and myeloid development
Common γ-chain[c]	Yes	Yes	Yes	N.D.	Marked deficiency in T-cell development, defects in B-cell and NK-cell development
Ikaros C[d]	NO	Yes	Yes	N.D.	Marked deficiency in B-cell and NK-cell development, defects in T-cell development, disrupted splenic marginal-zone organization
RelB[e]	NO	Yes	Yes	Yes	Myeloid hyperplasia, normal lymphoid development, reduced splenic white pulp and splenomegaly
PU.1[f]	NO	Yes	Yes	N.D.	Marked deficiency in B-cell and myeloid development
ICSBP[g]	Yes	NO*	NO*	Yes	Defects in plasmacytoid DC and myeloid development; defective DC maturation
Id2[h]	Yes	NO*	NO*	NO	Marked deficiency in NK-cell development, normal T- and B-cell development
TGF-β[i]	Yes	Yes	Yes	NO	Normal lymphoid and myeloid development

* Low numbers of CD8⁺ DCs can be detected in ICSPB and Id2 knockout mice. ICSBP, interferon consensus sequence binding protein; Id2, inhibitor of DNA binding 2; Ikaros C, Ikaros null mutation; Ikaros DN, dominant-negative Ikaros mutation; N.D., not determined

[a](103); [b](104); [c](105); [d](106); [e](107); [f](108, 109); [g](110, 111); [h](112); [i](113).

1.1.3.2. Human and Non-Human Primate DC Subsets

The pDC also has been defined in humans and is identified by lack of expression of CD11c, expression of IL-3Rα (CD123) [and the requirement for IL-3 in *in vitro* culture; reportedly, addition of IL-4 in precursor pDC cultures induces apoptosis in these DC (6, 114)], and the shared ability of mouse pDC to secrete IFNα (61-63). Also similar to mouse pDC, human pDC are considered the putative tolerogenic DC subset and are believed to induce the differentiation of T reg cells (92, 115). $CD11c^{+}CD123^{-}$ DC are generally considered to be immunogenic and essentially refer to any DC that is not a pDC. These subsets have been defined in the rhesus macaque as well, in both the blood and LN (42).

In humans, however, DC are often referred to not by their expression of specific cell surface molecules, but by the type of Th response they drive. Therefore, DC1 induce Th1; DC2 induce Th2. This method of classification is very useful when defining what type of an immune

response a specific DC can initiate, but is very misleading when used to define subsets. For example, human pDC have been referred to as DC2 (114, 116), but as this subset secretes IFNα (a Th1 cytokine, as mentioned) (7), and as human non-pDC have been known to initiate Th2 responses long before the pDC was discovered, this method of classification is flawed. By this same logic, it has been suggested that while tissue-isolated DC may in fact have some phenotypic differences (i.e., $CD11c^+CD8\alpha^+$ vs $CD11c^+B220^+$ in mice and $CD11c^+CD123^-$ vs $CD11c^-CD123^+$ in humans), what truly distinguishes DC subsets is their function *in vitro* and *in vivo* and that it is their microenvironment during differentiation from precursor to iDC and/or the kind of stimulation they receive to become mDC (either in culture or *in situ*) that defines their function (117-119). Thus LPS-stimulated DC induce Th1 responses (117), while DC matured in PGE_2 induce Th2 responses (120).

1.1.3.3. Follicular DC

There are two distinct populations of primary and secondary follicle-residing DC: follicular DC (FDC) (121) and germinal center DC (GCDC) (122, 123). Human Follicular DC have a fibroblast morphology and express CD14, the complement receptors CD35 (CR1), CD21 (CR2), and CD11b (CR3), the adhesion molecules CD54, CD106 (VCAM-1), VLA-3-6, and VLA-β chain, and CD40, CD19, CD20, and CD24 (38), although the high expression of Fc receptors on FDC result in a lot of background staining, making phenotypic analysis difficult. Though the phenotype of FDC has been well characterized in humans, they have not been studied as well in mice or rats. FDC are essential for the organization of primary follicles (123, 124) and are believed to be important facilitating IgG, IgA, and IgM secretion by CD40-activated B cells (125). Though they share a somewhat similar dendritic morphology to classic DC, they do not

share the classical DC functions (e.g., iFDC do not migrate to sites of inflammation in the periphery, take up Ag, and bear it so secondary lymphoid tissues for presentation to naïve T cells).

Germinal center DC, while sharing a similar phenotype to FDC (CD16, CD32, CD64, CR1-3), are more classic mDC, with the ability to induce allo $CD4^+$ T cell proliferation and enhance B cell growth and differentiation (38). It has been suggested that GCDC represent the mature form of $CD11c^+$ blood precursors (126, 127) and may correspond with the memory T and B cell-generating APC originally described by Szakal *et al* (128).

1.1.4. DC in Transplantation

Because of their strong Ag-presenting capacity (mDC are the most potent stimulators of T cell proliferation of all APC), DC are of extreme interest to cancer immunologists in their quest to develop tumor-specific vaccines. Therefore it almost seems counterintuitive to employ DC to diminish T cell responses to allogeneic Ag. However, DC are ideal target cells for regulating T cell responses to transplanted organs and tissues for several reasons: (i) DC are the only cells that can activate naïve (alloAg-reactive) T cells; (ii) DC are known regulators of central and (likely) peripheral tolerance; (iii) DC are easily influenced *in vitro* (and in some reported cases, *in vivo*) by exogenous factors (e.g., cytokines, viral transfection, lipofection, pharmacologic agents, blocking antibodies/antagonists); (iv) Some DC subsets have been purported to possess tolerogenic properties; (v) almost 10 years ago, it was discovered that iv administration of donor iBMDC one week prior to transplant results in significant prolongation of cardiac or pancreatic islet allograft survival, while mBMDC accelerate graft rejection (129, 130).

As mentioned, DC can arise from different progenitor cell populations (i.e., $CD34^+$, CLP, CMP, BM-precursors, etc), and the environment in which they are cultured can influence these progenitors. The knowledge that iBMDC can prolong allograft survival, indefinitely in one report (131), sparked a variety of investigations to maximize on the potential of using DC with similar properties to iDC to achieve tolerance to alloAg.

1.1.4.1. DC Plasticity

DC have been cultured in the presence of an assortment of pharmacologic and biologic agents, some of which are currently used clinically in transplant therapy protocols, which have been shown to regulate their ability to activate T cells, to differentiate into mature DC, and to migrate to secondary lymphoid tissues (Table 3; Fig 5a). For example, Hackstein *et al* (132) investigated the effects of clinically-relevant doses of aspirin on DC and found the drug maintained DC immaturity even after LPS-stimulation, due in part to impairment of NF-κβ activity.

The natural ability of DC to secrete cytokines make them ideal targets for genetic manipulation for secretion of cytokines that would most greatly influence the desired T cell response. Adeno- and retroviral vectors and lipofection have been used to transfect DC with IL-4, IL-10, TGFβ1, IDO, or FasL, for example (Fig 5b), although with varying success in prolonging allograft survival [i.e., (133)], and to date, no genetically-modified donor DC used as a mono-therapy have induced donor-specific tolerance.

As the enigmatic, rare (even for DC!), pDC has been suggested to be able to induce the production of T reg cells (92, 115), attempts are being made to optimize the propagation of this highly-sought after DC *in vitro* (115, 134). The recently-discovered murine "regulatory" DC (64, 93), so-called because of their supposed ability to induce T reg populations, is also found in extremely low numbers *in vivo*. Culture of BMDC precursors in GM-CSF, TNFα, and IL-10 leading to substantial numbers of regulatory DC *in vitro* (64) suggests these DC may be able to be studied more extensively, possibly leading to their manipulation for therapy.

Table 3. Pharmacologic, biologic, and genetic engineering approaches to potentiation of DC tolerogenicity

Biologic	**Non-viral**	**Anti-inflammatory cytokines**
Costimulation Blocking Agents (anti-CD40L/CTLA4Ig[Σ])	electroporation	IL-10 (mammalian and viral)
Anti-inflammatory Cytokines (IL-10/TGFβ)	lipofection	TGFβ
	biolistic (gene gun)	IL-4 (in murine collagen arthritis[§])
Pharmacologic	**Viral**	**Co-stimulation blocking agents**
Corticosteroids	retrovirus	CTLA4Ig, sCD40Ig[Ω]
Anti-metabolites (MMF*)	adenovirus	
Calcineurin Inhibitors	adeno-associated virus	**Death-inducing ligands**
TOR[#] Inhibitors (Rapamycin)	vaccinia	FasL (CD95L)
Deoxyspergualin	herpes simplex	TRAIL[ɕ]
Vitamin D_3	lentivirus	**Others**
		Serrate 1
		Single chain anti-CTLA4 mAb
		HLA-G

[Σ]CTLA4Ig = cytotoxic T lymphocyte Ag 4-immunoglobulin

*MMF = mycophenolate mofetil

[#]TOR = target of rapamycin

[§]Several independent reports have shown that DC transduced using various vectors to express IL-4 inhibit collagen-induced arthritis in mice.

[Ω]sCD40Ig = soluble CD40-immunoglobulin

[ɕ]TRAIL = TNF receptor apoptosis-inducing ligand

[Modified from (135)]

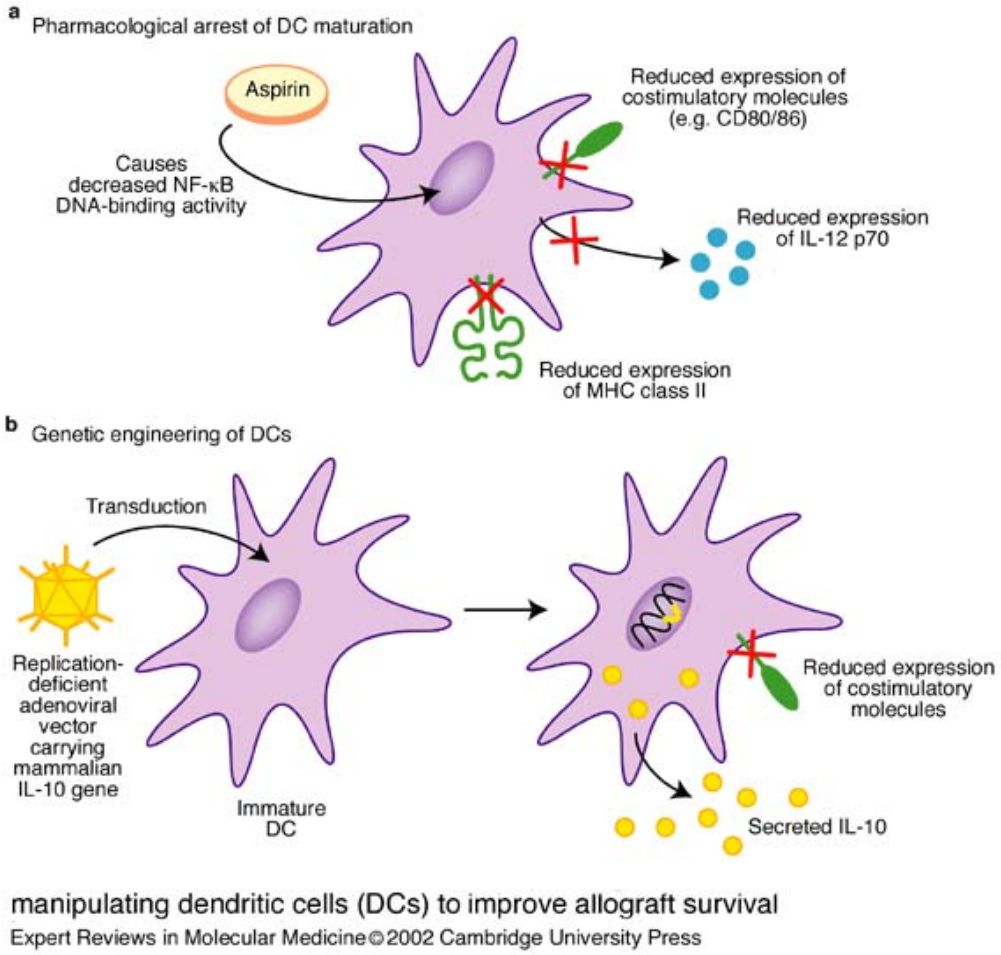


Fig 5. Manipulation of DC for enhancement of allograft survival.

(A) Aspirin exerts its inhibitory effects on DC differentiation/maturation by an NF- κB-dependent, cyclooxygenase-independent, pathway such that NF-κB DNA-binding activity is reduced (132, 136). Aspirin profoundly inhibits expression of major histocompatibility complex (MHC) class II and costimulatory molecules such as CD80/86, and impairs expression of the potent allostimulatory cytokine interleukin 12 (IL-12); explaining the subsequent failure of aspirin-treated DC to stimulate T-cell proliferation. (B) Transfection of DC by genetically manipulated virus, such as replication-deficient adenovirus, can induce overproduction of specific immunomodulatory proteins (here represented by IL-10, which elicits alloantigen-specific T-cell hyporesponsiveness) that may be beneficial for prolongation of graft survival. Human DC transduced with IL-10 show reduced cell-surface expression of costimulatory molecules, impaired production of IL-12 and are able to inhibit autologous human skin graft rejection in a human–murine skin graft model (137) [Fig modified from (48)] *Reprinted with the permission of Cambridge University Press*.

1.1.4.2. DC Antagonists

A number of cell surface molecules on DC are essential for regulating their response to inflammation (as well as steady state trafficking). Numerous methods to target these molecules have been employed. For instance, as the delivery of the second signal through CD80/CD86-CD28 interactions is essential for activation of naïve T cells, investigators have attempted to interrupt this exchange of information by blocking functional CD80 and CD86 expression on the DC cell surface through cytotoxic T lymphocyte Ag (CTLA) 4Ig molecules (138-142) - an immunoglobulin (Ig) fused with CTLA4. The alternate receptor for the B7 molecules, CTLA4 has about a 20-fold higher affinity for CD80 and CD86 than CD28 and reaches maximal upregulation on T cells 48-72 h after activation as a self-regulating mechanism (143, 144). Other attempts have been made through administration of blocking interactions of CD40 (expressed on mDC)/CD40L (CD154; expressed on activated T cells, the ligation of which with CD40 on DC activates DC to signal activation of $CD8^+$ T cells) (145), with some success in mouse transplant models (142, 146) and non-human primate models (147-149), but in primate renal transplants, for example, all grafts eventually were rejected after anti-CD154 treatment was stopped (150, 151). Further, there were thromboembolic complications in the human and primate renal models (152), which had not been evident in the murine studies. Currently, investigations are being undertaken to block chemokine receptors (important in directing leukocyte migration and discussed further in Section 1.2), [i.e., (153-155) and Fig 6] although analysis of the effects of chemokine antagonists on DC yet is to be explored.

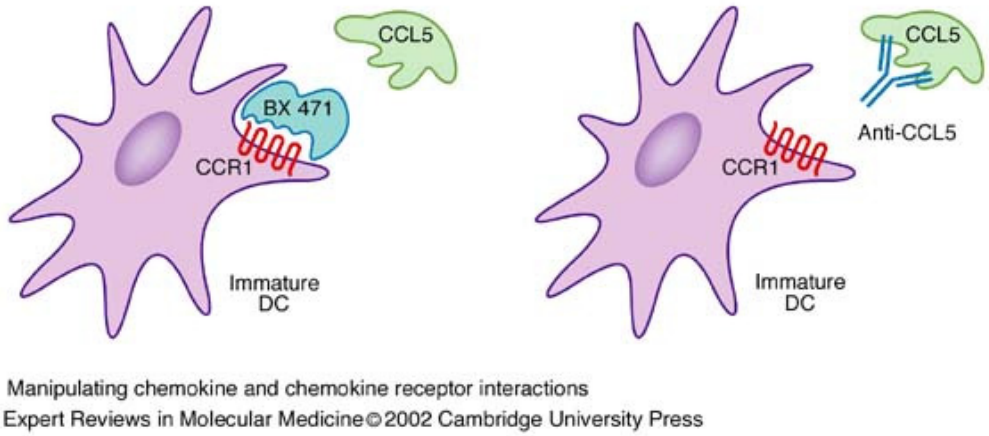


Fig 6. DC antagonists.
A chemokine receptor antagonist, such as BX 471 (155), might prevent immature DC expressing chemokine receptors (such as CCR1) for inflammatory chemokines from migrating to the graft and hence prevent the subsequent presentation of graft antigens to naive T cells. Similarly, binding of free chemokine by blocking antibody, such as anti-CCL5, can also potentially prevent inflammatory chemokine-induced migration of immature DC to the graft. *Reprinted with the permission of Cambridge University Press.*

1.1.4.3. DC Subsets in Transplantation

Donor iBMDC, used to prolong allograft survival in early DC/transplant studies (130, 131), do not express CD8α. Reports that $CD8\alpha^+$ DC are potentially tolerogenic led researchers in our laboratory to investigate whether these DC might be able to extend graft survival beyond that seen with iBMDC. In a comparative study between donor $CD8\alpha^-$ and $CD8\alpha^+$ splenic DC, both subsets of iDC significantly prolonged murine cardiac allograft survival (47), as previously reported for murine iBMDC. Also like BMDC, $CD8\alpha^-$ mDC accelerated graft rejection. Interestingly, mDC populations which were >95% $CD8\alpha^+$ by flow-sorting also significantly prolonged allograft survival. Both subsets were determined to be phenotypically mature before administration, and their maturation could not be further enhanced by exposure to LPS or

CD40L (indicating both subsets were terminally mature upon administration). Both mDC subsets were equally capable of stimulating allo T cell proliferation in primary MLR, but T cells from allograft recipients which were pre-treated with $CD8\alpha^+$ DC 7 days prior to transplant were less responsive to donor Ag than those from $CD8\alpha^-$ DC pre-treated animals. However, this prolongation of graft survival was found to be associated with neither immune deviation nor induction of T reg cells. Nor does it seem likely the tolerogenic effects of $CD8\alpha^+$ DC are due to IDO in this model as this effect was lost if the mDC population administered before transplant contained more than 5% $CD8\alpha^-$ DC [as only 3% of $CD8\alpha^+$ DC are needed to abrogate the ability of $CD8\alpha^-$ DC to activate naïve T cells against tumor/self-peptide (82)]. In early $CD8\alpha^+$ DC studies by Kronin *et al* (83), $CD8\alpha^+$ DC impaired $CD4^+$ T cell proliferation through a FasL-dependent mechanism, as evidenced by their lack of ability to suppress T cell allo-responses in *gld* (FasL-deficient) and *lpr* (Fas-deficient) mice. However, despite mRNA expression for CD95, vigorous attempts to detect this protein on the surface of either DC subset have met with failure thus far. Studies in our own laboratory using donor-derived apoptotic bodies injected 7 days prior to transplant showed prolonged allograft survival similar to injection of iBMDC (Morelli, AE, unpublished observations). That $CD8\alpha^+$ DC may be more susceptible to apoptosis than $CD8\alpha^-$ DC (Moser, M, personal communication) offers a possible explanation as to how these cells are able to prolong allograft survival in either the mature or immature state, but do not explain why this ability is FasL dependent. Nevertheless, though the mechanism(s) by which $CD8\alpha^+$ mDC prolong cardiac allograft survival remains unknown, new theories regarding their alleged tolerogenic abilities continue to come to light.

As mentioned, pDC have been proposed to promote T reg induction. Therefore our group, in collaboration with Dr. Pia Björck, investigated whether administration of freshly-isolated donor pDC prior to transplant would result in prolongation of graft survival. These iDC delayed murine cardiac allograft rejection significantly beyond that observed in control, untreated animals, and have in fact given the most marked extension of the mean graft survival of any unmanipulated donor iDC subset [MST 53 days for ipDC (156) vs MST 22 days for iBMDC (130)]. Experimental tests regarding T reg cell detection have been inconclusive, and further exploration of the mechanism by which ipDC prolong graft survival is necessary. The recent discovery of IDO expression (by RT-PCR) in freshly-isolated pDC suggests a possible suppression of recipient DC-mediated T activation (de Creus, unpublished observations).

1.2. Chemokines[2]

The immune response is strictly regulated. Though a diversity of inflammatory cytokines exists, each is specifically released in response to certain stimuli, resulting in elicitation of a tightly-controlled immune response. A major player in immune response regulation is the secretion of specific chemokines by inflamed tissue and immune cells. Chemokines have roles in leukocyte activation, selectin/integrin up-regulation, hematopoiesis, angiogenesis, and adaptive immunity, but were first recognized for their ability to function as chemoattractants (157-159). All known chemokines bind to seven-pass, transmembrane-spanning, Gi/Go protein-coupled, *Bordetella pertussis* toxin-sensitive receptors (157, 160, 161) (Fig 7). The specificity and complexity of the chemokine system stem from the regulated expression of their receptors (CR). Not only does each chemokine family recruit only specific cell types, as discussed below (Section 1.2.1), but also frequently the expression of CR is controlled further by regulation of expression according to cell subsets (e.g., Th1 cells express CCR5 and CXCR3, although Th2-cells express CCR3 and CCR4) and/or the state of cell activation (e.g., iDC express CCR1–6, although mDC down-regulate these six receptors and up-regulate CCR7 expression) (162-164).

[2] **Section 1.2** contains modified excerpts from reviews co-written by the author in the journals *Transplantation* and *Expert Reviews in Experimental Medicine*.

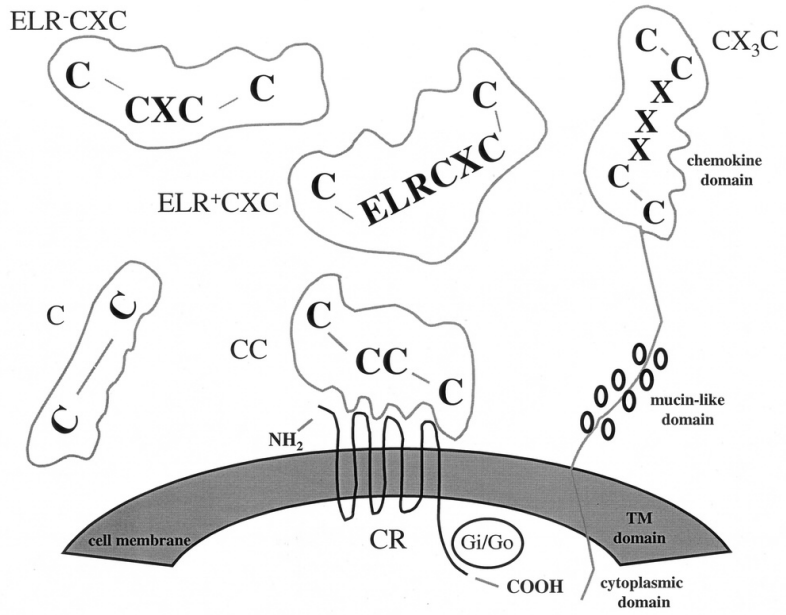


Fig 7. The four known chemokine families (C, CC, CXC, and CX_3C).
All known chemokines bind to Gi/Go protein-coupled, 7-pass transmembrane spanning receptors, as shown for a generic CC chemokine. Both subsets of the CXC family are shown, and the CX_3C chemokine, fractalkine, is depicted in its membrane-bound form (165).

1.2.1. Classification

Like most newly-discovered molecules, each chemokine was given a descriptive name implicating a biologic function(s) by its discoverer. As some were named according to structure, others to function, and still others according to favorite movie characters [Rantes, the protagonist from "Man Facing Southeast", 1986, Film Dallas productions, inspired *r*eleased upon *a*ctivation, *n*ormal *T* cell *e*xpressed and *s*ecreted (Tom Schall, personal communication and (166)], Yoshie and Zlotnik (167) suggested a uniform nomenclature for chemokines based on structure and classical nomenclature that was presented and approved at the Keystone Symposium on Chemokines and Chemokine Receptors in 1999. The new nomenclature for CR proposed by Murphy *et al* (157) was presented to and approved by the Nomenclature Committee of the International Union of Pharmacology in 2000. These nomenclatures will be used herein. Classic chemokine names will be included upon first appearance (followed by the new nomenclature in parentheses) and the new nomenclature will be used thereafter. Readers are referred to Table 4 [excerpted from (168)] for complete listings of chemokines and their receptors.

Table 4. CXC, C, CX3C, and CC chemokine/receptor families

Systematic name	Human chromosome	Human ligand	Mouse ligand	Chemokine receptor(s)
CXC chemokine/receptor family				
CXCL1	4q21.1	GROα/MGSA-α	GRO/MIP-2/KC?	CXCR2 > CXCR1
CXCL2	4q21.1	GROβ/MGSA-β	GRO/MIP-2/KC?	CXCR2
CXCL3	4q21.1	GROγ/MGSA-γ	GRO/MIP-2/KC?	CXCR2
CXCL4	4q21.1	PF4	PF4	Unknown
CXCL5	4q21.1	ENA-78	GCP-2/LIX?	CXCR2
CXCL6	4q21.1	GCP-2	GCP-2/LIX?	CXCR1, CXCR2
CXCL7	4q21.1	NAP-2	Unknown	CXCR2
CXCL8	4q21.1	IL-8	Unknown	CXCR1, CXCR2
CXCL9	4q21.1	Mig	Mig	CXCR3[a]
CXCL10	4q21.1	IP-10	IP-10/CRG-2	CXCR3[a]
CXCL11	4q21.1	I-TAC	I-TAC	CXCR3[a]
CXCL12	10q11.21	SDF-1α/β	SDF-1/PBSF	CXCR4[b]
CXCL13	4q21.1	BCA-1	BLC	CXCR5
CXCL14	5q31.1	BRAK/bolekin	BRAK	Unknown
(CXCL15)		Unknown	Lungkine/WECHE	Unknown
CXCL16	17p13			CXCR6
C chemokine/receptor family				
XCL1	1q24.2	Lymphotactin/SCM-1α/ATAC	Lymphotactin	XCR1
XCL2	1q24.2	SCM-1β	Unknown	XCR1
CX_3C chemokine/receptor family				
CX_3CL1	16q13	Fractalkine	Neurotactin/ABCD-3	CX_3CR1
CC chemokine/receptor family				
CCL1	17q11.2	I-309	TCA-3/P500	CCR8
CCL2	17q11.2	MCP-1/MCAF/TD CF	JE?	CCR2
CCL3	17q12	MIP-1α/LD78α	MIP-1α	CCR1, CCR5
CCL3 L1	17q12	LD78β	Unknown	CCR1, CCR5
CCL4	17q12	MIP-1β	MIP-1β	CCR5α
CCL5	17q12	RANTES	RANTES	CCR1, CCR3, CCR5
(CCL6)	Unknown	C10/MRP-1	Unknown	
CCL7	17q11.2	MCP-3	MARC?	CCR1, CCR2, CCR3
CCL8	17q11.2	MCP-2	MCP-2?	CCR3, CCR5
(CCL9/10)	Unknown	MRP-2/CCF18/MIP-1γ	MIP-1γ	CCR1
CCL11	17q11.2	Eotaxin	Eotaxin	CCR3
(CCL12)	Unknown	MCP-5		CCR2
CCL13	17q12	MCP-4	Unknown	CCR2, CCR3
CCL14	17q12	HCC-1	Unknown	CCR1, CCR5
CCL15	17q12	HCC-2/Lkn-1/MIKP-1	Unknown	CCR1, CCR3
CCL16	17q12	HCC-4/LEC/LCC-1	Unknown	CCR1, CCR2
CCL17	16q13	TARC	TARC/ABCD-2	CCR4
CCL18	17q12	DC-CK1/PARC/AMAC-1	Unknown	Unknown
CCL19	9p13.3	MIP-3β/ELC/exodus-3	MIP-3β/ELC/exodus-3	CCR7[d]
CCL20	2q36.3	MIP-3α/LARC/exodus-1	MIP-3α/LARC/exodus-1	CCR6
CCL21	9p13.3	6Ckine/SLC/exodus-2	6Ckine/SLC/exodus-2/TCA-4	CCR7[d]
CCL22	16q13	MDC-STCP-1	ABCD-1	CCR4
CCL23	17q12	MPIF-1/CKβ8/CKβ8-1	Unknown	CCR1
CCL24	7q11.23	Eotaxin-2/MPIF-2	MPIF-2	CCR3
CCL25	19p13.3	TECK	TECK	CCR9
CCL26	7q11.23	Eotaxin-2	Unknown	CCR3
CCL27	9p13.3	CTACK/ILC	ALP/CTACK/ILC/ESkine	CCR10
CCL28	5p12	MEC		CCR3/CCR10

[a]CD183
[b]CD184
[c]CD195
[d]CD_w197

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1.2.1.1. Family

Chemokines make up a superfamily consisting of 8 to 11 kDa proteins subdivided into four families (C, CC, CXC, and CX_3C) according to the position and separation of their first two amino-terminal cysteine (C) residues of a four-cysteine motif (157, 161) (Fig 7). The C family consists of only two members: lymphotactin-α [X chemokine ligand (CL)1] and lymphotactin-β (XCL2), which are homologous to the CC family at their carboxyl end, but lack the first and third cysteine residues found in the other three families. XCL1 and XCL2 recruit T lymphocytes only (169). The CC chemokine family evokes monocyte, lymphocyte, and DC migration (162, 164, 170). The CXC family is further separated into two sub"families": those that possess an additional glutamic acid(E)-leucine(L)-arginine(R) amino acid motif and primarily induce neutrophil migration, and those that are ELR^- and recruit a more lymphocytic population (158). The CXC family also contains a unique member, CXCL16, which is predicted to be the only one of its family to possess a mucin-like stalk (171-173). The CX_3C family contains only one member: fractalkine (CX_3CL1). CX_3CL1 was, previous to the discovery of CXCL16, the only known membrane-bound (via a mucin stalk) chemokine. Both CXCL16 and CX_3CL1 are predicted to function, then, as both an adhesion molecule (possibly aiding DC-T cell interactions) and a chemoattractant (for IL-2 activated natural killer [NK] cells and $CD8^+$T cells) (172-179).

1.2.1.2. Expression

Chemokines can be grouped again into categories reflecting their temporal and spatial expression: the inducible (or inflammatory) and the constitutive (or lymphoid) chemokines. The inducible chemokines are regulated by pro-inflammatory stimuli (e.g., IL-1, TNFα, IFNγ, LPS) and regulate innate and adaptive immune responses. These chemokines are released by injured endo- and epithelial cells, innate/early immune-responders such as neutrophils, monocytes/Mϕ, and NK cells, and adaptive immune-responders such as iDC and activated T cells (159, 180). The constitutive chemokines are important for tissue homing of leukocytes, as well as for immune surveillance (159, 162, 170, 181). Lymphatic and high endothelial vessel cells, peripheral epithelial cells, and DC and EC in T cell areas express the constitutive chemokines (182-184).

1.2.2. Chemokines & DC Recruitment

Ubiquitously distributed throughout the body, tissue-resident DC likely arise from circulating, blood-borne pre-DC. LC precursors are attracted to residency in the epidermis by chemokine gradients expressed by keratinocytes (185-187). The skin recruits $CD34^{+}CD14^{+}CCR6^{+}$ immature (i) DC through the constitutive expression of macrophage inflammatory protein (MIP)-3α (CCL20) (185, 188). The expression of TGFβ in the skin induces further differentiation of these precursors into LC (186) [and TGFβ KO mice lack LC (113)]. Less is known about DC recruitment into other tissues, but it has been suggested that recruitment into the kidney (for example) is by a similar method. Coates *et al* (190) have shown that blood iDC express CCR5 mRNA, respond to at least one CCR5 ligand *in vitro*, and enter the kidney (an organ that expresses CCR5 ligands) after iv injection. Interestingly, the same chemokines that (apparently) recruit pre-DC (*in vitro* or *in vivo*) do not recruit tissue resident iDC. Neither kidney nor skin

(LC) iDC migrate to CCR5 or CCR6 ligands, respectively (50, 185) and appear to be no longer functional despite receptor mRNA expression comparable to that of pre-DC. Mack *et al* (191) have reported CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) downmodulate CCR5 expression on murine NK cells *in vitro* (as well as CCR2 by CCL1 on monocytes) in a dose-dependent manner. This aids weight to the suggestion that CR may become desensitized to their chemokine ligands after prolonged exposure *in vivo*. This also may explain the pluripotent ability of blood- and BM-derived iDC to respond to nearly any of the inducible CC chemokines typically tested in *in vitro* chemotaxis assays (192-194) – it is unlikely they remain in any one area long enough to become desensitized, and it seems more immunologically efficient to have semi-differentiated (non tissue-specific) iDC be capable of responding to inflammation anywhere in the body (and thus to a wide panel of chemokines), instead of just in the tissue in which they reside.

Chemokines also are principal in directing DC migration during an immune response. CCL3, CCL4, and CCL5 are archetypal chemokines released by inflamed tissues (195), which have been reported to induce iBMDC migration in chemotaxis assays *in vitro* (192). The production of these chemokines by injured cells would eventually produce a chemokine gradient, with the maximal concentrations at the site of the inflammation. Circulating and resident peripheral iDC receiving these and other inflammatory signals (TNFα, IL-β, etc) likely follow this gradient to its source. At the site of injury, the iDC would phagocytose Ag as usual, receiving further signals from the wounded area to commence differentiation into mDC. During this process, activated, intermediately-matured (int) DC begin to downregulate Ag uptake as well as CR for inducible chemokines. As they upregulate their co-stimulatory and MHC I and II molecules, the DC also

upregulate CCR7, the CR for the constitutive chemokines MIP-3β (CCL19) and secondary lymphoid chemokine (SLC; CCL21) (192-194, 196-199), as well as CXCR4 [which binds stromal-derived factor (SDF)-1α (CXCL12)] (193, 194). intDC are directed into lymphatic/high endothelial vessels by a CCL21 gradient, leading the DC into secondary lymphoid tissues (184, 197, 198, 200, 201). Upon departure of the lymphatic vessel, int/mDC are directed to T cell areas by CCL19 (44, 197, 198) or to B cell areas, likely by CXCL13 (B lymphocyte chemoattractant; BLC) (202, 203).

All mDC (based on phenotypic and some functional criteria) studied to date, regardless of tissue [e.g., skin (185), Peyer's patches (44), BM (197), spleen (48), liver (54), kidney (50), and blood (204)] or species of origin [human (195), non-human primate (43), and mouse (192)] have been reported to migrate to either CCL19 and/or CCL21 (not all studies tested both chemokines). iDC, however, are not uniform in their response to inducible chemokines. As mentioned, iDC do not always migrate to the same chemokines as they do as pre-DC. Unlike mDC, iDC isolated/generated from different tissues have differing abilities to migrate to inducible CC chemokines [Chapter 1 and reviewed in (198)], although as yet no major differences have been reported between species. It has been reported, however, that human $CD11c^{+}CD123^{-}$ "myeloid" i and mDC and ($CD11c^{-}CD123^{+}$) pre- and mpDC both express different CR on their surface, as well as respond to different chemokines (193, 205). Further, it was determined in these studies that pDC appear to express some CR that are non-functional (as determined by *in vitro* chemotaxis assays and changes in intracellular Ca^{++} levels). No comprehensive comparative studies of any of the DC subsets have been performed in the mouse previous to this work.

Although it is well documented that iDC are directed to sites of inflammation by specific chemokines released by activated endothelial cells, and that mature DC are recruited to secondary lymphoid tissues by constitutively expressed chemokines, DC are also capable of influencing leukocyte trafficking via the same mechanisms important for their own localization. Chemokines secreted by DC ("dendrikines") typically are involved in T cell recruitment. mDC in particular express CCL19 (182, 206-208) and CCL22 (macrophage-derived chemokine; MDC) (182, 206), aiding in the localization of both mDC and naïve T cells in T cell areas.

Both human and mouse DC have been reported to secrete chemokines in response to inflammatory/maturation stimuli, important for initiating activated T cell migration, such as CCL9/10 (MIP-1γ) (mouse) (209), CCL18 [DC chemokine (DC-CK) 1] (human) (210), CCL3, CCL5 (human and mouse) (211, 212), CXCL8 (IL-8) (human) (212), CCL4 (human)(212), CCL22 (human and mouse) (179, 213, 214), CCL17 (thymus- and activation-regulated chemokine; TARC) (human and mouse) (163, 215, 216), and CXC_3L1 (mouse) (178, 179). DC can selectively recruit Th2 cells: CCL17-expressing DC may be important for the recruitment of activated Ag-specific $CD4^+$ Th2 cells but not naïve $CD4^+$ T cells (162, 215, 216). Th1 cell recruitment may be suppressed by up-regulation of Th2-specific chemokines (e.g., CCL22) by DC exposed to PGE2 (217). DC also produce IFNγ (10, 218, 219), which can elicit the production of CXCL9 (monokine induced by IFNγ; Mig) and CXCL10 (IFN-γ–inducible protein of 10 kDa; IP-10) -- chemokines important in graft rejection (220).

DC do not produce only T cell-attracting chemokines. CXCL12, CCL19, CCL20, and CCL21 (221), all of which recruit B cells, are made by (human) DC. As with T cells, DC can specifically

recruit B cell subsets. Dubois *et al* (221) have shown that supernatant from activated DC recruits naïve and memory B cells, but not germinal center B cells.

1.2.3. Chemokines in Transplantation

Organ transplant rejection is mediated largely by circulating peripheral leukocytes induced to infiltrate the graft by various inflammatory stimuli. The secretion of chemokines by inflamed graft tissues, as well as by early innate-responding leukocytes that infiltrate the graft, are responsible for the recruitment of phagocytic APC such as iDC and activated alloreactive leukocytes (165).

1.2.3.1. Chemokine Expression in Transplanted Tissues

Because leukocytic graft infiltration seems to be such a key event in graft rejection, recent work has investigated the expression of chemokines in rejecting versus accepted allografts (154, 161, 222, 223) (Table 5). Early expression of inducible/inflammatory chemokines is most likely induced as part of the innate [i.e., graft-infiltrating NK cells (224, 225)] immune response to damaged graft vessels, as chemokines expressed in the first 4 days (peaking at day 3) posttransplant are expressed at similar levels in murine allo- and syngeneic skin grafts [Table 5 and (220, 223)]. These early-expressed chemokines are no longer detectable by day 5 (with the exception of CCL2) and are replaced by a second set of chemokines (CCL5, CXCL9, CXCL10) in allogeneic grafts closer to the time of graft failure (3–4 days before rejection). This expression pattern is similar to that reported for rejecting human skin allografts, albeit in a human donor/SCID recipient model (226, 227).

Murine cardiac grafts also show a temporal chemokine expression pattern [(225, 228, 229) and Table 5], although in heart grafts, CXCL10 is produced both early and late (whereas it is only a late-expressed chemokine in skin grafts). The late-expressed chemokines in cardiac allografts likely are induced by adaptive immune responses of graft-specific leukocytes [i.e., CXCL9 and CXCL10 are produced by $CD8^+$ T cells (225)]. In rejecting human cardiac allografts, immunocytochemical analysis of endomyocardial biopsies for several chemokines/CR were found to have a high correlation between the incidence of $CD3^+$ T cells, CXRC3 and CXCL10 (a ligand of CXCR3) expression, and acute rejection (230).

Table 5. Summary of chemokine expression in transplanted organs

Organ	Syngeneic/nonrejecting	Allogeneic/rejecting	Allogeneic/accepted[e]	Refs
Skin[m,h]	**CCL2, CCL3, CCL4, CXCL1**[d] CCL2^{lo}[,d]	**CCL2, CCL3, CCL4, CXCL1** CCL2^{lo}, CCL5, CXCL9, CXCL10	ND	**a**
Heart[m,h]	**CCL2^{lo}, CXCL10^{lo}**	**CCL2, CXCL1, CXCL10** CCL3, CCL4, CCL5, CXCL9, CXCL10	CCL3^{lo}, CCL4^{lo}, CCL5^{hi}, CXCL1^{lo} CXCL10^{hi}	**b**
Kidney[r,h]	**CCL2^{lo}, CCL3^{lo}, CCL5^{lo}**	**CCL2, CCL3, CCL5, CXCL1** XCL1, CCL3, CCL4, CCL5, CXCL1^{lo}, CXCL10	ND	**c**
Lung[r,h]	**CCL5^{lo}, CXCL10^{lo}**	**CCL5^{lo} CXCL10^{lo},** CCL5^{hi}, CXCL10	ND	**d**

[m]Mouse; [r]rat; [h]human. [d]**Bold-faced chemokines**, early expressed; normal text chemokines=late expressed. [e]Long-term (>60 days) cardiac allografts in gallium nitrate-treated hosts. ND, Not determined.
[a](220, 226, 227); [b](225, 227-229); [c](231, 232); [d](233-235).

Though murine heart and skin allografts express similar chemokines, temporally the pattern is dissimilar. In rejecting heart allografts, CCL3 and CCL4 are observed late (in skin they are early) and CXCL9 and CXCL10 are expressed both late and early, while for skin they are expressed only late (close to and during the time of rejection) (Table 5). Other analyses of human kidney (231, 232, 236) and lung (233-235) transplants have focused predominantly on the expression of CCL5, but other chemokines, such as CCL2 and CXCL10, also are expressed in rejecting kidney and lung grafts. Interestingly, in gallium nitrate-treated mouse recipients, long-term surviving heart allografts expressed low levels of CCL3 and CCL4 60 days posttransplant, although levels of CXCL10 and CCL5 remained high, indicating expression of "rejection-associated"

chemokines does not necessarily indicate rejection (229). Clearly, the expression of chemokines in transplanted tissues and organs is complex, regulated both by time after transplant as well as by specific the type of tissue graft.

Chemokines also may facilitate chronic cardiac allograft rejection, e.g., CCL5 production was found to correlate with leukocytic infiltration as well as to precede intimal thickening of arterial vessels (237). In a heart allograft model using CCR1 (a receptor for CCL5) KO mice, mAb depletion of $CD4^+$ T cells resulted in permanent graft acceptance in both the KO and wild type (wt) recipients. Only CCR1 KO mice did not exhibit chronic rejection (238). Targeting of CCR5 (via KO or neutralizing mAb) also leads to marked improvement of murine cardiac allograft survival that can result in permanent graft acceptance with no chronic rejection when used with cyclosporine (CsA) treatment (239). Further, transgenic overexpression on endothelial cells of the Duffy Ag (a non-signaling chemokine-binding protein that acts as a "sink" for CC and CXC chemokines) has recently been shown to have a negative effect on angiogenesis (240). Theoretically, this occurs through prevention of chemokine interactions with functional CR. As a consequence, efforts to block early chemokine expression to alleviate acute rejection may also have to be directed toward the later stages of allograft rejection.

1.2.3.2. Chemokine Targeting Strategies in Transplantation

There are several approaches to targeting DC migration for therapy of graft rejection. Several chemokines, which bind to multiple CR, are expressed in the graft. Although it is known that BM- and blood-derived iDC can express any of CCR1 to CCR6, it is not clear which CCR are expressed on tissue-resident DC, making it difficult to target DC specifically. Further, blockade

of recipient iDC migration to the graft would block only the indirect pathway of graft rejection, as "passenger" donor-derived DC will mature under the influence of inflammatory signals in the graft and migrate to DLN, activating the direct pathway of rejection. Therefore, blockade of CCR7-CCL19/CCL21 interactions may be more suitable for effective targeting of DC migration for transplant therapy. However, some current strategies that block CCR (by means of KO mouse models for CCR1 or CCR5 or anti-CCR5 mAb) that bind inflammatory chemokines (blocking iDC, Th1 cells, monocytes, and NK cells) have succeeded in achieving indefinite graft survival in conjunction with CsA administration [(160, 170) and Table 6]. The advent of synthetic CR antagonists such as BX 471 (a CCR1 antagonist) may well prove to be an effective means for targeting specific DC-expressed CR [(155) and Fig 6]. Another strategy has been to use virally-manufactured proteins ["virokines": MC418 and vMIP-II, for example, (241, 242) further reviewed in (243-245)] that antagonize CR activity to block leukocyte–chemokine interactions within the graft for modest, but significant, prolongation of both non-vascularized and vascularized heart allograft survival (246). Strategies to enhance tolerogenic DC migration [i.e., through over-expression of important DC CR such as CCR7 (199)] may become important for maximizing DC–T cell interactions.

Table 6. Summary of chemokine-targeting strategies in transplantation

Organ/tissue transplanted	Chemokine(s)/CR targeted	Method	Cell type(s) potentially targeted	Graft outcome	Refs
Bone marrow	CCL3	MIP-1 and CCL3 KO recipient mice	Th1 cells, iDC, monos	[a]$CD8^+$ T cell infiltration; [a]GVHD	a
Heart	CXCL10	CXCL10 KO recipients and donors	Th1 cells, B cells	KO to wt=prolongation wt to KO=rejection	b
	CCL3	CCL3 KO recipients	Th1 cells, iDC, monos	Normal rejection	c
	CCL5	CCL5 KO recipients	Th1/Th2 cells, iDC, monos	Normal rejection	c
	CCR1	CCR1 KO recipient	T cells, iDC, monos	Indefinite w/CsA	d
	CCR5	CCR5 KO/anti-CCR5 mAb recipient	Th1 cells, iDC, monos, NK cells	Indefinite w/CsA and no chronic rejection	c
	CXCR3	CXCR3 KO recipient	Th1 cells, B cells	Indefinite w/CsA	e
	CXCL9	CXCL9 antiserum	Th1 cells, B cells	Prolongation	f
	CX_3CR1	CX_3CR1 poly Ab	NK cells, T cells	Prolongation	g
	CX_3CR1	CX_3CR1 KO recipients	NK cells, T cells	Prolongation w/CsA	h
	Various chemokines	vMIP-II (virokine)	Neutrophils, NK cells, T cells, B cells, iDC, monos?	Prolongation; better prolongation w/vIL-10	i
	CCR8	MC148 (virokine)	T cells, B cells, monos	Prolongation; vIL-10=no effect	i
Skin	CXCL1	CXCL1 antiserum	Neutrophils, monos	[a]CXCL9 and [a]CXCL10; prolongation	j
	CXCL9	CXCL9 antiserum	Th1 cells, B cells	Prolongation	k
	CXCL9/CXCL10	IFNγ KO recipients	Th1 cells, B cells	[a]CXCL9 and [a] CXCL10; prolongation	k

All studies were done in mice. KO=knockout. wt=wild type.
[a] reduction in severity or level of expression. CsA, Cyclosporine; iDC, immature DC.
a(235); b(228); c(239); d(238); e(247); f(248); g(249); h(250); i(242); j(251); k(220)

Despite the undisputed importance of DC migration to transplantation outcome and the responses of DC to a large number of CC chemokines, very little attention has been afforded to date to manipulating DC/chemokine interactions as a basis for antirejection therapy. The investigations mentioned above and in Table 6 regarding the effects of blocking chemokine/leukocyte interactions focus on preventing graft infiltration by activated T cells. Most discussions of DC immunobiology in transplantation, although acknowledging the necessity of DC reaching T cell areas to induce naïve T cell activation, refer instead to genetic/environmental manipulation of potential "stimulatory" DC to render them "tolerogenic" (170, 198, 222, 252, 253). Alternatively, they focus on the T cell aspect of chemokine-directed DC/T cell interactions (161). Previous to this work, no attention had been given to the role specific chemokines play in DC migration and in DC initiation of the activated T cell response against the allograft.

1.2.3.3. DC, Chemokines, and Allograft Immunity

Depending on their tissue of origin, iDC express several CR, most of which bind to inflammatory chemokines (170). Importantly, the early-expressed chemokines detected in skin and heart allografts are among those reported to induce iDC migration (170). Infiltration of graft tissues by iDC allows these DC to take up and process graft Ag for presentation to naive T cells in DLN, alerting graft-specific T cells to the presence of transplant Ag. Both donor- and recipient-derived, graft-Ag-bearing mDC are directed to DLN by CCL21 (and then to T cell areas by CCL19) upon maturation for direct and indirect activation of naïve, alloreactive recipient T cells.

Strategies to prevent migration of (recipient and donor) mDC from organ allografts could allow would-be donor-specific T cells to remain immunologically "ignorant" of the "foreign" tissue (Fig 8) until the inflammatory phase of the transplant passes. Theoretically, in the absence of "danger" signals (254, 255), DC induce tolerance to peripheral Ag. Thus, mDC migration-blocking therapies eventually could be withdrawn, putatively leaving the recipient immune system unimpaired while achieving donor-specific tolerance due to alloAg presentation in the absence of inflammation. The *plt/plt* mouse (*p*aucity of *l*ymph node *T* cells; which has spontaneous mutations in the expression of CCL19 and CCL21) (256), and the CCR7 KO mouse (the receptor for CCL19 and CCL21) (257), both of which have deficiencies in DC and T cell homing to LN, are ideal models for testing the effects that blocking mDC migration may have on allograft survival. No data using either mouse model have been reported before initiation of the present study.

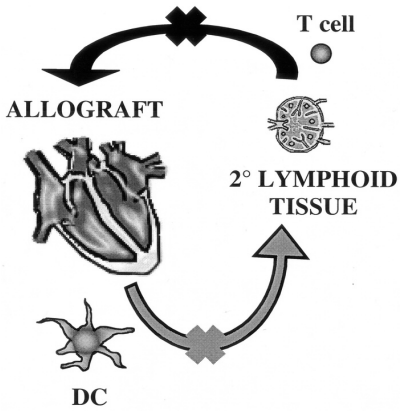


Fig 8. Targeting DC migration.

Current efforts to prolong graft survival by targeting chemokines attempt to interdict activated T cell infiltration of the graft (black arrow and X). However, manipulating migration of alloAg-bearing DC (either donor or recipient) from the graft to 2° lymphoid tissue (e.g., by targeting CCR7/CCL19/21) could potentially block both the direct and indirect pathways of allorecognition by preserving T cell ignorance of the graft. Alternatively, overexpression of DLN homing receptors [as accomplished by Takayama *et al.* (199) with retrovirally induced overexpression of CCR7 in murine DC] could enhance migration of specifically engineered/tolerogenic DC to the 2° lymphoid tissue to promote their interaction with/subversion of allospecific T cells (gray arrow and X). From (165).

1.3. Migration

As discussed in Section 1.2, leukocyte migration is essential for detecting and transporting Ag and initiating immune responses. Though this activity is largely directed by chemokines, other factors play equally important roles. Vascular EC regulate the trafficking of leukocytes from the blood into tissue through selective expression of chemokines, as discussed, but also through the expression of intercellular adhesion molecules, which bind to selectively-expressed ligands on blood vessel-trafficking leukocytes. In the absence of inflammation, during steady state conditions, there is normal turnover of blood-borne to peripheral tissue to DLN DC that monitor for possible incoming pathogens/Ag for maintenance of peripheral self-tolerance. When EC become activated during inflammation, depending on the type of inflammatory signals released by pathogens and/or the injured cells, they release specific chemokines and upregulate the expression of specific selectins and other ligands for integrins expressed on the appropriate leukocytes. This system allows tightly-regulated tailoring of an immune response for the recruitment of the appropriate leukocytes needed to eliminate specific foreign/pathogenic Ag. The emigration of leukocytes from the blood into peripheral tissues is a three-step process that involves rolling on selectins along the vessel wall, tethering to Ig superfamily members, and then extravasating (also called diapedesis) through the EC wall.

1.3.1. Transendothelial migration

The selectins are a three-member family of adhesion molecules that are key in the initial rolling and tethering steps of transendothelial migration. E-selectin (CD62E) is expressed on EC, P-selectin (CD62P) is found on platelets as well as EC, and L-selectin (CD62L) was originally discovered on lymphocytes, but has since been determined to be expressed by monocytes, neutrophils, and granulocytes as well [reviewed in (258-260)]. CD62P and CD62E are

constitutively expressed at low levels on EC and aid in iDC and memory T cell immunologic monitoring of peripheral tissues. Both are upregulated in response to inflammatory stimuli, and their increased expression aids in binding to P-selectin glycoligand (PSGL)-1 and E-selectin ligand (ESL-1) expressed on passing leukocytes, respectively, slowing down leukocytic travel through the blood, until the leukocytes literally are rolling along the blood vessel wall. This rolling allows ligation of CD62L with any of its 5 glycoprotein ligands on EC, signaling upregulation of integrins on leukocytes (260).

The integrins are a family of heterodimeric α and β proteins [e.g., LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), VLA-4 (CD49d/CD29)] expressed on leukocytes that act together in varying combinations to bind to members of the Ig superfamily [e.g., ICAM-1 (CD54), ICAM-2 (CD102), VCAM-1 (CD106)] expressed on EC constitutively, but upregulated in response to inflammatory signals (258). Interestingly, chemokines also play a role in migration before direction of chemotaxis – the secretion of chemokines by EC [or the transport of chemokines secreted within peripheral tissues across EC barriers (261)] and the sequential anchoring of chemokines onto the EC surface (262) triggers stronger adhesion of Ig superfamily molecules [i.e., VLA-4 (263) and ICAM-1(264)] to rolling leukocytes. Binding of integrins to Ig superfamily members slows rolling to a stop, allowing leukocytes to adhere to EC long enough to start the extravasation process.

The exact mechanisms involved in diapedesis (discussed further in Section 1.3.1.1) have been long debated in the field of transendothelial migration. Some reports indicated passage of leukocytes *through* EC into inflammatory sites (265), but now most investigators agree that

leukocytic emigration from the blood into peripheral tissues involves migration *between* EC (266). While heterophilic interactions (binding between two different molecules, i.e., CD54-CD11b/CD18) between adhesion molecules on leukocytes and EC are essential for rolling and tethering of leukocytes, homophilic interactions (binding between two identical molecules, i.e., CD31-CD31) of adhesion molecules on the two cells types are key for facilitating the actual process of extravasation.

Platelet-endothelial-cell adhesion molecule (PECAM)-1 (CD31), CD99, junctional adhesion molecule (JAM)-A, -B, and -C, and vascular endothelial (VE)-cadherin are believed to be involved in some way during the transendothelial migration process. These molecules are known to bind homophilically between either leukocytes and EC (all but VE-cadherin) or between EC only (all six molecules), although all three JAMs may also bind either of the molecules LFA-1, Mac-1, and/or VLA-4 (259). CD99 is a unique molecule (267), while VE-cadherin is a cadherin, and CD31 and the JAMs are members of the Ig superfamily. While VE-cadherin appears to be involved in regulating the adherens junctions between EC (268, 269), its absence apparently is essential during leukocytic emigration between EC (270, 271), as it is transiently relocated away from the EC surface at the site of contact with the transmigrating leukocyte. The presence of CD31 and CD99 at the site of leukocyte extravasation, however, is essential to transendothelial migration. Migration experiments in which CD31 was blocked resulted in a 90% reduction in leukocytic diapedesis *in vitro* and *in vivo* [reviewed in (259)]. Blocking both CD99 [blocking CD99 alone reduces transendothelial migration by >90% (272)] and CD31 has been shown to completely abolish transendothelial migration (272), although blocking of neither impairs the rolling or tethering of leukocytes along EC (272, 273). The role(s) of the JAMs in

transendothelial migration is(are) less clear. All are reported to bind either heterophilically or homophilically to adhesion molecules involved in any of the three steps of transendothelial migration, but reports regarding their significance in emigration of leukocytes from the blood to sites of inflammation are varied [reviewed in (259)]. As the involvement of these molecules in transendothelial migration has only recently come to light, it is likely that as more in depth *in vitro* and *in vivo* studies emerge, their roles will become more clear.

1.3.1.1. DC diapedesis

As discussed earlier, pre- and iDC also can express CR appropriate for promoting the establishment of tissue residency ($CCR6^+$ LC precursors leave the bloodstream in response to CCL20 expression in the skin) or for entrance into inflamed tissues for Ag uptake [blood-isolated and BM-cultured iDC chemotax in response to inflammatory chemokines *in vitro* (198)]. However, although CD11c (a ligand of iC3b and fibronectin) is the most common DC marker in mice, monkeys, and humans, and DC also commonly express CD11b (although the expression of this molecule is markedly decreased on $CD8\alpha^+$ DC, compared to other DC subsets [Table 1, Figs 10 and 21, (47, 48, 78)]), very little attention has been given to the function of these adhesion molecules on DC [with de la Rosa *et al* (205) being the only current report of the effects of blocking CD11b on human monocyte-derived DC]. CD31 and CD54 are expressed at mid to high levels on DC, but little attention has been given to the importance of these molecules in DC/EC interactions. No doubt the expression of CD31 and CD54 on iDC facilitates entrance of pre- or iDC into peripheral tissues for steady state and immunologic monitoring, but while it is known which molecules are essential for T cell, neutrophil, B cell, and even monocyte [reviewed in (258, 259)] egress the bloodstream, studies on what adhesion molecules are critical for DC

rolling, tethering, and extravasation are noticeably lacking (205, 274, 275). While it might be argued that as DC express CD31, for example, a key molecule in neutrophil and monocyte extravasation (276), and interactions between CD31 on DC and EC are highly likely to be fundamental in facilitating DC diapedesis. However, the expression of this same molecule by T cells has been shown to be unnecessary for their migration through EC (277). There have yet to be any accounts in the literature as to whether the difference in expression of specific adhesion molecules (CD11b, for example) between murine DC subsets may play a role in the reported differences in their migration. Studies later in this work have attempted to elucidate which, if any, of the aforementioned adhesion molecules play a role in DC migration *in vivo*.

1.3.2. Reverse Transmigration

For immune monitoring (by DC or memory T cells, for example) to be complete, leukocytes must be able not only to enter peripheral tissues, but to exit them as well. Reverse transmigration has been much understudied compared to transendothelial migration (37, 278). Leukocytes first interact with extracellular matrix proteins, rather than EC. CD54 is believed to be involved early in the process (279), similar to transendothelial migration, although, interestingly, CD31 is not (274). Tissue factor, a transmembrane member of the coagulation cascade (280, 281), also appears to be important in the navigation process of leukocytes from peripheral tissue into lymphatic vessels (280-282). Though not directly tested in vitro, it is likely CCL21 plays an important role in mediating mDC and T cell trafficking into lymphatic vessels. This supposition is further strengthened by the severe lack of T cells and DC in lymph nodes (which requires traffic through lymphatic vessels), but not the spleen (cells enter the spleen through the bloodstream), of CCR7 KO (257) and *plt* mice (200).

Metalloproteinases [specifically matrix metalloproteinases (MMP) 2 and 9], enzymes important for "opening" the passageways between EC to facilitate leukocyte transendothelial migration (allowing exposure of intercellular adhesion molecules) (259, 266, 283), reportedly are expressed on the cell surfaces of human DC which have migrated out of skin explants, but not on human Mo-derived or $CD34^+$ blood stem cell-derived mDC (284). Not surprisingly, then, metalloproteinases have been reported to be essential for mouse LC (284, 285) and dermal DC (284) and human LC (284, 286, 287) and dermal DC (284) exodus from the periphery into the lymphatic vessels, aiding their passage into secondary lymphoid tissues. Only one study has been reported utilizing non-skin-derived DC (288). In these experiments, investigators noted human Mo-derived DC expression of MMP 9, the upregulation of which was concurrent with an increase in mDC *in vitro* migration (both in response to CCL19). Thus, though DC migration (chemokine-directed, at least) patterns appear to vary according to tissues of origin/derivation [reviewed in Section 1.2 and Chapters 1 (Section 2) and 2 (Section 3)], as most reverse transmigration *in vivo* likely occurs with int/mDC and mDC appear to have similar migratory properties regardless of their ontogeny, it is possible that mDC from other tissues will also be determined to rely on metalloproteinases to aid in their exit from peripheral tissues, though this theory has yet to be tested in either the mouse or human DC model system.

1.3.2.1. DC departure from peripheral tissues

Peripheral int or mDC that have upregulated CCR7 acquire the ability to respond to CCL21, allowing them to leave the tissue enter draining lymphatic vessels to traverse to the nearest lymph tissue (43, 192, 194, 195, 198, 206, 257). As DC transmigration has been shown to rely on multidrug resistance I protein (274) and CD31 (205, 283), these adhesion molecules may play a

role in their exit from peripheral tissues. Interestingly, there are no reported differences in the expression of adhesion molecules on ("resting") i vs ("activated") mDC suggesting, (i) a role for DC as environmental sentinels in both the steady and inflammatory states and (ii) that an increase of DC in peripheral tissues during inflammation is likely facilitated by upregulation of adhesion molecules on EC. Reverse transmigrating monocytes can be stimulated, purportedly by EC, to differentiate into DC during/following the process of reverse transmigration (37). Although several factors have been determined to be important for this *in vitro* [e.g., phagocytic material (37); IL-4 and IL-13 (289); CD40 ligation (290, 291); extracellular matrix components (292, 293)], the mechanism(s) by which monocyte to DC differentiation occurs *in vivo* is not yet known. Some of these same alleged factors involved in monocyte to DC progression also may be involved of the differentiation of pre-pDC into pDC *in vivo* [i.e., CD154 (6, 42, 57)], although to date only pre-pDC have been detected *in vivo* (6, 42, 57, 62, 89, 193). Not surprisingly, the presence of inflammatory cytokines (e.g., IL-1, TNFα) has been shown to enhance DC emigration from tissue both *in vitro* (279) and *in vivo* (294). It is not clear whether DC which have extravasated into peripheral tissues reverse transmigrate back into the blood, but at least one report has shown that passenger leukocytes detected in donor cardiac allografts (tissue resident DC) can be found in the spleen during the first four days after transplant, necessitating a re-entrance of these DC into the bloodstream [(295) and Fig 35].

Though there have been a few reports regarding pDC migration *in vitro* (193, 205), the *in vivo* migratory patterns of pDC have remained much understudied, with the singular report on their trafficking patterns into inflamed tonsils being speculative at best (89). It was recently determined that human blood pDC require CD62L expression to enter secondary lymphoid

tissues (58), and that they require β1 and β2 integrins for transendothelial migration, at least *in vitro* (205). However, whether reverse transmigration influences differentiation of pre-pDC into pDC or monocytes into pre/pDC is yet to be determined.

1.3.3. EC and Transplantation

The first interaction alloactivated-T cells have with graft tissue is with EC. EC that have been activated by IL-1 and TNFα (as alloEC are during the surgical, ischemia, and reperfusion processes imposed on grafts during transplantation) rapidly upregulate the expression of chemokines (296, 297) and adhesion molecules (298, 299) that recruit and secure activated T cell entrance into the transplanted organ. Alloactivated $CD8^+$ T cells themselves further stimulate alloEC to produce CXCL9 and CXCL10, chemokines highly-selective for activated $CXCR3^+$ T and NK cells (300), as well as CCL2-5, chemokines which recruit $CD4^+$ T cells and monocytes (159). $CD4^+$ T cells also induce chemokine secretion in alloEC, although a much more restricted set compared to $CD8^+$ cells – only CCL5 and CXCL10 of the chemokines tested (300). Thus, interactions between activated T cells and EC facilitate the production/upregulation of factors essential for infiltration of peripheral tissues by the T cells, which, in the case of transplantation, also facilitate initiation of acute graft rejection. In addition, it has been suggested that the expression and anchoring of chemokines to EC could lead to the continual recruitment of passing immune effector cells, contributing to chronic rejection (238, 264, 301, 302).

1.3.4. DC Migration and Transplantation

Effector cells have specific routes by which they traverse the body to monitor for immunological targets, and they express specific adhesion molecules and CR according to their migratory needs. For example, as mentioned, memory and activated T cells express CD62L, while naïve T cells do

not, allowing the former to travel through the blood and enter tissue during either steady state or inflammatory conditions and impairing the latter from doing the same. As discussed, pre- and iDC travel from the blood into tissues as well (although they do not express CD62L), and they leave peripheral tissues and enter the lymph as int or mDC. Therefore the expression of specific CR and adhesion molecules on DC is likely tightly-regulated according to their origin and tissue of residence. Thus it may be essential to consider these factors when determining the optimal route of injection of DC for immunotherapy, as not all DC express them same CR or the same level of adhesion molecules.

1.3.4.1. Delivery of DC

Although further discussed in Chapter 1, it is important to note that while donor iDC (47, 130, 131) prolong allograft survival if administered iv 7 days prior to transplant, comparatively few reach their intended secondary lymphoid tissue (the spleen) (48). It is interesting to consider whether optimizing the delivery of exogenous DC to the thymus is an effective means of promoting tolerance. Inaba *et al* (303) injected donor DC into the thymus of allograft recipients differing in minor lymphocyte-stimulating Ag, resulting in anergy of alloreactive T cells and no evidence of graft versus host disease. Although we have detected some mDC in the thymus 24 h after sc injection (52), the reported lack of function of thymi in adult humans may delegate this type of therapy to pediatric transplant recipients only. Thus, efforts to manipulate DC *in vitro* in order to maintain their immaturity *in vivo* may be in vain if the injected iDC will never reach essential T cell areas. mDC, on the other hand, which express high levels of CCR7 are fairly efficient at trafficking to T cell areas of secondary lymphoid tissues (48, 304), although this ability is greatly affected by their route of administration [Chapters 1 and 4 and (48)]. However,

(CD8α$^-$) mDC also express high levels of co-stimulatory molecules and accelerate graft rejection (52, 130, 131).

STATEMENT OF THE PROBLEM

DC not only prime the immune system against transplanted tissues through presentation of alloMHC by donor (indirectly) and by recipient DC, initiating acute rejection, but they also are thought to be key players in chronic rejection, through indirect donor alloAg presentation. The ability of DC to regulate central and (putatively) peripheral tolerance also suggests a role for these APC in educating the immune system about the benignity of an allograft. We have been investigating the use of "tolerogenic" DC to prolong allograft survival and to achieve donor-specific tolerance. Possible candidates are iDC, genetically-, pharmacologically-, or biologically-modified DC, or discrete DC subsets, especially (at the time which these studies began) $CD8\alpha^+$ DC (in mice). Currently, in nearly all transplant studies in which DC are used as immunotherapeutic agents, the DC are administered via iv injection. In most of these studies, the donor heart is heterotypically transplanted into the abdomen, making the spleen the draining lymphoid tissue. Thus iv injection seems the most likely route for delivering the maximal number of DC into T cell areas. Studies with "tolerogenic" DC have had variable degrees of success in prolonging allograft survival utilizing this method of administration.

Early reports of $CD8\alpha^+$ DC, identified initially as the constitutive DC within mouse thymus ($\leq$ 5% $CD8\alpha^-$ DC), showed this subset of splenic APC to suppress both $CD4^+$ and $CD8^+$ T cell proliferation in an MLR, making them "ideal" candidates for transplant therapy. However, some groups reported that while (typically sc-) injected $CD8\alpha^+$ DC could prime an immune response *in vivo* (most likely through cross-presentation to migration-competent $CD8\alpha^-$ DC), these DC were seriously impaired in their ability to migrate to secondary lymphoid tissues. Other groups, showed that (iv-injected) $CD8\alpha^+$ DC could migrate in small numbers to T cell areas of secondary

lymphoid tissue, but each of the groups analyzed CD8α$^+$ DC using different methods (Table 7). These differences no doubt highly influenced the results in each report, as aside from their few reported functional differences, both DC subsets have similar phenotypes and reportedly co-habitat the same tissues (suggesting similar expression of CCR/adhesion molecules). Thus, we hypothesized that **CD8α$^+$ and CD8α$^-$ DC subsets have an equal ability to migrate *in vitro* and *in vivo***.

If CD8α$^+$ DC are candidate cells for use in transplant immunotherapy, it is essential to know whether they can reach T cell areas in order to exert their immunosuppressive effects on naïve alloreactive (and/or central memory cross-reactive) CD4$^+$ and CD8$^+$ T cells.

Therefore we proposed to compare routes of injection, cell numbers injected, state of DC maturation, and time of detection of migrated cells. As we believe that CD8α$^+$ DC are potential tolerogenic DC, capable of delaying, if not preventing, allograft rejection in an alloAg-specific manner, we also wanted to identify which chemokines, CR, and adhesion molecules play key roles in regulating their migration *in vivo*. These data will potentially allow specific augmentation (or impediment, if they are determined to be explicitly immunogenic) of (injected) CD8α$^+$ DC migration to T cell areas in a transplant setting. Other groups have failed to repeat the work showing CD8α$^+$ DC to suppress T cell proliferation. However our own work showing these DC to prolong allograft survival, coupled with reports that CD8α$^+$ DC express IDO and may be key players in peripheral tolerance (through uptake and presentation of self-Ag from apoptotic bodies), indicate they remain at the forefront of immunological interest. In the studies presented and discussed herein, we employ a variety of experimental methods to resolve the question as to

whether this important APC subset can traffic *in vitro* and *in vivo*, and which chemokines and adhesion molecules are principal in facilitating the migratory process. We also have applied these data in an experimental transplant model to evaluate whether our findings have potential clinical relevance.

Table 7. Disparities between CD8α$^+$ DC migratory studies.

	FL treatment	DC maturity	Cell separation	# of DC injected❖	Type of Recipient	Route of Injection	Time of Detection❋	Migration Detection Method	CD8α$^+$ Migration?	% Migration CD8α$^-$	% Migration CD8α$^+$
1	no	i	magnetic beads	0.1-0.7	syn	sc	2d	CMFDA/FACS	LN: no SPL: N/A	0.31	0.059
2	yes	m	FACS	0.1-0.25	allo	sc	2d	PCR & immunohistochemistry	LN: yes SPL: yes	N/A	N/A
3	no	i	FACS	1.0 0.2	syn	sc/iv	1d	CMFDA/FACS	LN: no SPL: yes	0.07	0.03
4	yes	m	unsorted	2.5	syn	sc	1d	CMFDA/FACS	LN: yes SPL: N/A	59.6*	28.5*

[1]Smith & Fazekas de St. Groth, J Exp Med, 1998; [2]O'Connell *et al*, J Immunol, 2000; [3]Ruedl & Bachman, Eur J Immunol, 1999; [4]Drake *et al*, J Virol, 2000.

❖(x 10^6) ❋post injection *% CMFDA-labeled cells LN: lymph node SPL: spleen

2. CHAPTER ONE[3]

FACTORS INVOLVED IN SPLENIC DC MIGRATION *IN VITRO*

The CC chemokines, CC chemokine receptors, and adhesion molecules likely to play a role in directing $CD8\alpha^+$ and $CD8\alpha^-$ splenic DC trafficking *in vivo* are evaluated in this chapter utilizing an *in vitro* chemotaxis migration assay (novel to our lab previous to this work). These findings provide several possible explanations for splenic DC *in vivo* migration patterns, as detailed in the Chapter Discussion and in Chapter Two, which follow.

[3] Data from this chapter are excerpted from (48) where indicated.

2.1. ABSTRACT

Murine $CD11c^+CD8\alpha^-$ and $CD11c^+CD8\alpha^+$ DC differentially regulate T cell responses. Although specific chemokines that recruit immature (i) or mature (m) classic $CD8\alpha^-$ DC have been identified, little is known about the influence of chemokines on $CD8\alpha^+$ DC. iDC and mDC isolated from spleens of FL-treated B10 mice were compared directly for migratory responses to a panel of CC chemokines. *In vitro* assays were performed using unmodified Transwell® filter chambers. iDC did not respond to any CC chemokines tested. Both subsets of mDC migrated to CCL19 and CCL21, with consistently lower percentages of $CD8\alpha^+$ DC migrating. Chemokine receptor (CR) mRNA and protein expression were analyzed, but no correlation between expression and function was demonstrated. Changes in intracellular Ca^{++} were not seen for iDC, suggesting that either CR are expressed only at the message level or that surface-expressed CR are non-functional on splenic iDC. mDC showed a striking morphologic response to exposure to chemokine *in vitro*, and correlative with their migration, $CD8\alpha^+$ DC showed a qualitatively reduced change in dendrite extension compared to $CD8\alpha^-$ DC. These findings provide insight into the specific CC chemokines most likely to direct $CD8\alpha^-$ and $CD8\alpha^+$ splenic mDC trafficking *in vivo* and suggest CCR7 may be at least partly essential for facilitating their differential migration, although the correlation between expression and function of CCR7 as well as that of CCR1-6 has yet to be determined.

2.2. INTRODUCTION

The immunobiology of $CD11c^{+}CD11b^{lo}CD8\alpha^{+}$ murine DC (32, 47, 80, 305) has attracted much attention since these cells were first described (60, 78, 306). The high expression of the typically-"lymphoid" marker CD8α (expressed as a homodimer, unlike on $CD8^{+}$ T cells, which commonly express CD8αβ on their cell surface) coupled with the low expression of the "myeloid" CD11b led DC biologists to conclude initially that these DC were of lymphoid origin. Since then, various investigators have sought the elusive origin of this DC subset, invariably with contradictory reports [for exemplary reviews, see (66) vs (94)]. Presently, there still is no conclusive evidence to fully support either a lymphoid lineage for these DC or that the expression of CD8α is a marker of activation. Of particular interest to transplant- and auto-immunologists, early reports suggested that $CD8\alpha^{+}$ DC might be specialized for tolerance induction since, unlike their so-called "myeloid" counterparts ($CD11c^{+}CD11b^{hi}CD8\alpha^{-}$), they suppressed both $CD4^{+}$ and $CD8^{+}$ T cell proliferation (83, 84) and enhanced the apoptotic death of activated T cells (305). Several groups have reported diverse functions for these cells, including selective Th cell subset activation *in vivo* in response to soluble (ovalbumin, keyhole limpet hemocyanin) or viral antigens (Ags) (75, 81, 304, 307), suppression of $CD8\alpha^{-}$ DC-induced delayed-type hypersensitivity to tumor/self-peptide (87, 308), and prolongation of organ allograft survival (47). As with classic murine $CD8\alpha^{-}$ DC, the ability of these APC to affect immune reactivity also depends on their tissue distribution and migratory properties, both of which are influenced by chemokines.

The *in vitro* chemotactic responses of $CD8\alpha^-$ ("myeloid") i and mDC derived from murine bone marrow (BM) (192), skin (197), or lymph node (LN) (197), or from human skin (181, 185) or blood (201, 204, 207, 309), or from non-human primate blood (43) have been well documented. The few reports regarding $CD8\alpha^+$ DC have examined either only their *in vivo* trafficking (71, 86, 304) or their *in vitro* migration -- $CD11b^-(CD8\alpha^+)$ (Peyer's patch; PP) DC in response to a restricted set of chemokines (CCL19, CCL20, and CCL21) (44) -- and have not adequately considered all the factors that might influence their migration. Until the studies discussed in this chapter [(48) and two others from our laboratory (50, 54) stimulated by these investigations] and aside from one PPDC study (44), the majority of DC migration assays have been performed on *in vitro*-propagated DC. The relationship between maturation status and migratory potential of murine DC *in vivo* has not been resolved, - a discrepancy underlined by the fact that in non-human primates, iDC and mDC migrate with equal efficiency to DLN after intradermal injection (despite the fact that rhesus mDC express CCR7 and iDC do not) (43). Nor have there been any reports investigating the adhesion molecules involved in murine DC transendothelial migration, the two studies having been performed with human monocyte-derived and blood-isolated DC (205, 283). Furthermore, there has there been no thorough investigation of the chemokine receptor expression of murine secondary tissue derived-DC, although this likely is partly due to the paucity of reliable reagents for murine studies.

Leukocyte recruitment *in vivo* is mediated largely by chemokines. Each of the four families of chemokines (C, CC, CXC, and CX_3C) attract specific leukocytes (159, 164, 167, 169, 177, 178, 195, 310, 311). Inducible CC chemokines recruit iDC to inflamed peripheral tissues, while constitutive CC chemokines attract mDC to secondary lymphoid organs. There is also recent

evidence that chemokines within organ allografts may influence the outcome of transplant survival (220, 229, 230, 247, 251). In this context, it appears that chemokines elicit migration of cells important in facilitating rejection (i.e., iDC or alloAg-specific T cells); conversely, chemokines may recruit cells for suppression of rejection (i.e., T reg cells or "tolerogenic"/regulatory DC). Manipulation/targeting of those chemokines may promote graft survival. In this chapter, we have analyzed factors that influence the *in vitro* trafficking of splenic DC subsets. We show for the first time that murine spleen iDC fail to respond to the same panel of inducible CC chemokines as has been reported for iBMDC, despite a similar expression pattern of mRNA for CCR (192, 196). Additionally, we show that this lack of migration is due to a lack of functional CCR expression on splenic iDC (through a failure of these DC to show a change in intracellular Ca^{++} in response to CC chemokines). Ca^{++} flux studies with mDC also revealed that $CD8\alpha^{+}$ DC do not respond as positively to CCR7 ligands as do $CD8\alpha^{-}$ DC, suggesting one possible explanation for the lower percentage of migrating $CD8\alpha^{+}$ DC.

2.3. MATERIALS AND METHODS

2.3.1. Mice

Male C57BL/10J (B10; $H2^b$), 8-12 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). They were maintained in the specific pathogen-free facility of the University of Pittsburgh Medical Center and fed Purina rodent chow (Ralston Purina, St. Louis, MO) and tap water *ad libitum*. Experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC).

2.3.2. DC isolation and maturation

Spleen DC were isolated as described (47). Briefly, B10 mice were treated with r huFL (Immunex; now Amgen, Seattle, WA) for 10 days (10 μg mouse/day intraperitoneally) to increase the number of DC (60). This approach allowed isolation of a greater number of DC from fewer mice than isolation of DC from untreated animals. On day 11, spleens were excised, gently mascerated through wire mesh, and red blood cells lysed. No digestive procedures were employed in order to minimize possible perturbation of cell surface Ag expression. To generate mDC, cells were incubated overnight (18 h) in RPMI-1640 (Life Technologies Inc, Gaithersburg, MD) supplemented with antibiotics, 10% heat-inactivated FBS (Life Technologies, Inc.), and r mouse GM-CSF (1000 U/ml, Schering Plough, Kenilworth, NJ) at 37°C, 5% CO_2 in air. BM-derived DC (BMDC) were generated as previously described (10). Briefly, BM was extracted from the femurs and tibias of FL-treated B10 mice, and iBMDC were harvested after one day of culture and defined by the same criteria as spleen iDC. For enrichment of iDC and mDC for phenotypic analyses, chemotaxis assays, and RPA, a 16% or 14.5% v/v metrizamide (Sigma-Aldrich, St Louis, MO) solution were used, respectively. Spleens from untreated mice were digested with 100U collagenase (which did not affect their phenotype as

assessed by flow cytometric analysis; data not shown), teased apart, and then further digested in 400U collagenase for two 30 min incubation periods in order to maximize DC recovery. Bulk spleen cell cultures were then enriched for i and mDC with 16% or 14.5% w/v metrizamide as for cells from FL-treated mice. Too few cells were recovered from spleens of untreated animals to sort the subsets by fluorescence activated cell sorting (FACS) or magnetic beads, so bulk populations were used in experiments with these cells.

2.3.3. DC purification

Bulk DC were incubated with magnetic beads coupled to anti-CD8α mAb (Miltenyi Biotec, Auburn, CA) at 4°C for 15 min (according to the manufacturer's instructions). Half of the bulk DC was then passed through a positive selection column (Miltenyi Biotec) to obtain a purified population (60-90%) of magnetically labeled ($CD8\alpha^+$) DC; the remainder was passed through a depletion column (Miltenyi Biotec) to select for a purified population of unbound ($CD8\alpha^-$) DC (75-95%). Freshly-isolated $CD8\alpha^-$ iDC were further enriched for CD11c by incubation of depletion column-eluted DC with magnetic beads coupled to anti-CD11c mAb at 4° for 15 min, then passed through a positive selection column. iBMDC were further purified via anti-CD86 (depletion) and anti-CDllc (selection) mAb bead separation after metrizamide enrichment. DC were then used for both phenotypic and functional analyses.

2.3.4. Extracellular flow cytometric analyses

Cell surface phenotypic analysis was performed by flow cytometry using an EPICS Elite ESP analyzer (Beckman Coulter, Hialeah, FL). All flow cytometric experiments were done at 4°C. Leukocytes were first blocked with 10% normal goat serum for 10 min and then stained with mAb for 30 min. DC purity after immunomagnetic bead sorting was assessed by determining the expression of CD11c and CD8α using flow cytometry. Phenotypic analysis of cell surface markers was performed with the following Abs: phycoerythrin (PE)-CD11b, -CD40, -CD54, -CCR5, -IA^b, -$H2^k$, -CD80, -CD86 (mAb, 1-2 μg/ml, BD PharMingen, San Diego, CA); biotinylated-CCR1 (C-terminus reactive), -CCR1 (N-terminus reactive), -CCR5 (C-terminus reactive) -CCR7 (C-terminus reactive) (goat anti-mouse polyAbs), secondary fluoroscein isothiocyanate (FITC)-conjugated donkey anti-goat (Santa Cruz Biotechnology Inc, Santa Cruz, CA); FITC-CCR3, -CCR6 (mAbs, R&D Systems, Minneapolis, MN); biotinylated-CCR4 (donkey anti-mouse polyAb), secondary FITC-conjugated goat anti-donkey (Capralogics Inc, Hardwick, MA) [Attempts to titrate CCR Abs (1-50 μg/ml) failed to render reproducible results in >25 experiments]. Cells stained with the appropriate isotype-matched Ig (BD PharMingen; Santa Cruz; Capralogics; R & D Systems) were used as negative controls. After staining, the cells were fixed with 4% paraformaldehyde (PFA). For some experiments, $CD11c^+CD8\alpha^+$ and $CD11c^+CD8\alpha^-$ iDC and mDC were flow-sorted according to their positivity for CD11c and CD8α.

2.3.5. Intracellular flow cytometric analyses

Intracellular staining was performed according to the standard PharMingen protocol: after Fc-block with CD16/32, DC were surface stained with PE-CD11c and Cy-chrome CD8α (all mAbs, 1-2 μg/ml, PharMingen) for 30 min and then fixed for 20 min with 4% PFA. Cells were then

permeablized in the presence of biotinylated-CCR5 or -CCR7 (polyAbs, 3-5 μg/ml, Santa Cruz) for 30 min, washed, and then resuspended in permeablization buffer in the presence of the FITC-conjugated secondary goat anti-donkey polyAb (1-2 μg/ml, Santa Cruz). Cells stained with the appropriate isotype-matched Ig (Santa Cruz) were used as negative controls. After staining, cells were fixed again with 4% PFA.

2.3.6. Chemotactic agents

Mouse recombinant CC chemokines CCL2, CCL3, CCL4, CCL5, CCL9/10, CCL7, CCL12, CCL19, CCL20, CCL21, CCL22 and CCL25 for the chemotaxis assay experiments (please refer to Table 4 for original nomenclature). Complement factor (C)5a and the bacterial formyl peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) (both from Sigma), both non-chemokine chemoattractants, also were tested.

2.3.7. Chemotaxis assays

Assays were performed as previously described (194), with minor modifications. Purified $CD8\alpha^+$ or $CD8\alpha^-$ iDC or mDC, iBMDC, or bulk iDC or mDC from untreated mice (1-4 x 10^5) were resuspended in 100 μl 0.5% BSA RPMI 1640 (no FBS, without chemokine) in Transwells® (5 μm pores for mDC, 5 or 8 μm pores for iDC; Costar, Cambridge, MA) were placed in 24-well plates with 600 μl of chemokine dilution in 0.5% bovine serum albumin RPMI 1640 per well and incubated for 2 h at 37° C in 5% CO_2 in air. After the incubation period, the Transwells® were removed and migrated DC from the 24-well plates were collected and enumerated using a Coulter Counter (Coulter). For accurate comparison between experiments, results were expressed as the percentage of migrated DC. Migration assays were performed in duplicate; experiments were repeated at least 3 times.

2.3.8. Calcium flux

Ca^{++} flux was analyzed as described previously (196), with minor modifications. DC were loaded with 2 μM fura-2/AM (Molecular Probes), incubated for 30 min at 37°C in PBS without Mg^{++} or Ca^{++} supplemented with 4 [mM] probenecid (Sigma) in the dark, washed twice in PBS (BioWhittaker), and resuspended in PBS containing 1.2 [M] Ca^{++} and 1.5 [M] Mg^{++} at 2-3 x 10^6 cells/ml. For iDC (BM and splenic), chemokines were added at a concentration of 10 [nM] at indicated times in a continuously stirred cuvette at 37°C in a Model MS-III spectrofluorimeter (Photon Technology, South Brunswick, NJ). Fig 15 only: after fura-labeling, mDC were placed on poly-D-lysine coated coverslips for 30 min at room temperature in PBS containing 1.5 [mM] Ca^{++} + 1% BSA. DC-coated coverslips were mounted on a ACAS 570c confocal microscope (Meridian Instruments, Okemos, Michigan) for measurement and data was collected and analyzed by the C*IMAGING Systems-Simple PCI program (Compix Inc, Imaging Systems, Cranberry Township, PA). Basal levels of intracellular Ca^{++} were measured for approximately 1 min, then Ca^{++} PBS + 1% BSA was removed and 10 [nM] chemokine of CCL19 or CCL21 was added. The imaging system used in these studies consisted of a Nikon Diaphot 300 microscope (Nikon, Melville, NY) fitted with a 40quartz objective, a Dage-MTI cooled-CCD camera with 640 480-pixel resolution (Dage-MTI, Michigan City, IN). For both iDC and mDC, the relative ratio of fluorescence emitted at 510 nm following sequential excitation at 340 and 380 nm then was recorded.

2.3.9. RNase protection assay

The RNase protection assay (RPA) was performed as described (69). Briefly, RNA was isolated from ~5x10^6 flow-sorted DC using a total RNA Isolation Kit (BD PharMingen). RPA was

conducted using the RiboQuant mCR-5 Multi-Probe Template Set with anti-sense RNA probes that target CCR1, 1b, 2, 3, 4, and 5, and the housekeeping genes L32 and GAPDH (BD PharMingen). CCR6 and 7 probes were included in a customized RiboQuant Multi-Probe Template Set (BD PharMingen). The corresponding antisense RNA probe set was included as molecular weight standards. Mouse RNA and RNA degradation controls were included. Yeast tRNA served as a negative control. Quantification of bands was performed by densitometry followed by assessment using Image QuantNT software (Molecular Dynamics, Sunnyvale, CA). The signals from specific mRNA were normalized to signals from the housekeeping genes run on each lane to adjust for loading differences.

2.3.10. Statistical analysis

Results are expressed as means ±1 SD. Comparisons between means were performed by analysis of variance and then by the Newman-Keuls test. Comparison between 2 means was performed by the Student *t*-test. A "*P*" value ≤ 0.05 was considered to be significant.

2.4. RESULTS

2.4.1. Isolation and identification of splenic DC subsets

Although most murine DC *in vitro* studies have utilized BM-derived DC, this culture system does not produce the CD8α-expressing DC subset. Therefore, for these studies, we chose to isolate DC from the spleens of FL-treated mice, as the ratio of $CD8\alpha^-$ to $CD8\alpha^+$ DC averages 1:1 [(46, 47) Colvin: unpublished observations] and FL treatment greatly increases both DC subsets without apparent significant differences in phenotype or function (as compared to DC from non-FL-treated spleens) [Fig 9 and (47, 70)]. While flow sorting typically results in highly-purified cell populations (> 95%) at the cost of time and limited cell recovery, we employed immunomagnetic bead sorting to recover $CD8\alpha^+$ and $CD8\alpha^-$ freshly-isolated (iDC) or overnight-GM-CSF-cultured (mDC) B10 splenic DC enriched on a 16% (iDC) or 14.5% (mDC) metrizamide solution from bulk spleen cells, a method recently adopted by other groups for DC subset isolation (82, 304). Metrizamide separation yielded >60% of $CD11c^+CD86^{-/lo}$ iDC (Figs 9A and 10) and >90% of $CD11c^+CD86^{hi}$ mDC (Figs 9B and 10). Forty to 60% of iDC or mDC were $CD8\alpha^+$ (Fig 9A and B). Purification of DC by immunomagnetic bead sorting yielded ~80% i and >90% m $CD8\alpha^+$ DC and ~80% i and ~90% m $CD8\alpha^-$ DC populations (Fig 9C and D). As expected, both subsets of iDC exhibited immature DC morphology, were $CD40/80/86^{-/lo}$ and MHC II^{lo}, and were poor stimulators of allogeneic naïve T cells in MLR [Fig 10 and (47, 52)], while mDC of both subsets showed abundant dendritic prolongations, were MHC II^{hi} and $CD40/80/86^{hi}$ (Figs 6 and 11 and data not shown), and exhibited potent naïve T cell allostimulatory capacity, as previously reported by our group (47, 52).

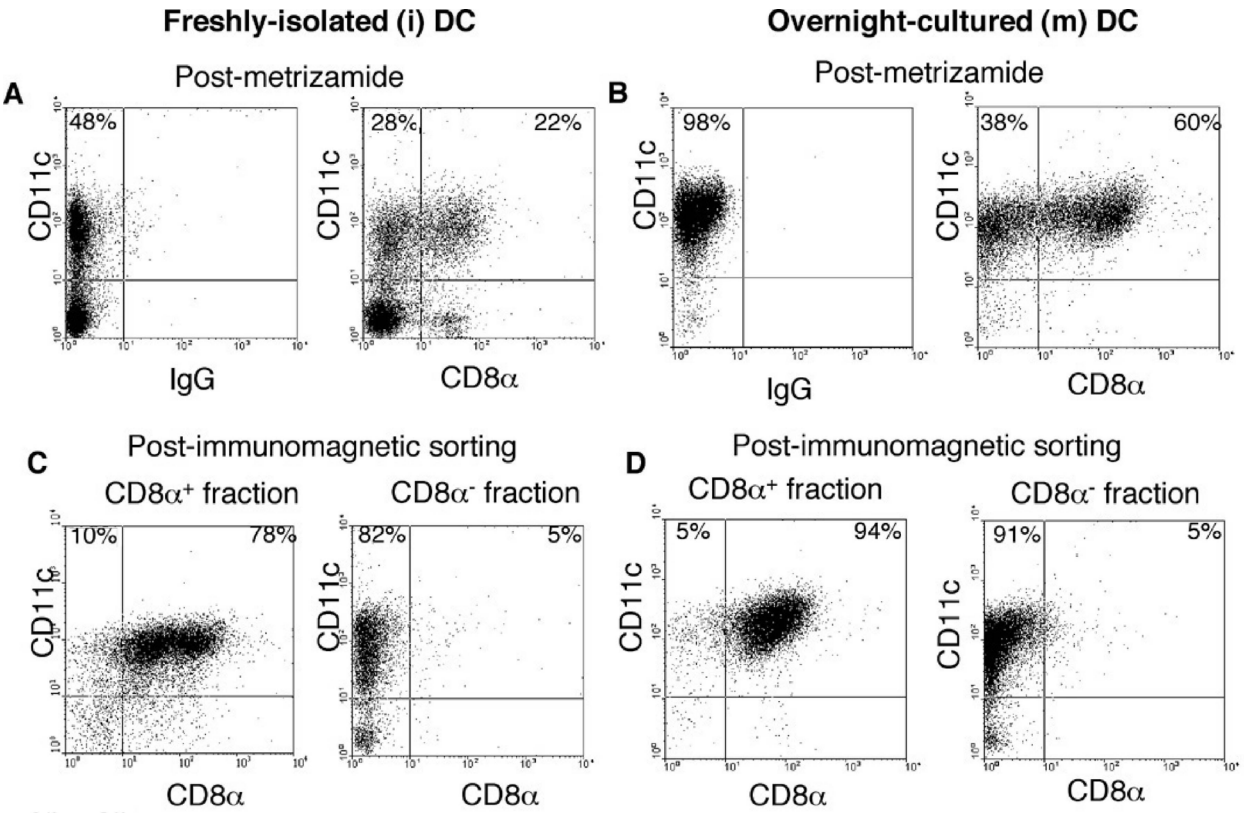


Fig 9. Immunomagnetic bead sorting yields ample numbers of highly-purified DC populations.
(A, B) Freshly-isolated or overnight (18h), GM-CSF-cultured spleen cells from 10 d-FL-treated B10 mice were layered on 16% w/v or 14.5% w/v (for iDC or mDC, respectively) metrizamide/PBS. (C, D) This $CD11c^+$ DC-enriched population was then immunomagnetic bead-sorted according to CD8α expression, typically yielding ~80% $CD8\alpha^+$ and ~80% $CD8\alpha^-$ iDC (C) and >90% $CD8\alpha^+$ and >90% $CD8\alpha^-$ mDC (D). Appropriate isotype controls (Ig) were use for gating (data not shown); numbers indicate percentage of cells (48).

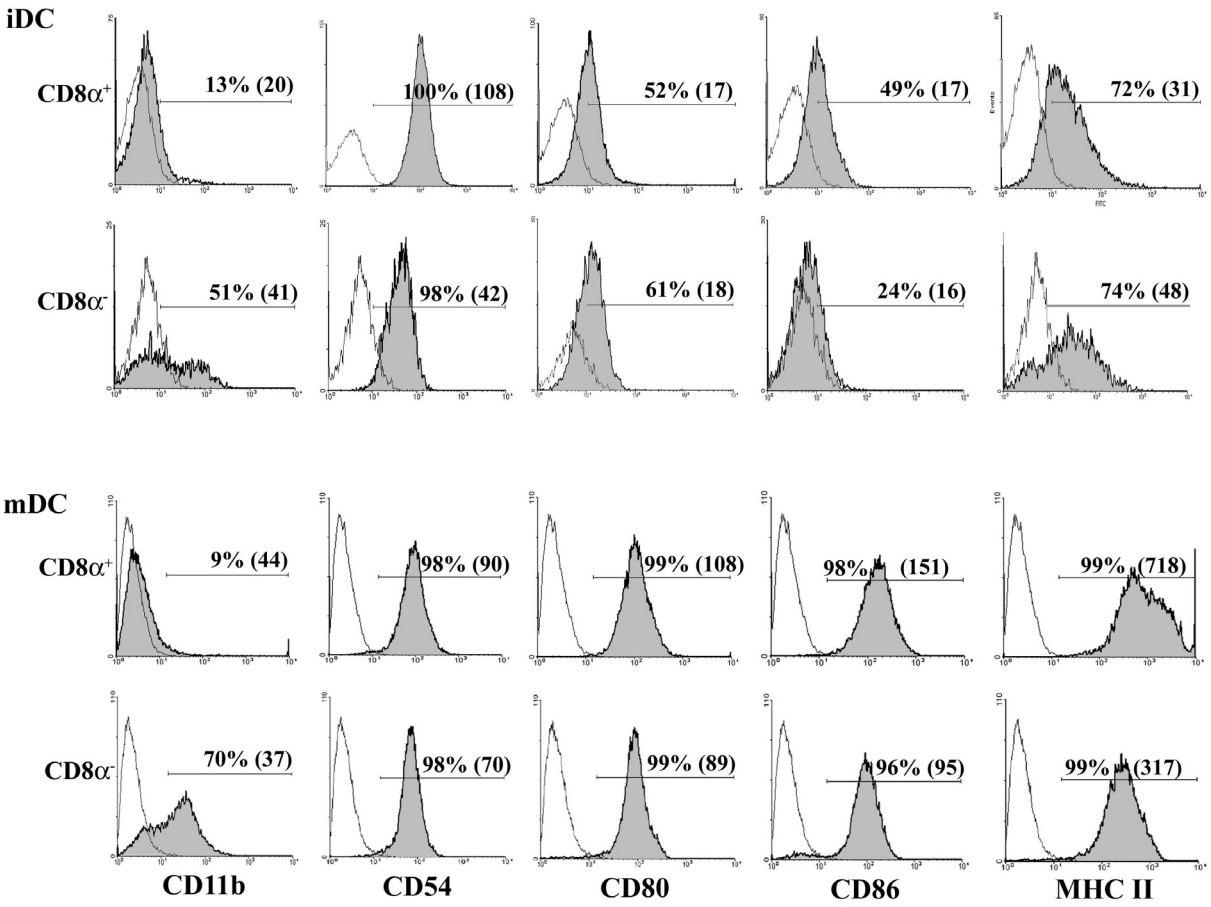


Fig 10. Phenotype of splenic iDC and m DC.

Metrizamide-enriched, magnetic bead-sorted i and mDC were gated on CD11c and CD8α and stained for the adhesion molecules CD11b and CD54, the co-stimulatory molecules CD80 and CD86, and the Ag-presenting molecule MHC II. $CD8\alpha^+$ DC are low, but not completely negative for CD11b and express high levels of CD205, while $CD8\alpha^-$ DC express high levels of CD11b, but low levels of CD205 (CD205 data not shown). Both subsets expressed (typical for iDC) low levels of co-stimulatory and MHC molecules when freshly-isolated (top two rows), and upregulated these after overnight culture (showing typical mDC phenotype) (bottom two rows) (312). Mean fluorescence intensity (MFI) follows the percent expression of each molecule in parentheses. Open histograms represent isotype control Ig; filled histograms represent protein expression.

2.4.2. Freshly-isolated spleen CD8α$^+$ and CD8α$^-$ DC do not migrate *in vitro* in response to previously-defined iDC migration-inducing CC chemokines

Various mouse CC chemokines were tested in chemotaxis assays to determine the *in vitro* migratory response of CD8α$^+$ and CD8α$^-$ iDC. Surprisingly, although both human and mouse BM- and blood-derived and skin iDC have been reported to respond to several CC chemokines (44, 192, 204, 309), neither murine spleen iDC subset migrated to any of the CC chemokines tested (CCL2, CCL3, CCL4, CCL5, CCL7, CCL9/10, CCL12, CCL19, CCL20, CCL21, CCL22, or CCL25) (positive migration was considered ≥10% migration above control wells without chemokine) (Table 8, Fig 11A, and data not shown). We also tested the non-chemokine chemoattractants C5a and fMLP, which have been reported to elicit human skin (313) and blood (314) iDC migration *in vitro* (although blood iDC responded to higher concentrations than those tested by us: 1000 and 10,000 [nM]), but these factors did not induce migration of either splenic iDC or mDC (data not shown). To control for possible impairment of migration due to the presence of anti-CD8α or anti-CD11c magnetic beads on iDC, iDC subsets were sorted by fluorescence activated cell-sorting (FACS), but this did not increase iDC migration (data not shown). Finally, we increased the incubation up to 4 h, but this increased migratory period merely resulted in an equal increase of iDC kinesis in all wells, including the control well (data not shown).

To confirm that our assay was appropriate for analysis of chemotaxis of immunobead-sorted, FL-treated, spleen iDC migration, we replaced the spleen iDC with BM-derived iDC as positive controls. The iBMDC exhibited reproducible migration to CCL4 (MIP-1β) and CCL5 (RANTES) (Fig 11B), as reported previously (164, 195, 204, 315). As expected, neither CCL19

(MIP-3β) nor CCL21 (SLC) (both chemoattractants for mDC) elicited migration of splenic CD8α$^+$ or CD8α$^-$ iDC or iBMDC (Table 8, Fig 11A and B, and data not shown).

Table 8. Assessment of migratory responses of immature and mature CD8α$^+$ and CD8α$^-$ DC to CC chemokines.

DC Subset	**CCL4** (MIP-1β)	**CCL5** (RANTES)	**CCL19** (MIP-3β)	**CCL21** (SLC)
iBM	+[a]	+	-	-
iCD8α$^+$	-[b]	-	-	-
iCD8α$^-$	-	-	-	-
untreated iDC*	-	-	-	-
mCD8α$^+$	-	-	+	+
mCD8α$^-$	-	-	+	+
untreated mDC*	-	-	+	+

i - immature; m - mature; ND - not done.
[a]Positive DC migration was considered 10% ≥ control well migration.
[b]Negative DC migration was considered 10% < control well migration
*Untreated spleen DC isolation procedures did not yield enough DC to sort into subsets for these experiments.

(48)

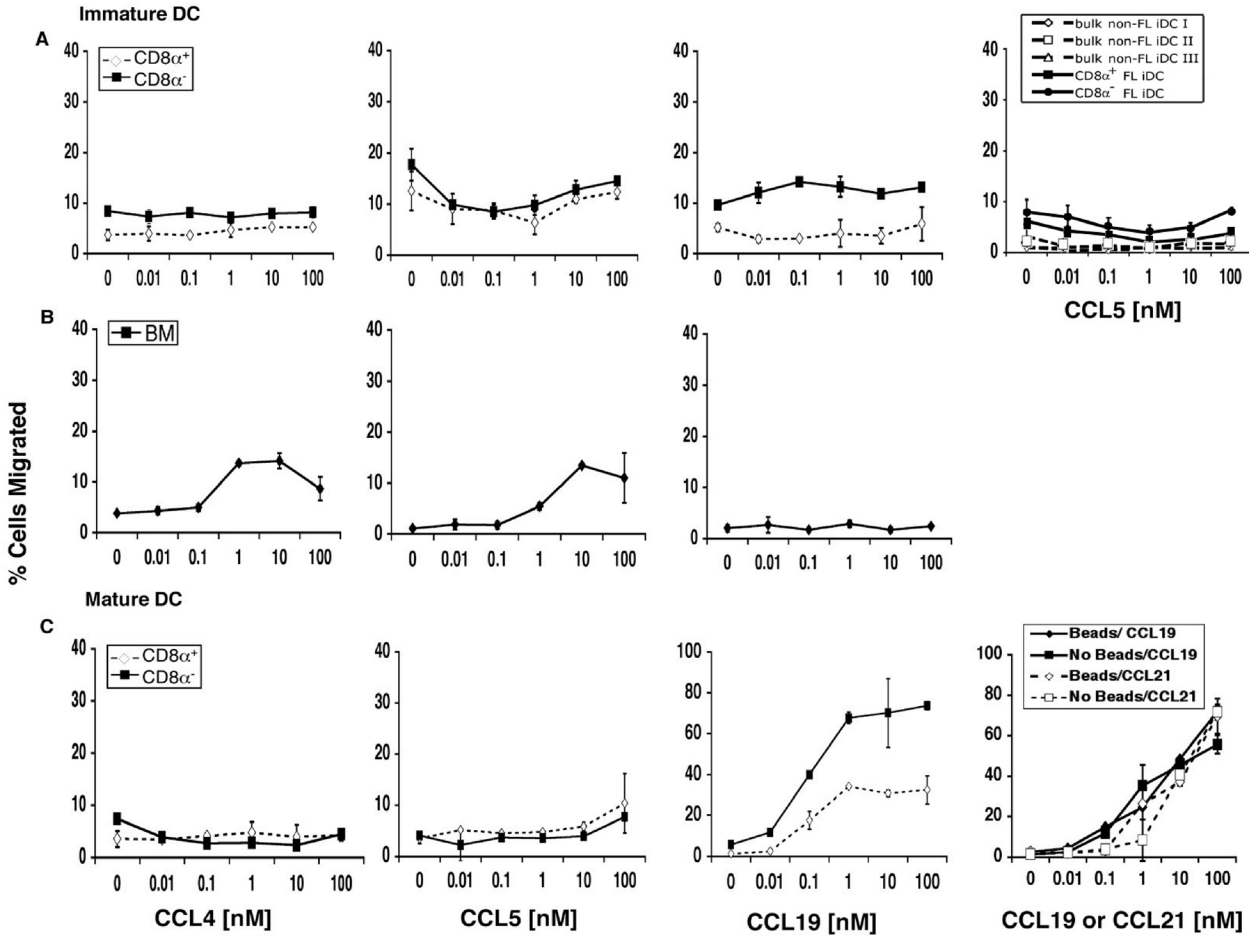


Fig 11. DC migration to specific CC chemokines *in vitro* is dependent on both tissue of origin and state of maturation.

Immunobead-sorted (unless otherwise noted) DC were placed in the upper wells of Transwell® chambers over graded concentrations of CC chemokines. (A) Freshly-isolated CD8α$^+$ or CD8α$^-$ iDC from either non-FL- (non-immunobead-sorted) or FL-treated spleens failed to migrate to any CC chemokines tested (positive chemotaxis was considered ≥10% migration over control without chemokines). (B) iBMDC migrated to CCL4 and CCL5, but to neither of the constitutively expressed chemokines CCL19 and CCL21 (data not shown). (C) Splenic CD8α$^+$ or CD8α$^-$ mDC migrated only to CCL19 and CCL21 (data not shown). To assess whether immunomagnetic beads interfere with DC chemotaxis, the migratory response to CCL19 and CCL21 of negatively-selected CD8α$^-$ mDC (unbeaded) was compared to that of negatively-selected CD8α$^-$ mDC incubated with anti-CD11c magnetic beads. CD8α$^-$ mDC migrated equally well with or without magnetic bead attachment. Three examples of the 12 CC chemokines tested are shown (for a complete list of chemokines tested, the reader is referred to the text). Data are from a single experiment representative of at least 3 performed; each chemokine dilution was tested in duplicate. (48)

2.4.3. Spleen CD8α$^+$ and CD8α$^-$ mDC migrate *in vitro* in response to mDC migration-inducing chemokines

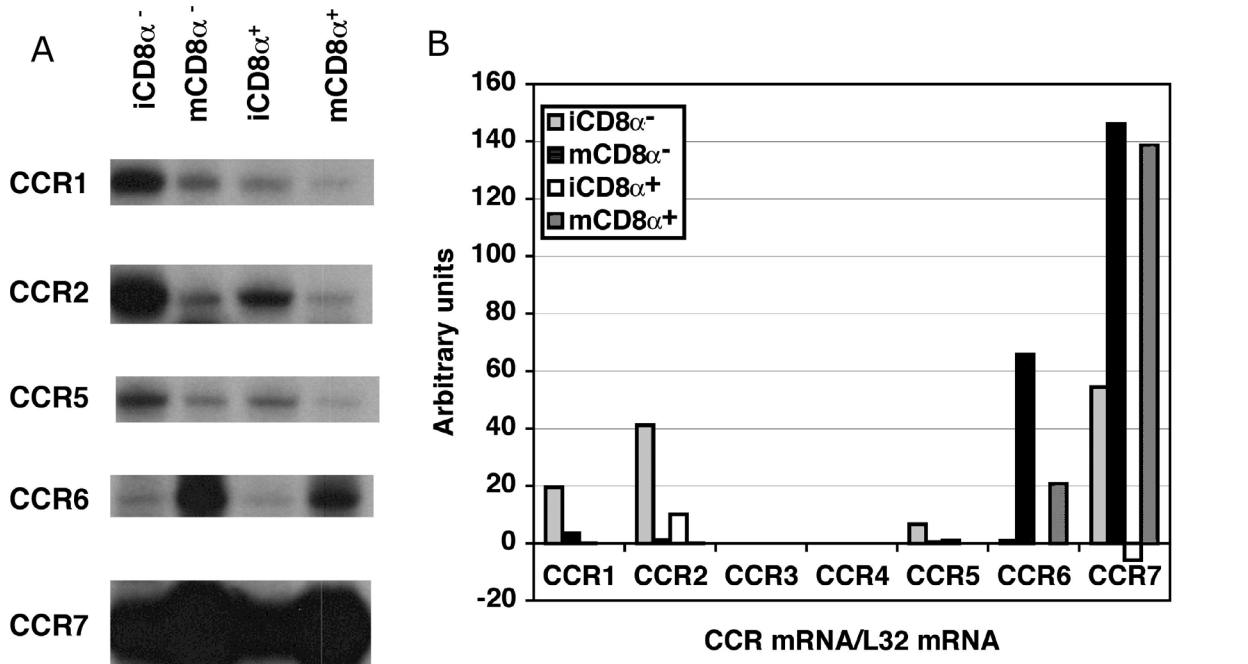
The constitutively-expressed chemokines CCL19 [by DC and endothelial cells (EC) in secondary lymphoid tissues] and CCL21 [by EC in lymphatics and high endothelial venules (HEV)] (182-184) have been reported to induce migration of human and murine mDC (44, 192, 197, 204, 315). In keeping with these reports, both CD8α$^+$ and CD8α$^-$ splenic mDC migrated in response to CCL19 and CCL21, but not to the inflammatory CC chemokines tested (CCL2, CCL3, CCL4, CCL5, CCL7, CCL9/10, CCL12, CCL20, CCL22, CCL25) (Table 8 and Fig 11C, and data not shown). CD8α$^+$ mDC consistently migrated in fewer numbers in response to CCL19 and CCL21 compared to CD8α$^-$ DC (Fig 11C). Since after cell purification, CD8α$^+$ DC (unlike CD8α$^-$ DC) may have retained magnetic beads on the cell surface, we ascertained whether the presence of magnetic beads on the cell surface would affect CD8α$^+$ DC migration. As shown in the last panel of Fig 11C, CD8α$^-$ DC bearing anti-CD11c magnetic beads migrated equally well to both CCL19 and CCL21 as compared to unbeaded CD8α$^-$ DC. Therefore, the presence of immunomagnetic beads on the cell surface did not affect migration.

To ensure FL-mobilization of the spleen DC was not a factor influencing their ability to migrate, bulk spleen i and mDC were isolated from untreated mice and their chemotactic responses were tested to a select group of CC chemokines. Because it required a large number of mice to isolate enough iDC for one chemotaxis assay, and because only a very few non-FL-treated iDC could be isolated, even from a large number of spleens, the iDC were not separated into CD8α$^+$ and CD8α$^-$ populations. Like iBMDC, whose migration in response to CC chemokines is not affected by exposure to FL (192, 196), spleen iDC from non-FL-treated mice did not migrate to CCL4, CCL19, CCL20, CCL21 (data not shown), or CCL5 (Table 8 and Fig 11A), and non-treated

spleen mDC migrated to CCL19 and CCL21 (but not CCL4, CCL5, or CCL20), in a similar manner to their FL-mobilized counterparts (Table 8 and data not shown).

2.4.4. CCR mRNA and protein expression in spleen CD8α$^+$ and CD8α$^-$ DC does not correlate with (apparent) functional CCR expression

The lack of available cell surface-staining murine CCR Abs coupled with the absence, *in vitro*, of migration to inflammatory CC chemokines, led us to analyze the pattern of CCR mRNA expressed by splenic DC using RPA. CD8α$^+$ and CD8α$^-$ iDC showed the typical pattern of down-regulation of inflammatory CCR mRNA expression (CCR1, 2 and 5), especially CCR2 upon maturation, with concomitant upregulation of CCR7 (Fig 12A and B), similar to that reported for murine BMDC [both in the presence (192) and absence (196) of FL]. Despite the expression of mRNA for CCR 1, 2, and 5, both spleen iDC subsets failed to migrate in response to any of their ligands (Table 8 and Fig 11), although iBMDC migrated to the CCR1 and CCR5 ligands CCL4 and CCL5. Interestingly, expression of mRNA for CCR6, a CR typically expressed on precursor/iDC populations (44, 181, 185), was low in iDC but increased in mDC. We therefore then analyzed untreated spleen CD8α$^+$ and CD8α$^-$ i and mDC mRNA to determine if this unexpected CCR6 expression pattern was due to FL-treatment. i and m spleen CD8α$^+$ and CD8α$^-$ DC from non-FL-treated mice expressed an identical pattern of mRNA for CR receptors 1-7 (data not shown) as compared to all spleen DC subsets from FL-treated mice, indicating that the pattern of CCR6 mRNA was not due to treatment of the mice with the growth factor. Nevertheless, neither subset at either stage of maturation (with or without FL-mobilization) exhibited a positive migratory response to the CCR6 ligand CCL20.



	CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7
iCD8α-	19.58	41.14	0	0	6.606	0.815	54.37
mCD8α-	3.486	1.255	0	0	0.451	65.77	146.2
iCD8α+	0.034	10.08	0	0	1.03	0.004	-5.808
mCD8α+	0.001	0.021	0	0	0	20.65	138.5

Fig 12. CCR mRNA expression in spleen i and mDC.

CD8α$^+$ and CD8α$^-$ iDC and mDC were flow-sorted for maximum purity (>95%). (A) Both iDC subsets downregulated CCR1, CCR2, and CCR5 mRNA expression and upregulated CCR6 and CCR7 message upon maturation into mDC. (B) Densitometric analysis for quantitation of cytokine mRNA expression. Analysis of each lane was performed on scanned autoradiographs, and all values are expressed relative to corresponding housekeeping gene transcripts (L32). Data are from 1 experiment representative of 3 performed. (316)

As CCR mRNA expression did not correlate with spleen iDC migration, and since CD8α$^+$ mDC migrated in consistently fewer numbers than CD8α$^-$ DC (possibly indicating lower expression of CCR7), we attempted to measure DC surface protein expression of CCR 1, 3, 4, 5, 6, and 7 by flow cytometry. However, in >25 experiments, CCR detection was not reproducible. We

therefore endeavored to measure intracellular protein production using anti-CCR1, -CCR5, and -CCR7 polyAbs. Though these CCR were detected consistently in iDC and mDC of both subsets, no conclusions could be drawn regarding their differential surface expression, as apparent intracellular protein production showed little variation among the cell subtypes analyzed (Fig 13).

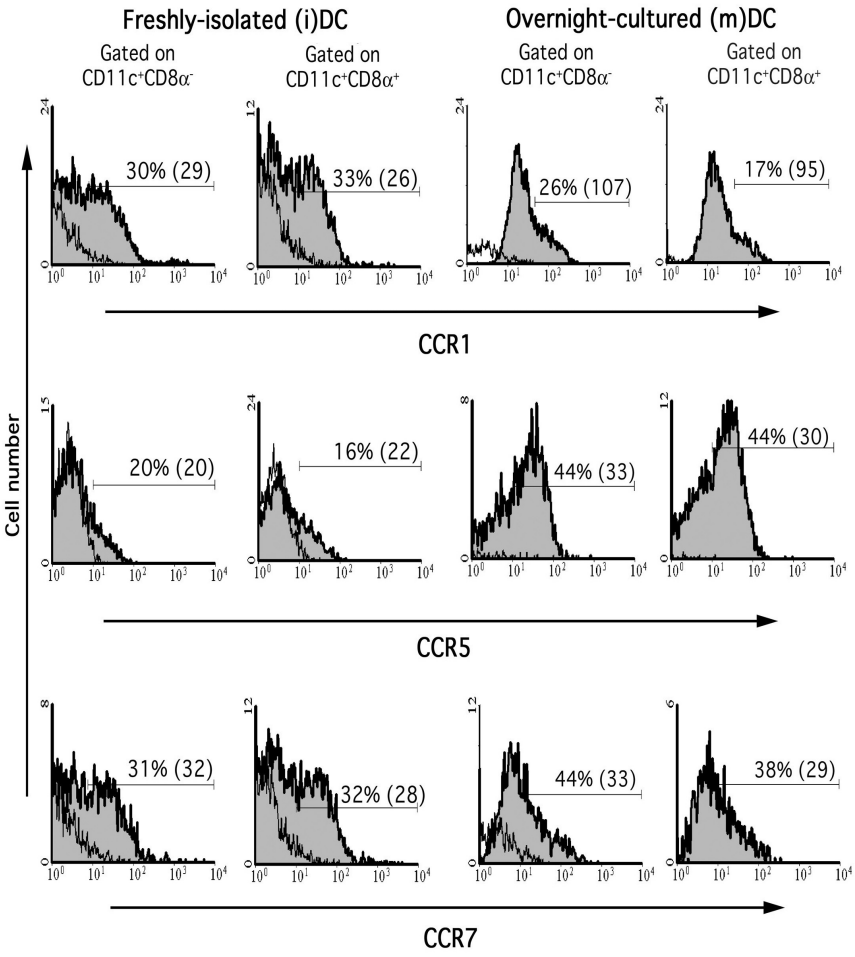


Fig 13. Intracellular staining of CCR1, 5, and 7 revealed no significant difference in the production of CCR between either subset of either state of maturation. Metrizamide-enriched i or mDC were stained for CD11c (PE) and CD8α (Cy-chrome), washed, permeablized, and then stained for intracellular protein production of CCR (FITC). MFI is presented in parenthesis after the percent expression of each molecule. (316)

iDC contained mRNA and protein for CCR1 and 5, but did not migrate to any of the ligands for these CR. We performed a Ca^{++} flux assay to determine if the DC expressed CR that could be activated, even if they were unable to induce migration of DC after binding their ligand(s). Spleen iDC did not exhibit any Ca^{++} flux in response to CCL4, CCL5, CCL19, CCL20, or CCL21, although they did release Ca^{++} in response to a non-specific Ca^{++} flux inducer,

ionomycin (Fig 14 and data not shown), somewhat similar to human blood-isolated plasmacytoid iDC (193). Freshly-isolated human blood pDC express CXCR3, and CXCR4 (for example) but migrate only to SDF-1α (CXCL12), the ligand for CXCR4, and to no ligands for CXCR3. Nor do they exhibit changes in intracellular Ca^{++} levels in response to the CXCL10 (a ligand for CXCR3), but they do flux in response to CXCL12 (193). iBMDC, however, which migrated in response to CCL5, also fluxed after exposure of the DC to CCL5 (Fig 14).

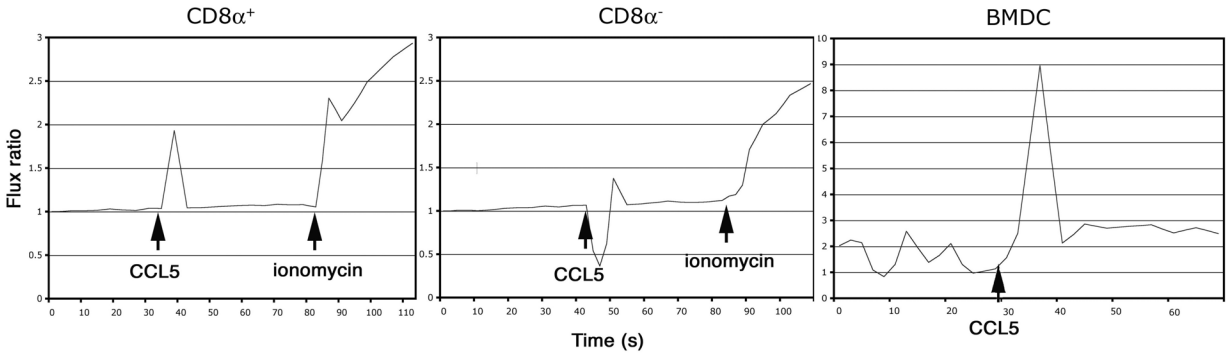


Fig 14. Despite mRNA and intracellular protein production of CCR1 and 5, but in correlation with *in vitro* migration data, spleen iDC do not show a change in intracellular Ca^{++} in response to CCL4 or CCL5.

Neither $CD8\alpha^+$ (left) nor $CD8\alpha^-$ (middle) spleen iDC exhibited a change in intracellular Ca^{++} after exposure to CCL4 (data not shown) or CCL5, although a Ca^{++} flux was seen after exposure to ionomycin (left and middle). However, iBMDC (right) responded to exposure to CCL5. A positive change in Ca^{++} flux is indicated by a change in the overall flux ratio, as seen after spleen iDC exposure to ionomycin or iBMDC exposure to CCL5: an initial spike in the flux ratio was observed initially each time chemokine or ionomycin was added to the DC, but immediately returned to baseline in negative reactions. (316)

mDC expressed similar levels of CCR7 mRNA and intracellular protein, although $CD8\alpha^-$ DC consistently migrated in greater numbers than $CD8\alpha^+$ DC. One possibility for this difference is a lesser ability of $CD8\alpha^+$ DC to respond to CCR7 ligands. The current lack of Abs which bind the extracellular portion of CCR7 prevents direct detection of protein levels on the surface of DC,

therefore we were unable to ascertain whether $CD8\alpha^+$ DC simply express less CCR7 than $CD8\alpha^-$ DC. However, as both mDC subsets showed changes in intracellular Ca^{++} levels upon exposure to CCL19 and CCL21, we attempted to compare the function of CCR7 on $CD8\alpha^+$ and $CD8\alpha^-$ DC indirectly. Yanagawa and Onoé (317) reported that LPS-matured BC1 cells (a spleen-derived murine iDC line) extend their dendrites in response to CCL19 after an hour, but not to CCL21. Interestingly, we found that both mDC subsets rapidly (less than 1 min) extended their dendrites in response to 10 [nM] CCL19 and CCL21. While the morphologic response of $CD8\alpha^-$ DC to these chemokines was more striking (compare Fig 15A and B to C and D), the mDC subsets showed a similar change in intracellular Ca^{++} levels after exposure to chemokine (Fig 15E), making quantitative comparison of their expression of CCR7 unachievable.

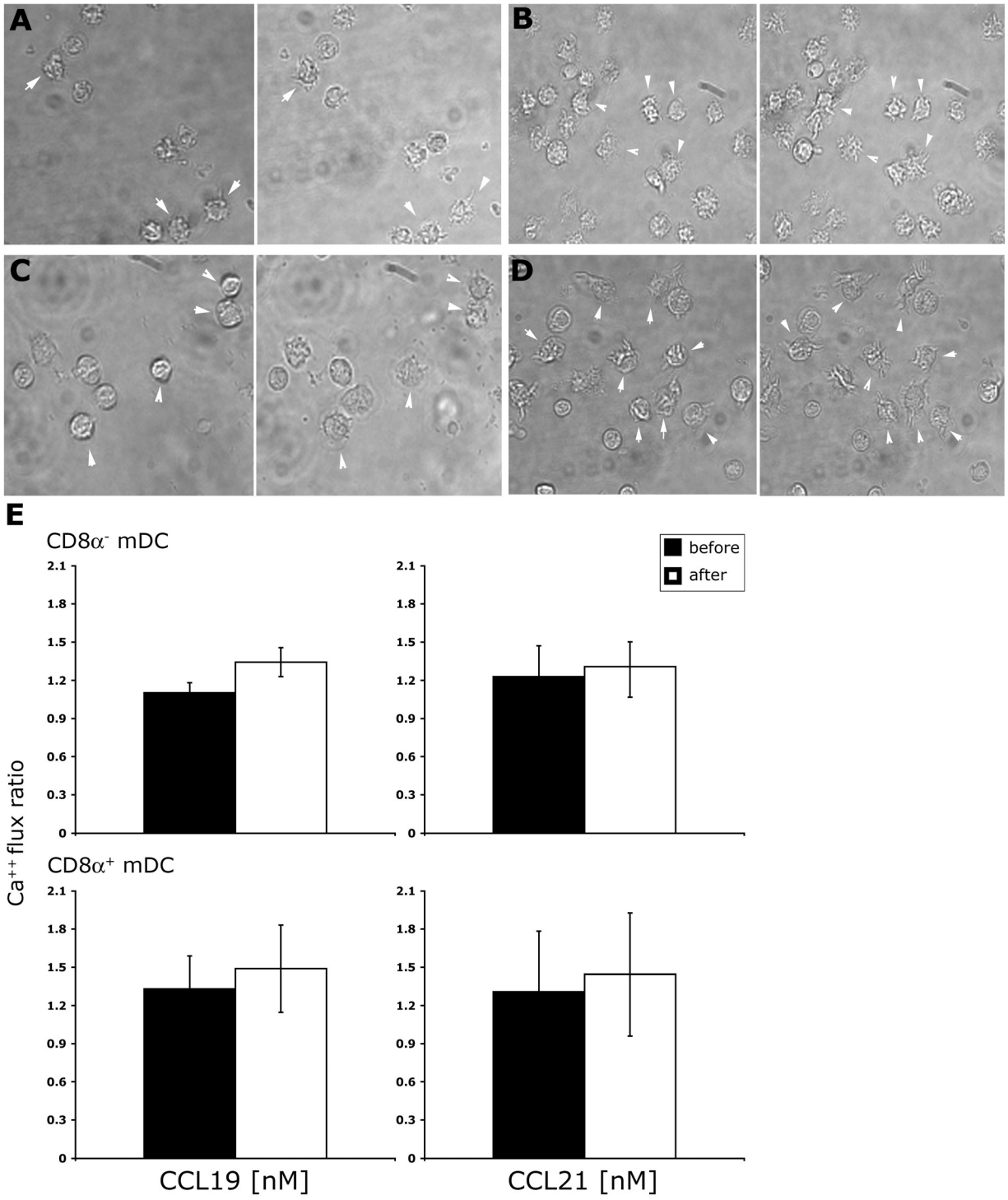

A
B
C
D
E
CD8α- mDC
CD8α+ mDC
before
after
Ca++ flux ratio
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
CCL19 [nM]
CCL21 [nM]

Fig 15. Splenic mDC morphologic and intracellular responses to CCR7 ligands.

Immunomagnetic bead-sorted $CD8\alpha^+$ and $CD8\alpha^-$ mDC were labeled with fura-2 and allowed to adhere to poly-D-lysine-coated glass coverslips. Basal levels of Ca^{++} flux were measured in DC immersed in PBS + Ca^{++} and 0.1% BSA (A-D first panels; E black bars), and the change in flux was measured again after addition of 10 [nM] chemokine (A-D second panels; E white bars). (A-D) DC exhibited dendrite extension after 1 min of exposure to either chemokine, with slightly more drastic extension in response to CCL21 (B and D), and with $CD8\alpha^-$ mDC showing slightly more apparent extension than $CD8\alpha^+$ mDC (compare A and B to C and D). Arrowheads highlight mDC that exhibit an increase in dendrite extension after exposure to CCR7 ligand. (E) Despite a more obvious extension of dendrites by $CD8\alpha^-$ mDC, neither subset appeared to have a more drastic quantifiable change in intracellular Ca^{++} levels. Bars represent the mean ± SD change in intracellular Ca^{++} in n = 20 DC averaged from three separate experiments.

2.5. DISCUSSION

In this study, we have compared the migratory ability of murine CD8α$^+$ spleen DC to their more extensively studied CD8α$^-$ counterparts. Previous data regarding "classic" myeloid DC, generated from BM or blood precursors or isolated from the skin or LN, suggest that iDC are capable of responding to diverse inflammatory CC chemokines. This is not surprising, given the role iDC play during immune responses. However, unlike *in vitro* generated BM- or blood-derived DC, here we have shown that iDC isolated directly from spleen are more restricted in their chemotactic reactivity. While iBMDC exhibited positive (albeit weak, compared to mDC) migration to iDC migration-inducing CC chemokines, as previously reported (159, 167, 192, 195, 204, 309, 311, 315), freshly-isolated spleen CD8α$^+$ and CD8α$^-$ iDC failed to respond to any of the CC chemokines tested. Preliminary studies in our laboratory with FL-mobilized murine liver (54) and kidney DC (49) reveal this phenomenon is not restricted to spleen iDC, as iDC isolated directly from these non-lymphoid/parenchymal organs also appear to have restricted chemotactic responses to CC chemokines. We consider it unlikely that this phenomenon is related to DC mobilization by FL treatment, as FL-DC have been reported by several groups to be phenotypically and functionally identical to DC from non-treated animals (47, 60, 307). Studies using iBMDC from FL-treated mice showed iBMDC responded to the same CC chemokines as those reported by groups using non-FL-mobilized murine BMDC (192), and our own data using spleen iDC from non-FL-treated mice were comparable to those from FL-treated DC (Fig 11A). Further, our *in vivo* migration data for freshly-isolated, FL-mobilized DC (Figs 26 and 27) are consistent with those reported by Ruedl and Bachmann (304), who used untreated iDC and with those reported by Drake *et al* (71), who used FL-treated mDC.

It seems likely that spleen iDC do not express functional CR, as evidenced by their lack of Ca^{++} flux (Fig 12D) and lack of migration to their ligands (Fig 11A). At least one study has reported CCR5 expression on murine $CD8\alpha^+$ iDC [through binding of FITC-labeled CCL4 (MIP-1β)] (318), although we did not observe spleen $CD8\alpha^+$ iDC migration or Ca^{++} flux to CCR5 ligands (e.g., CCL4 or CCL5; Fig 11A and 13D). A possibility for the lack of detected activity is that the CCR on iDC have been downregulated by constitutive expression of CC chemokines in the spleen. By RPA, we have detected low levels of CCL3 and CCL4 and high levels of CCL5 in the spleens of FL-treated mice (50). Mack *et al* (191) have shown that these three chemokines downmodulate CCR5 expression on murine NK cells *in vitro* (as well as CCR2 by CCL1 on monocytes) in a dose-dependent manner, suggesting that CCR expression downmodulation on iDC *in situ* is a distinct possibility. No studies to date have reported CR expression on murine DC using poly- or mAb. Despite the availability of several such reagents, we were not able to demonstrate differential expression of CCR on mouse DC subsets, as has been reported for mouse Th1 and Th2 cells (159) and human DC (193, 205). The eventual advent of reliable Abs for detection of mouse extracellular CR will aid in determining whether iDC express no, non-functional [like human i pDC (193)], desensitized (or any combination of the three) CCR.

Whereas spleen iDC responses to the CC chemokines essentially were lacking, spleen mDC reacted as predicted from all other studies with mDC, regardless of tissue or species of origin (44, 49, 50, 54, 163, 164, 190, 195, 198, 309, 316). Perhaps analogous to *in vivo* data (86, 304) however, $CD8\alpha^+$ mDC migrated in consistently fewer numbers, a fact apparently unaffected by the chemokine tested (Figs 11C, 16-18) or method of sorting (Fig 11C). Without reliable anti-

CCR7 mAbs, it is not possible to know for certain whether fewer CD8α$^+$ DC overall express CCR7, or whether less CCR7 is expressed by CD8α$^+$ DC overall, only that fewer migrate (as compared to CD8α$^-$ mDC) and the change in intracellular Ca^{++} is (surprisingly) similar to CD8α$^-$ mDC. Though these data do not provide conclusive evidence as to why CD8α$^+$ DC migrate in fewer numbers, they give further insight into this consistent pattern: though both DC subsets may receive the same intracellular message to migrate, CD8α$^+$ mDC exhibit decreased prolongation of dendrites as compared to CD8α$^-$ mDC. As we discuss in Chapters 2 and 3, both mDC subsets migrate with equal agility to secondary lymphoid tissues *in vivo* (Figs 29 and 30), but not through EC-layered Transwell filter chambers (Figs 20, 21, 23-26), suggesting CD8α$^+$ mDC may require further stimulus (beyond CCL19 or CCL21) for full extension of their dendrites that has not been provided in our *in vitro* models. We have shown DC matured overnight in culture rapidly (in less than 1 min) extend their dendrites in response to both CCL19 and CCL21, unlike the LPS-matured iDC cell line previously investigated by Yanagawa and Onoé (317) (which exhibit prolongation of dendrites after 1 h exposure to CC19 only). Perhaps indeed the rapidity/ability with which mDC extend their dendrites depends on more factors than just chemokine. Thus, the advent of cell surface-specific murine anti-CCR mAbs will benefit greatly the investigations of CCR expression on both i and mDC.

In conflict with previous studies, we report here that spleen DC upregulate CCR6 mRNA expression after overnight culture (concomitant with phenotypic and functional maturation), although neither iDC nor mDC responded to CCL20 (the natural ligand of CCR6). Iwasaki and Kelsall (44), on the other hand, demonstrated that CD11b$^+$(CD8α$^-$) freshly-isolated PPDC migrated in response to CCL20, while neither CD8α$^+$ PPDC nor either subset of spleen DC did

so. Despite the fact that the freshly-isolated DC in their experiments appeared to be phenotypically immature, all the DC subsets evaluated migrated to CCL19 and CCL21, inconsistent both with the present findings and those of others [Fig 11A and B and (164, 185, 192, 194, 196, 197, 204, 309, 315)]. Kucharzik *et al* (319) detected EGFP/CCR6 on $CD11b^{+}(CD8\alpha^{-})$ PP and spleen freshly-isolated (untreated or FL-mobilized) DC, although they did not attempt in either case to determine whether the receptor was functional or whether its expression changed with maturation. Varona *et al* (320) also describe a lack of $CD11b^{+}$ PPDC in $CCR6^{-/-}$ mice, although they did not investigate whether this population was also absent in the spleen. Further, in the studies of Kelsall *et al* (44), DC were isolated from spleens of BALB/c mice, while our DC were isolated from the spleens of B10 mice. It has been reported that DC from C57BL/6J mice express mRNA for TRL9 only, while DC from BALB/c mice express mRNA for TLR2, 4, 5, and 6 (321). Perhaps CR mRNA expression is strain- as well as tissue of origin-dependent. If indeed CCR6 is expressed on mouse spleen iDC or mDC, it appears to be non-functional and unable to be activated (as assessed by Ca^{++} flux and migration assays) (Table 8, Fig 12, and data not shown).

In summary, here we have compared, for the first time, the *in vitro* migration of $CD8\alpha^{-}$ and $CD8\alpha^{+}$ (spleen) DC, taking into consideration the effect the state of DC maturation has on migratory capacity. Here we also show the first Ca^{++} flux assays performed to determine CR activation in response to specific chemokines with *in vitro*-generated (iBMDC) and tissue-isolated (spleen i and mDC) mouse DC. All other DC Ca^{++} flux studies previous to this one have been undertaken with human DC (193, 322) or a murine iDC cell line (196). Taken together with the migration data, these findings strongly suggest that unlike peripheral iDC, (i)DC within

secondary lymphoid tissues (in particular, the spleen) do not respond to (inflammatory) CC chemokines. They also offer the beginnings of an understanding as to why some groups have reported poor CD8α^+ mDC migration *in vivo*.

3. CHAPTER TWO[4]

MIGRATION OF SPLENIC DC SUBSETS THROUGH EC: MIMICKING THE *IN VIVO* ENVIRONMENT

In Chapter One, CCL19 and CCL21 were determined to selectively induce the migration of splenic mDC *in vitro* through unmodified Transwells®. Thus we analyzed whether these same chemokines were still able to induce mDC migration through a more "*in vivo*-like" environment by layering resting and activated endothelial cells (EC) across the Transwells®. Because of the differential migration pattern seen for $CD8\alpha^+$ vs $CD8\alpha^-$ mDC through both unmodified and EC-layered Transwells®, in this Chapter we attempted to determine which, if any, adhesion molecules expressed on mDC might be responsible for this difference. These findings also were important in determining which intercellular adhesion molecules may be important in facilitating splenic mDC migration *in vivo*, as considered in the Chapter Discussion.

[4] Data from this chapter have been submitted independently in manuscript form for peer review and publication (312) where indicated.

3.1. ABSTRACT

Immature DC are induced to migrate to sites of inflammation by inducible ("inflammatory") proteins called chemokines. Upon uptake of antigen and maturation, DC then are directed by constitutively-expressed ("lymphoid") chemokines to draining secondary lymphoid tissues, wherein they present these antigens to naïve and memory T cells, initiating the immune response. We have previously shown that mature murine CD8α$^+$ mDC are able to migrate, albeit poorly compared to their CD8α$^-$ counterparts, in response to CCL19 and CCL21 (constitutively expressed in T cell areas and HEV, respectively) using *in vitro* chemotaxis assays through unmodified Transwell® filters. One reason for this decreased migration may be the lower surface expression (compared to CD8α$^-$ mDC) of the adhesion molecule CD11b, which may hamper the ability of CD8α$^+$ mDC to adhere to/extravasate through filter pores *in vitro* and/or endothelial cells *in vitro/in vivo*. To test whether expression of this adhesion molecules plays a role in regulating the ability of CD8α$^+$ DC to migrate, we analyzed whether incubating CD8α$^-$ mDC with blocking monoclonal antibodies against CD11b would reduce their migration to the level of CD8α$^+$ DC. We further tested whether blocking adhesion molecules reported to be expressed on mouse and human DC (e.g., CD31, CD54, CD62L) would have an effect on their transendothelial migration. Resting or TNFα-activated mouse endothelial cells (EC) were layered on 5 μm Transwell® filters, and overnight-matured splenic, magnetic bead-sorted DC were placed into the upper wells of Transwell® filters for 2 h (with resting EC) or 4 h (with activated EC). Migrated DC then were removed from the lower chamber and enumerated using a Coulter counter. Both DC subsets had slightly impaired migration to CCL19 through resting EC (compared to absence of EC), but both DC subsets migrated through TNFα-activated EC as well

as in the absence of the EC monolayer. Contrary to reports concerning human DC, transendothelial migration of the murine DC subsets was not dependent on CD11b, CD31, or CD62L expression by these cells. CD54, however, was determined to be at least partly involved in DC/EC interactions. This is the first report to examine adhesion molecules involved in transendothelial migration of murine DC subsets. The data implicate CD54 in facilitating DC transendothelial migration, suggesting a potential target for manipulation of DC trafficking *in vivo*.

3.2. INTRODUCTION

DC trafficking *in vivo* requires interactions between DC and EC. In both steady-state and inflammatory situations, iDC leave the bloodstream, aided by interactions between integrins on DC [reportedly, for human monocyte-derived DC, CD11b/CD18 but not CD11c (283)] with Ig superfamily members on EC [e.g., CD54 (37, 283)] for rolling and tethering, and likely through homophilic engagement of CD31 for transendothelial extravasation (273, 276, 283). Upon entrance into the peripheral tissue, the iDC endo/phagocytose Ag, begin to mature, and modify their CCR expression. The concurrent downregulation of CR for inflammatory chemokines and the upregulation of CR for lymphoid chemokines initiates recruitment of the DC to secondary lymphoid tissues, requiring their reverse transmigration through EC into lymphatic vessels, a process which reportedly requires CD29 (β1-integrin), CD49d (VLA-4), and CD49e (VLA-5) (283), and tissue factor and multidrug resistance protein 1 (274, 278, 282).

Leukocyte rolling and tethering have been studied comprehensively in both the mouse and human. The adhesion molecules that regulate these processes have been elucidated for T cells, NK cells, and monocytes (258, 259). Recently, even the inscrutable transendothelial migration process has been illuminated (259) by studies that revealed both the physical means by which leukocytes transverse EC [most researchers now agree leukocytes (with the exception of one study with NK cells, which suggests migration *through* EC) wedge *between* two EC (265)] and the adhesion molecules [as mentioned above (259)] involved. However, these studies have not been extensive regarding DC. Presently, very few reports have considered which adhesion molecules may be important in directing DC extravasation, and these studies have been limited to human DC that differ in state of maturation as well as in origin of the DC analyzed (205, 283).

As observed in Chapter One, CD8α$^+$ mDC appear to have a reduced ability to migrate *in vitro* as compared to CD8α$^-$ mDC. As also discussed, one possibility for this discrepancy may be related to their expression of functional CCR7. Another possibility is their expression of adhesion molecules. CD8α$^+$ DC frequently are further characterized by their lack/low expression of CD11b, while CD8α$^-$ DC are classically identified as "myeloid" by their high expression of this integrin [Fig 10 and (46-48, 52)]. CD54, constitutively expressed on resting (albeit low/moderate levels) and (upregulated on) activated EC (258), is a major ligand of CD11b. Therefore it is conceivable that the reduced expression of CD11b on CD8α$^+$ DC may correlate with their reduced migration *in vitro* (Fig 11C) and reported *in vivo* trafficking (86, 304). Data concerning murine DC/EC/adhesion molecule interactions currently are lacking, leaving a discernible gap in the knowledge of a potential means by which to manipulate DC migration *in vivo*. In addition to questions concerning differences in CD8α$^+$ and CD8α$^-$ DC migration, basic information regarding which adhesion molecules permit DC to exit from the bloodstream into peripheral tissues is essential for optimizing their therapeutic delivery.

In Chapter Two we describe our creation of a more "*in vivo*-like" chemotaxis environment through layering of resting or activated EC over the Transwell® filters. This system allowed further evaluation of the ability of CCL19 and CCL21 to regulate mDC migration and provided a controlled environment in which the specific adhesion molecules thought to be important for DC extravasation could be examined. Thus in this Chapter, we evaluate, also for the first time, which adhesion molecules are essential for facilitating murine splenic DC subset transendothelial migration.

3.3. MATERIALS AND METHODS[5]

3.3.1. DC isolation

mDC were prepared as described in Chapter One (Section 2.3.2), except that 14.5% w/v nycodenz (Sigma) was used for DC enrichment (instead of 14.5% w/v metrizamide).

3.3.2. Endothelial cells

EC from the Mile Sven 1 (MS1) SV40-transformed pancreatic cell line derived from a C57BL/6 (B6; $H2^b$) background were generously donated by Timothy Carlos MD, PhD, Department of Medicine, University of Pittsburgh Physicians Faculty (who acquired the cells from ATCC). EC were grown to confluence in normal, uncoated cell culture flasks in EC media: RPMI-1640 supplemented with 5% FBS, antibiotics, fungicide® (an anti-fungal supplement; Sigma), and 1% sodium bicarbonate, as recommended by Dr. Carlos. The MS1 cell line requires no exogenous growth factors/cytokines (ATCC). EC were split at confluence (to prevent overgrowth) and for phenotypic and chemotaxis assays. Excess EC cultures were frozen down as available to provide backup cultures: EC were frozen in 90% dimethyl sulphoxide (9 parts DMSO: 1 part EC media) in liquid N_2. The MS1 cell line is not susceptible to activation by IL-1β, but overnight exposure to TNFα (10 ng/ml) elicits the expression/upregulation of typical activated EC markers (with the exception of mucosal addressin cell adhesion molecule (MadCam)-1, which is not expressed by this cell line, T Carlos, personal communication). For chemotaxis assays, EC confluence was determined by Giemsa staining of 5-7.5 x 10^4 EC layered on uncoated 5 μm Transwells® filters for 24-48 h. Optimal EC numbers were found to be 6.5 x 10^4 overnight or 5 x 10^4 for 48 h.

[5] The reader is referred to Chapter One Materials & Methods for any repeat methods not covered here.

3.3.3. Extracellular flow cytometric analysis

Confluent EC were exposed to 0, 5, 10, or 20 ng/ml TNFα in EC media for 0, 4, 8, 18, or 24 h in uncoated 6 well plates, washed thoroughly with PBS, trypsinized, washed in PBS, and then stained by normal flow cytometric analysis protocol. EC were first blocked with 10% v/v normal goat serum and then stained for the following cell surface molecules: FITC-/biotinylated-CD11a (LFA-1), CD11b (Mac-1), CD11c (p150/95), CD31 (PECAM-1), CD54 (ICAM-1), CD62L (L-Selectin), CD62E (E-Selectin), CD102 (ICAM-2), and CD106 (VCAM-1) (all from PharMingen). Cells stained with the appropriate isotype-matched Ig (BD PharMingen, San Diego, CA) were used as negative controls. After staining, cells were fixed in 4% v/v PFA.

3.3.4. Chemotaxis assays

For chemotaxis assays with EC, 5 (activated) -6.5 (resting) x 10^4 EC were layered over Transwells® with 5 μm pores 48 or 24 h, respectively, before the experiment. In assays with activated EC, 5 x 10^4 EC were layered 48 h preceding the migration assay, so that 10 ng/ml TNFα could be added 24 h after layering and 24 h before the chemotaxis assay. DC + EC were incubated for 4 h for resting EC and 2 h for activated EC. After the appropriate incubation period, the Transwells® were removed and migrated DC from the 24-well plates were collected and enumerated using a Coulter Counter. For accurate comparison between experiments, results were expressed as the percentage of migrated DC. Migration assays were performed in duplicate; experiments were repeated at least 3 times.

3.3.5. Statistical analysis

Student's unpaired t-test was performed to determined statistical significance using Statview and Microsoft Excel for the Macintosh. A "*P*" value < 0.05 was considered to be significant.

3.4. RESULTS

3.4.1. Phenotypic analysis of resting and activated EC

Primary mouse EC cell cultures are difficult to procure as well as to maintain. Therefore, we employed an established EC cell line. Although they appeared to be morphologically normal EC, we analyzed their cell surface phenotype (namely, adhesion molecules) to confirm there "resting" EC status. As expected, they expressed low levels of CD31, CD54, and CD62L, with high levels of CD62E, CD102, and CD106 (Fig 16), similar to normal EC (323, 324). Since EC are activated by exposure to TNFα (258, 279, 294), we used this cytokine to analyze CD11b (negative control), CD31, CD54, and CD106 expression after 0, 4, 8, 16, and 24 h exposure to TNFα. This allowed us to determine the minimum period of exposure of EC to TNFα for optimal upregulation of these adhesion molecules (Fig 17). Twenty-four h of exposure to 10 ng/ml TNFα resulted in maximal upregulation of all the molecules analyzed except CD102, which already was expressed by nearly 100% of the EC at 0 h (Fig 16). Flow cytometric analysis of adhesion molecule expression after 24 h exposure of EC to either 5 or 20 ng/ml of TNFα revealed no significant upregulation of adhesion molecules compared to that seen in the presence of 0 or 10 ng/ml, respectively (data not shown).

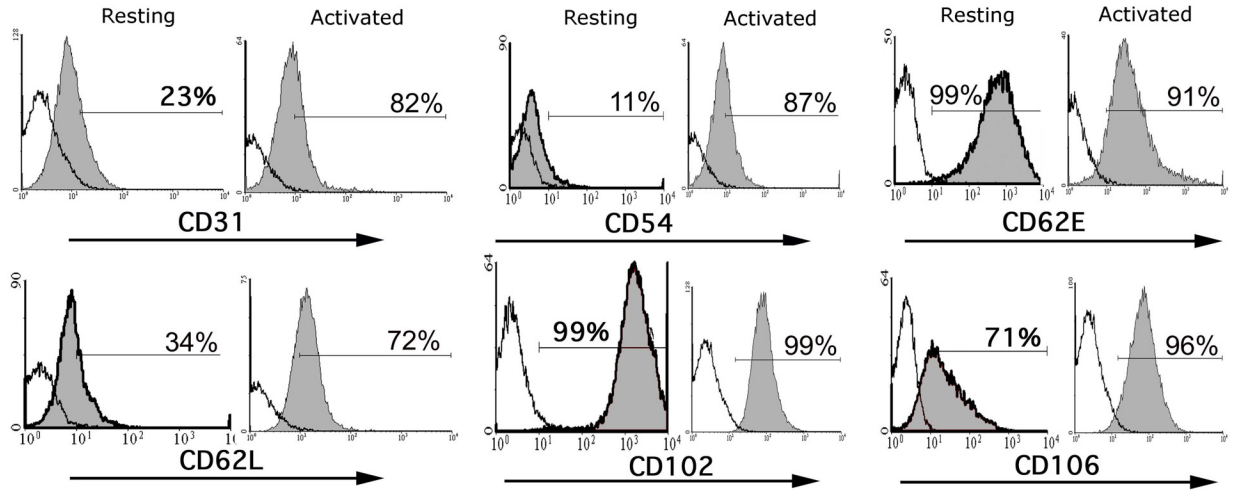


Fig 16. TNFα induces upregulation of various integrins and Ig superfamily members on EC.
EC were washed in PBS, trypsinized, and removed from plates after overnight culture (18h) with ("activated" EC; right histograms) or without ("resting" EC; left histograms) 10 ng/ml TNFα for staining of cell surface adhesion molecule expression. Open histograms represent appropriate isotype controls; grey histograms represent adhesion molecule expression (312).

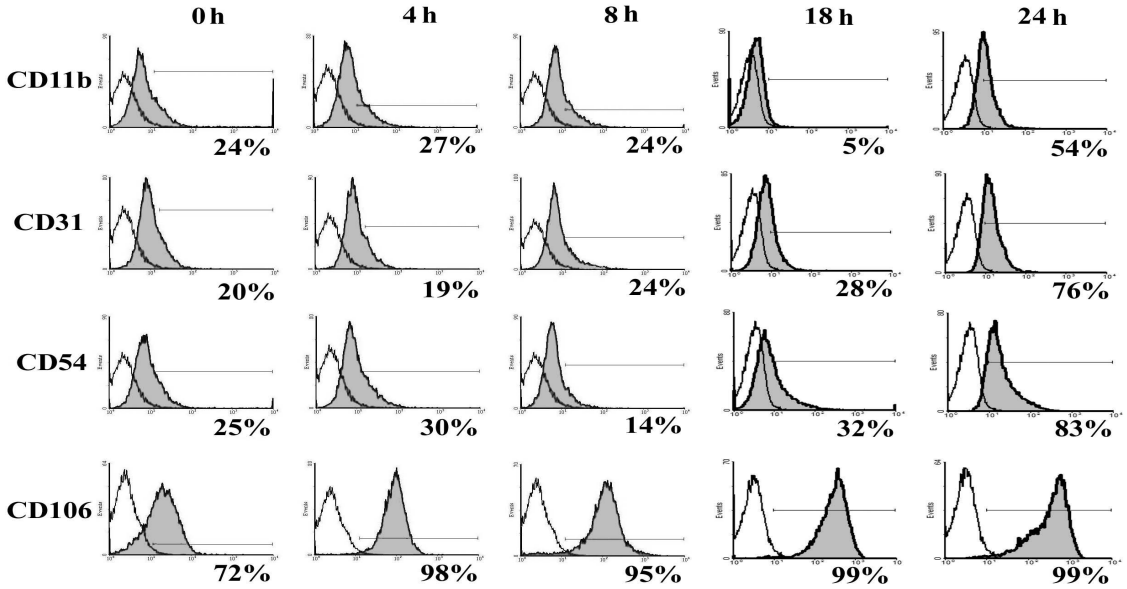


Fig 17. Optimal expression of adhesion molecules on EC occurs after 24 h exposure to 10 ng/ml TNFα.
EC were grown to confluence in EC media in 6-well plates and exposed to 10 ng/ml TNFα for 0, 4, 8, 18 or 24 h. EC were then washed in PBS, trypsinized, washed in fresh media, and then stained with appropriate adhesion molecule (grey histograms) or isotype control mAbs (open histograms) (312).

3.4.2. Spleen mDC subset migration is affected by the state of activation of EC

Normally it is iDC, not mDC, which extravasate through EC-walled blood vessels into peripheral tissues. However, our *in vitro* chemotaxis experiments with splenic iDC revealed that they were unable to migrate in response to any of the chemoattractants tested (either the CC chemokines or C5a or fMLP) (Table 8 and Fig 11A). Nor did they exhibit a change in intracellular Ca^{++} levels in response to any of the CC chemokines tested (Fig 14) or migrate in significant numbers to T cell areas of secondary lymphoid tissues in *in vivo* trafficking studies (as discussed further in Chapter Three). Thus, we chose not to pursue analysis of iDC *in vitro* migration through EC. Rather, we focused attention on overnight-cultured DC subsets ($CD8\alpha^+$ and $CD8\alpha^-$) since these "mature" DC both migrate to secondary lymphoid chemokines *in vitro* (Table 8 and Fig 11C) and migrate to secondary lymphoid tissue following their iv administration (Fig 28).

Maximal chemotactic mDC migration occurs between 2 and 2.5 h after addition of DC to the upper wells of the Transwell® filters (after 2.5 h, random DC movement in wells with no chemokine increases, giving a false positive migratory response: original time course performed by Dana Faratian, MD, Summer 1999; data not shown). Because the EC layer adds an additional barrier for mDC to transverse, we did time course assays to determine the optimal time to evaluate mDC transendothelial migration. As expected, maximal mDC migration through resting EC required an extended incubation period (after approximately 4 - 4.5 h of incubation) (Fig 18A). Somewhat unexpectedly, maximal mDC migration through activated EC occurred with slightly more rapidity than through unmodified Transwell® filter chambers (after approximately 1.5 - 2 h) (Fig 18B).

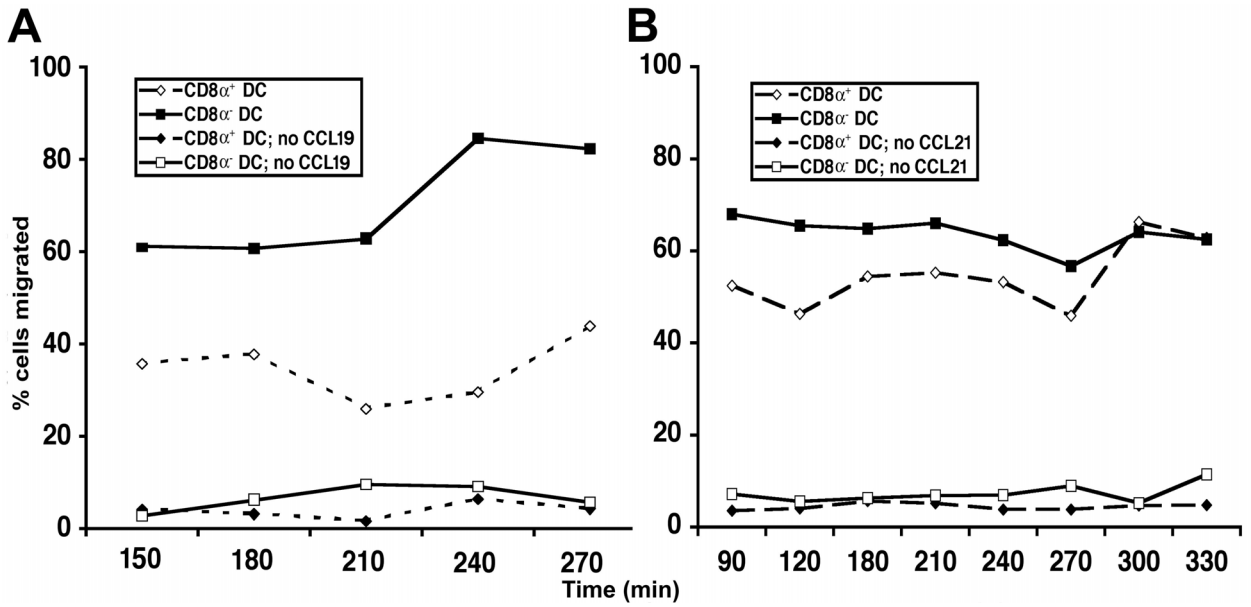


Fig 18. Time course of mDC migration to CCL19 through EC-layered Transwells®.
10 [nM] of (A) CCL19 or (B) CCL21 was added to the lower wells, and immunomagnetic bead-sorted $CD8\alpha^+$ or $CD8\alpha^-$ mDC were added to the upper well of Transwells® layered with (A) resting/(B) activated EC. The plates were incubated at 37° C in 5% CO_2 for the times indicated, at which point the Transwells® were removed and cells in the bottom well enumerated. To control for random movement of DC through EC over time, DC were also seeded in the upper wells of Transwells® that had no chemokine in the lower well. (A) Maximal mDC migration occurred after 4 - 4.5 h through resting EC. (B) Although from these data it appears maximal mDC migration occurred as early as 90 minutes, further chemotaxis assay experiments revealed 2 h to be the optimal incubation period for mDC migration through activated EC. Data are from a single experiment for each time course assay; one well per time point.

As expected from previous reports of their reactivity to the CC chemokines tested (e.g., Chapter 1), mDC migrated through unmodified Transwell® filter chambers only in response to CCL19 and CCL21 (Table 8 and Fig 11). We found both mDC subsets retained this ability, even in the presence of resting EC (Fig 19). Interestingly, despite the increased incubation period (from 2 h to 4 h), both mDC subsets displayed an overall decrease compared to migration through unmodified Transwell® filters in the percentage of cells that migrated through the EC barrier into the lower chamber (compare Fig 19 A to B and C).

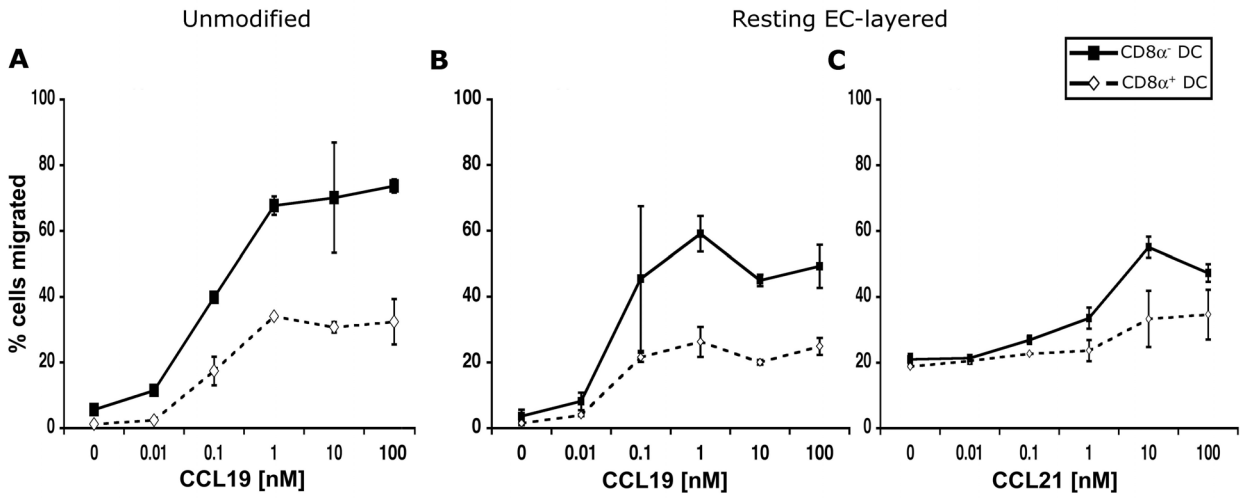


Fig 19. mDC subset migration through resting EC displays a similar pattern to mDC migration through unmodified Transwells® filter chambers, but with an overall decrease in the number of migrating DC.

Immunobead-sorted CD8α^- and CD8α^+ DC were placed in the upper wells of unmodified (A) or resting EC-layered (B, C) Transwell® chambers over graded concentrations of CC chemokines. DC migration through unmodified Transwells® solely to 10 [nM] chemokine was analyzed in parallel, to ensure that the decreased percentage of migrating DC was due to the presence of EC and not to a decreased ability of the DC to migrate in general (data not shown). Data are from a single experiment representative of at least 3 performed; each chemokine dilution was tested in duplicate (312).

mDC migration through activated EC (Fig 20) was greater than that through resting EC (Fig 19B and C) and equal or superior to that through unmodified filters (Fig 19A). This was likely due to the upregulated expression of intercellular adhesion molecules on the surface of activated EC (Fig 17), thus facilitating their migration through interaction with counter-receptors expressed on the DC.

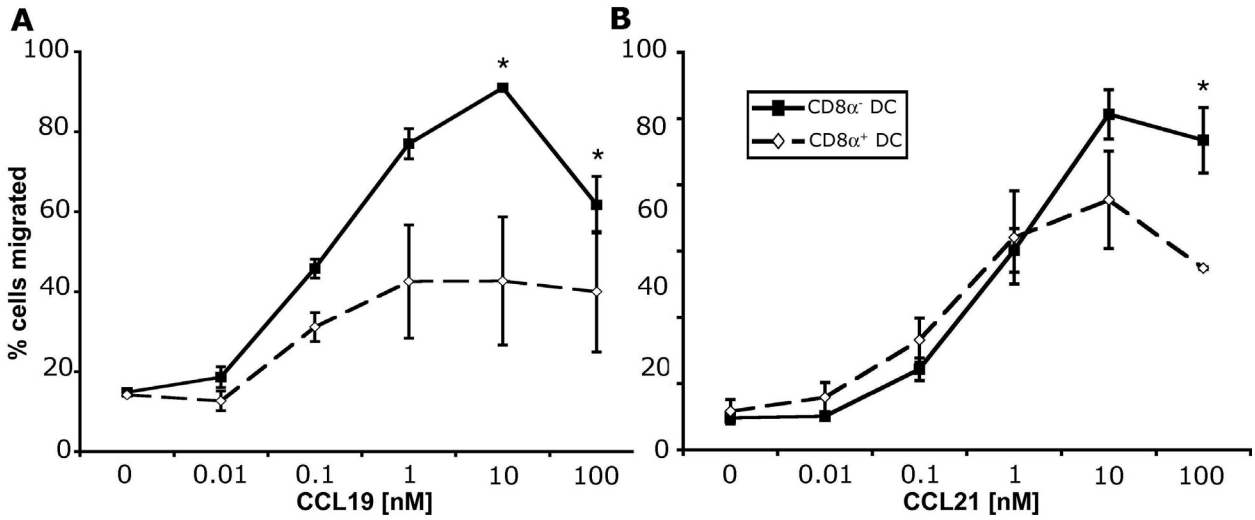


Fig 20. mDC subset migration through activated EC mimics mDC migration through unmodified Transwells® filter chambers and does not exhibit the decrease in migration seen through resting EC.

Immunobead-sorted CD8α$^-$ and CD8α$^+$ DC were placed in the upper wells of activated EC-layered Transwell® chambers over graded concentrations of CCL19 (A) or CCL21 (B). DC migration through unmodified Transwells® solely to 10 [nM] chemokine was analyzed in parallel, to ensure that the decreased percentage of migrating DC was due to the presence of EC and not to decreased ability of the DC to migrate in general (data not shown). Data are from a single experiment representative of at least 3 performed; each chemokine dilution was tested in duplicate. *, $P < 0.05$, significant difference between CD8α$^-$ and CD8α$^+$ DC (312).

3.4.3. Murine spleen mDC transendothelial migration *in vitro* does not require the same adhesion molecules as reported for human monocyte-derived and blood DC

CD31, CD54, and CD62L were all expressed at low levels on resting EC and upregulated upon EC activation (Fig 17). Both LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) (on CD8α$^-$ DC) expressed on human and murine DC are ligands of CD54, whereas CD31, its own homophilic ligand, is expressed on some human DC (205, 275). There is evidence that CD11b and CD31 are involved in human monocyte-DC/EC binding (CD11b and CD18) and extravasation (CD31) (278, 283) to/through EC. CD62L is involved in homophilic binding between EC and activated and memory T cells, and may facilitate T cell exit from the blood into lymph (325, 326). Therefore we investigated whether blocking interactions between these adhesion molecules and their counter-receptors would affect transendothelial migration of murine splenic DC subsets.

Since the influence of blocking specific molecules on EC is dependent on the state of activation of the EC barrier (205, 283), we chose to analyze both resting and activated EC conditions in these experiments.

3.4.4. Blocking of CD11b expression does not effect transendothelial migration of CD8α⁻ mDC

CD8α⁺ DC express lower levels of CD11b than do CD8α⁻ DC [Figs 10 and 21 and (46, 47, 52, 190) (316)]. Thus, another possible explanation for the apparent difference in mDC subset migration may be the level of expression of CD11b on CD8α⁺ DC. We incubated CD8α⁻ mDC with anti-CD11b mAbs to determine whether blocking this adhesion molecule would reduce CD8α⁻ mDC migration to the level of CD8α⁺ mDC. Despite reports that blocking CD11b or CD18 on human DC impairs their adhesion to/transmigration through EC (205, 283, 327), CD8α⁻ DC were not impaired in their migration through resting or activated EC (Fig 22).

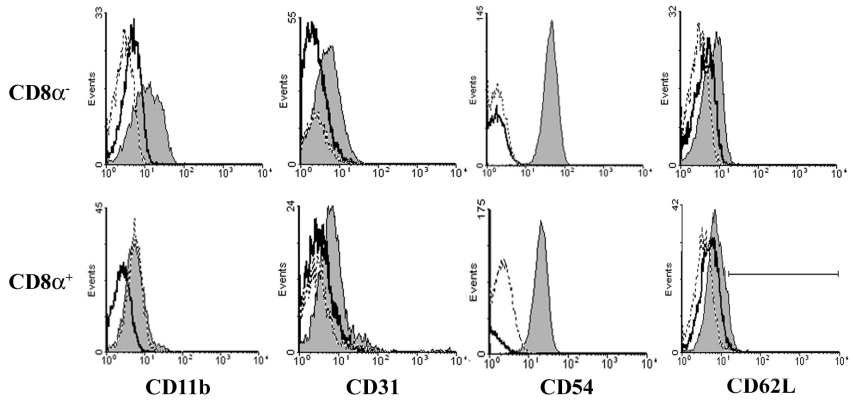


Fig 21. Blocking of specific adhesion molecules on mDC subsets.

Nycodenz-enriched, immunomagnetic bead-purified, overnight-cultured, CD8α⁻ and CD8α⁺ mDC were incubated with appropriate (purified or biotinylated) blocking or isotype control Ig for 30 min at 4° C. mDC were then washed and split for staining with FITC-conjugated mAbs to measure the extent of blocking or used in chemotaxis assays. Events were gated for CD11c⁺CD8α⁻ (top row) or CD11c⁺CD8α⁺ (bottom row) cells. Shaded histograms indicate detection of protein expression in the presence of isotype control Ig; bold outlined histograms represent isotype controls; dotted histograms represent detection of protein expression in the presence of blocking mAb. Data are from one experiment representative of at least 3 performed (312).

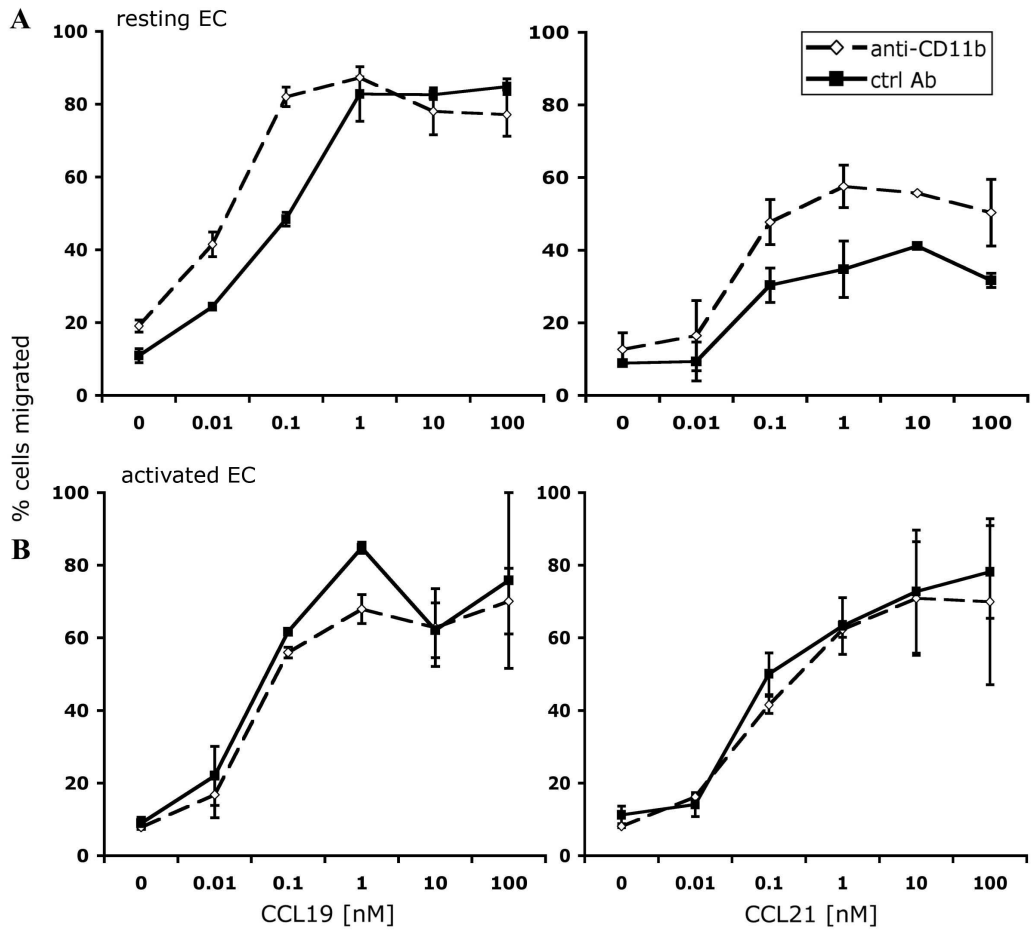


Fig 22. Blocking of CD11b expression does not influence CD8α⁻ DC migration *in vitro*.

Nycodenz-enriched, bead-sorted CD8α⁻ mDC were treated with either blocking anti-CD11b or isotype control Ig for 30 min at 4° C. Blocking was confirmed by both phenotypic (Fig 21) and functional analyses [phagocytosis of apoptotic bodies as described (31); data not shown]. After washing to remove excess mAb, DC were placed in the upper well of (A) resting or (B) activated EC-layered Transwells® and allowed to migrate for either 4h (resting EC) or 2 h (activated EC). Results are expressed as percent of transmigrated cells, mean ± SD of at least two experiments (two replicates per experiment) performed (312).

3.4.5. Lack of CD31 expression on murine spleen DC subsets precludes the necessity of this adhesion molecule in their transendothelial migration

CD31 also has been implicated in human DC (205, 283) and monocyte (278) transendothelial migration. Despite preliminary data suggesting a moderate level of expression of this adhesion molecule on mDC, incubation with blocking mAbs against CD31 did not affect the migration of $CD8\alpha^+$ or $CD8\alpha^-$ mDC through resting (Fig 23) or activated EC (data not shown), which we later determined correlated with the low-to-no CD31 expression on spleen mDC (Fig 21).

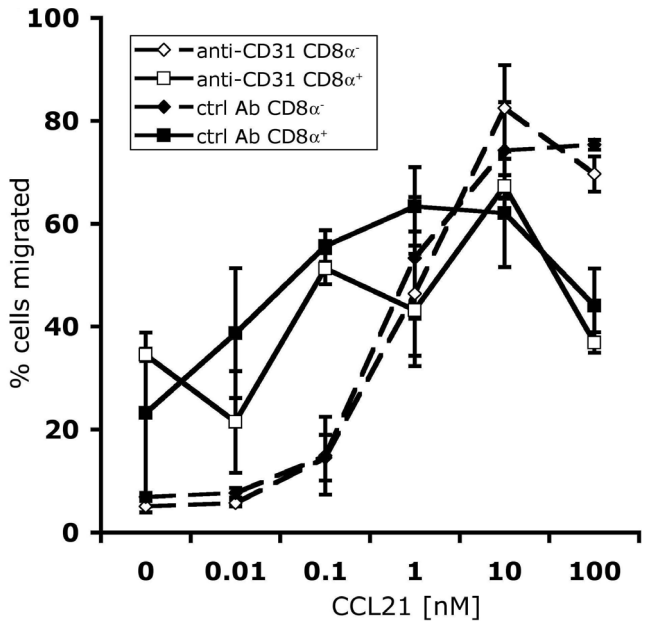


Fig 23. Unlike human monocyte-derived and blood-isolated DC, CD31 does not facilitate murine spleen DC transendothelial migration.

Nycodenz-enriched, bead-purified $CD8\alpha^+$ and $CD8\alpha^-$ mDC were incubated with purified anti-CD31 blocking or control isotype mAb for 30 min at 4° C (Fig 21). DC then were washed, resuspended in migration media, and their ability to migrate through resting and activated (data not shown) EC was analyzed as described. Data are from a single experiment representative of at least 2 performed; each chemokine dilution was tested in duplicate.

3.4.6. Blocking of CD54 expression inhibits transendothelial migration of DC subsets in response to CCL19

Although blocking of CD11b had no apparent effect on the migration of CD8α$^-$ mDC through resting or activated EC, blocking of CD54 significantly reduced the migration of both mDC subsets through resting EC (Fig 24A) and, to a lesser extent, through activated EC (Fig 24B). Interestingly, whereas DC migration through either resting of activated EC in response to CCL19 was impaired after blocking CD54 (Fig 24A), CD54 blocking appeared to affect mDC subset migration to CCL21 only through resting EC (Fig 24B).

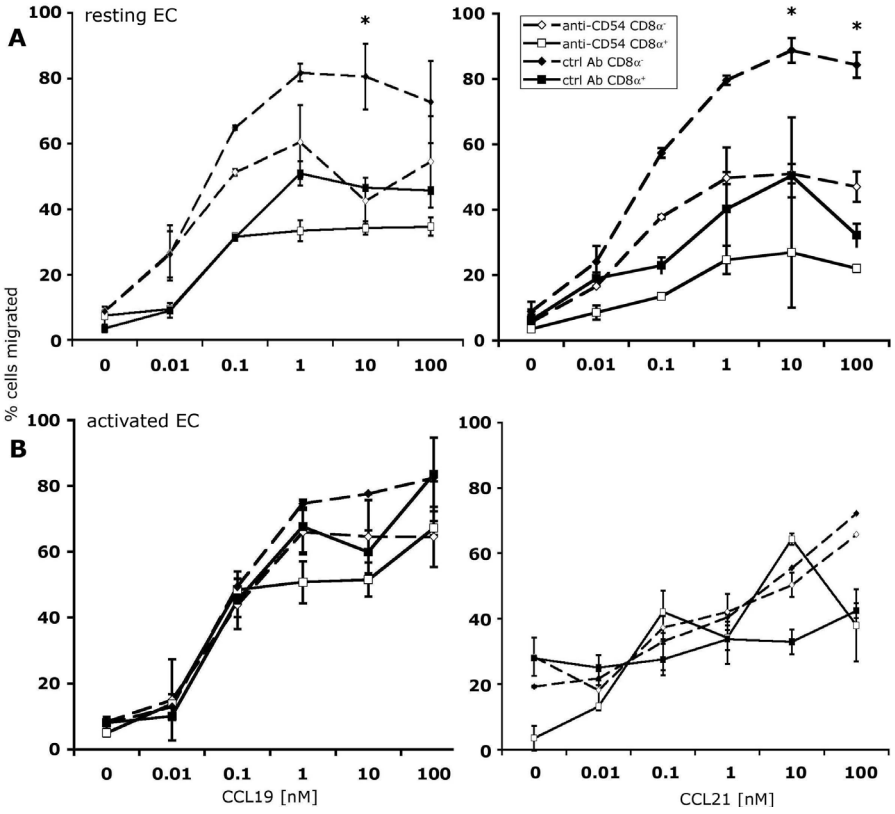


Fig 24. Blocking of CD54 expression reduces murine spleen CD8α$^-$ and CD8α$^+$ mDC subset chemotaxis through resting or activated EC.

mDC incubated with control isotype Ig or CD54-blocked mDC (Fig 21) were washed, resuspended in migration media, and then incubated for 4 h (for resting EC) or 2 h (for activated EC). Both subsets exhibited reduced migration through resting (A) and activated EC (B), although the effect was more marked through resting EC. Results are expressed as percent of transmigrated cells, mean ± SD of at least two experiments (two replicates per experiment) performed. *, $p < 0.02$, compared with control Ig (312).

3.4.7. Blocking of CD62L expression does not impair transendothelial migration of DC subsets

Although CD62L-blocking studies have been performed primarily on T cells and neutrophils (325, 326, 328-330), a recent report showed impaired *in vivo* migration of human blood $CD123^+$ DC after G-CSF-induced downregulation of this selectin (58). We show here for the first time that it also is expressed in moderate levels on FL-treated murine spleen DC (Fig 21). Thus we investigated the effect of blocking this selectin on DC on their chemotaxis through resting and activated EC (Fig 25A and B). Despite blocking of CD62L on both DC subsets with anti-CD62L mAb (Fig 21), neither their migration through resting nor activated EC was consistently impaired (Fig 25).

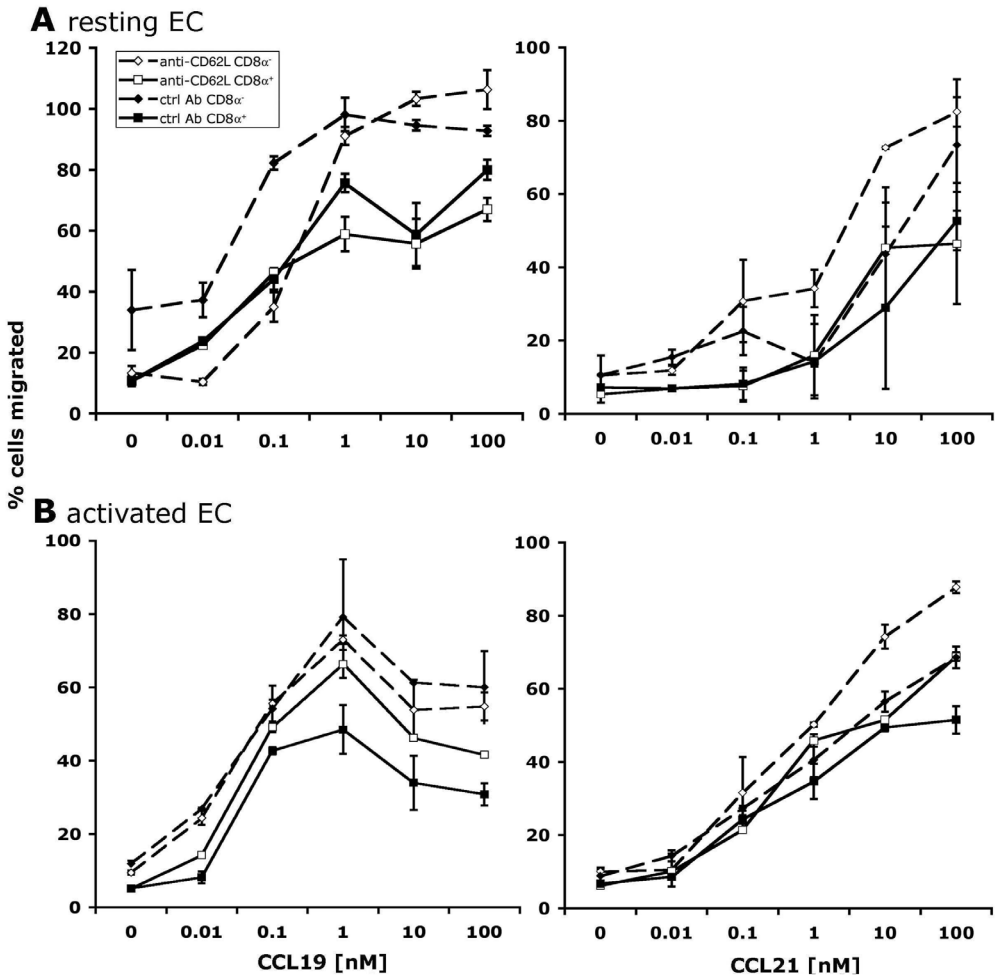


Fig 25. Blocking CD62L on CD8α$^-$ and CD8α$^+$ mDC does not impair their migration through resting or activated EC.

Nycodenz-enriched, bead-sorted CD8α$^-$ and CD8α$^+$ mDC were treated with either blocking anti-CD62L or isotype control IgG for 30 min at 4° C. Blocking was confirmed by phenotypic analysis (Fig 22). After washing to remove excess mAb, DC were placed in the upper well of (A) resting or (B) activated EC-layered Transwells® and allowed to migrate for either 4 (resting EC) or 2 h (activated EC). Results are expressed as percent of transmigrated cells, mean ± SD of at least two experiments (two replicates per experiment) performed (312).

3.5. DISCUSSION

In the course of these studies, we have set up an environment in which to examine the cell surface-expressed molecules involved in murine mDC transendothelial migration *in vivo,* while still using a tightly-controlled system *in vitro*. We first established that mDC indeed were capable of extravasation through resting EC in response to CCL19 and CCL21, but that their migration to these chemokines is visibly less compared to through unmodified Transwell® filter chambers or activated EC, despite a more prolonged (doubled) incubation period (Figs 18 and 19). Likely due to the upregulation of adhesion molecules on the EC (Fig 17), mDC trafficked with little to no impairment through activated EC, and did so within the same time frame as DC migrating in the absence of EC (Figs 11C, 19, and 20). Though it seemed initially that the results of our adhesion molecule blocking experiments were in direct conflict with reports of human DC-EC interaction, in depth review of these reports in comparison to the present findings (as discussed below) yielded insightful conclusions. As we have shown for BM-derived and spleen-isolated iDC responses to chemokines, differences in adhesion molecule requirements may be related as much to the state of maturation of the DC as to their tissue of origin/method of isolation/propagation.

Ca^{++} flux experiments with $CD8\alpha^{+}$ and $CD8\alpha^{-}$ mDC implicated a difference in their extension of dendrites (Fig 15), which may provide a partial explanation for the disparity seen in $CD8\alpha^{+}$ mDC migration. However, evidence that blocking of CD11b inhibits human monocyte-derived DC adhesion to EC by >60% (283) coupled with the differential expression of the integrin CD11b [Fig 10 and (46-48, 52)] by the two subsets suggested an alternative explanation as to why the CD8α-expressing subset showed a decreased migratory ability to the CC chemokines

CCL19 and CC21 compared to their CD8α$^-$ counterparts (Figs 11C and 19-25). Despite a lower expression of the adhesion molecule CD11b by CD8α$^+$ mDC, it does not appear that this integrin plays a critical role in regulating murine splenic mDC migration. Thus blocking CD11b on CD8α$^-$ mDC had no apparent effect on their transendothelial migration through either resting or activated EC (Fig 22), similar to human skin explant emigrated-DC, which also express CD11b but do not exhibit impaired migration in the presence of anti-CD11b blocking Ab (274). The differential pattern of migration seen for CD8α$^+$ and CD8α$^-$ mDC indeed may be explicitly related to their expression of functional CCR7/prolongation of dendritic extensions. A third explanation (not mutually exclusive) may be the high expression of CD205 by CD8α$^+$ DC. CD205 (DEC-205) is a comparatively large glycoprotein and might prevent interactions between with adhesion molecule on CD8α$^+$ DC and EC. Unfortunately, as we know of no way to induce CD205 expression on CD8α$^-$ DC (without genetic modification), and coating CD205 on CD8α$^+$ DC would merely increase its size, furthering increasing its interference, we were unable to test the degree to which this molecule may be involved (or not) in CD8α$^+$ DC migration within the scope of these studies.

In contrast to these findings, blocking of a major ligand of CD11b (CD54) considerably reduced CD8α$^-$ mDC extravasation and even further reduced an already weak migratory response of CD8α$^+$ mDC through resting EC. The same effect (though not as marked) was seen with activated EC (Fig 23B). While the overall numbers of mDC that migrated through resting EC were lower than those that traversed activated EC (one explanation for the less apparent reduction), the differences between control IgG-blocked and CD54-blocked DC groups in each system was greater in the presence of resting (~20% average inhibition) vs activated EC (~10%

average inhibition) (Fig 23A and B). Thus it is possible that one or more adhesion molecules on the activated EC may have compensated for the loss of functional CD54 on the DC (i.e., CD102/CD11a interactions were not investigated, as CD102 expression on EC does not change after activation, however, it may have facilitated DC migration in the absence of CD54).

While CD11b and CD54 likely are involved almost exclusively in leukocyte rolling and tethering *to* EC (the blocking of which would prevent further attachment to and extravasation through EC), CD31 has been reported to be a significant molecule in leukocyte (T cells, neutrophils, monocytes, human DC) migration *between* EC (37, 205, 273, 276, 277). Therefore it seemed surprising that it apparently had no involvement in murine spleen DC transendothelial migration. However, while a preliminary experiment indicated moderate expression of CD31 on both mDC subsets, consequent analyses proved this not to be the case. Indeed CD31 is expressed at very low levels on murine spleen mDC (4-5%; Fig 22), which makes it an unlikely candidate for involvement in DC/EC interactions after all. This low expression of CD31 on murine spleen DC likely is due to tissue rather than species of DC origin, as mouse BMDC express CD31 (~25%; Yuk Yuen Lan and An de Creus, unpublished observations). Human skin explant-emigrated DC do express CD31, but have no decrease in their migration in the presence of blocking Ab (274), while human blood-isolated $CD11c^+$ and $CD123^+$ have reduced migration when CD31 is blocked through activated but not resting EC (283). Thus the role of CD31 in DC transendothelial migration appears to be tissue specific.

It has been reported (259) that a leukocyte to EC ratio >10:1 can result in the destruction of integrity in an EC layer, due to the disproportionately high number of transendothelial migrators. Such an effect could eliminate any measurable influence of blocking mAb on DC migration. We typically use between 2.5-4 x 10^5 DC for our chemotaxis experiments to minimize deviations between paired wells. In our early blocking experiments, this resulted in a DC to EC ratio close to 10:1. These first experiments were with anti-CD11b and anti-CD31 mAb, and we saw no impairment of DC migration. The experiments were repeated with 1-2 x 10^5 DC, and as no change in the percent of DC migration through the EC was seen, we utilized this lower ratio in subsequent experiments to ensure the DC:EC ratio would not be a factor in other Ab blocking experiments.

Between 50% (58) and 90% (205) of human blood-isolated DC express CD62L, but there is little information on expression of CD62L by murine DC. It recently has been shown that administration of G-CSF induces upregulation CCR7 on human blood pDC, with a concomitant downregulation of CD62L, effectively preventing the exodus of the pDC from the blood into lymphatic vessels (58). Prior to this latter study, it was believed that G-CSF selectively enhances the generation of the $CD11c^-CD123^+$ pDC over the "classic myeloid" $CD11c^+CD123^-$ DC (57). In fact, it selectively impairs their emigration, resulting in an accrual of pDC in the blood, while $CD11c^+$ DC continue their normal trafficking patterns (not affecting their numbers in blood). Thus we felt it appropriate to investigate the expression of this molecule on murine spleen DC. In fact, approximately 50% of both $CD8\alpha^+$ and $CD8\alpha^-$ mouse spleen DC subsets express CD62L, and its expression is not subset specific, as in humans (although the expression of CD62L murine pDC has not yet been investigated). Similar to CD11b and CD31, shown to be

important in human (205, 283) but not murine (Figs 22 and 23) DC transendothelial migration, blocking of CD62L on either mDC subset did not impair their migration through resting or activated EC.

Upon first assessment, the results from our blocking Ab experiments appear to be in conflict with those reported for human DC. However, as for freshly-isolated spleen and immature BM-derived (murine) DC, it appears the factors that regulate DC migration may be specific to the source/subset/state of activation of DC and the activation status of the EC being analyzed. Blocking of CD31 on resting EC before their interaction with human monocyte-derived DC (mature DC; as indicated by their strong induction of naïve T cell proliferation in MLR) has been reported to reduce transmigration by 40% (283). However, incubation of CD31-blocked human blood-isolated immature DC subsets ($CD11c^+$ and $CD123^+$) with EC showed significant reduction only in transendothelial migration through activated EC,- their migration through resting EC was unaffected after blocking of CD31 on their cell surface (205). Moreover, whereas human $CD11c^+$ iDC transmigrate normally through resting and activated EC after CD54 is blocked on their cell surfaces, $CD123^+$ iDC show impaired migration through activated but not resting EC (205). Blood-isolated human $CD11c^+$ and $CD123^+$ (i)DC also apparently need a chemotactic agent to elicit significant migration through EC (205), while monocyte-derived DC migration through EC was detected even in the absence of exogenous chemoattractants (283). Thus while some of our data may not match exactly those reported for human DC from different tissue compartments, it appears likely that as more DC/EC interactions in each species are examined, patterns will emerge regarding DC tissue source, subset, and state of maturation, as well as EC state of activation. Further understanding of the influence of these variables will prove invaluable in optimizing appropriate means by which to administer target DC

therapeutically. In summary, herein we demonstrate for the first time adhesion molecules that appear to be important in regulating murine spleen DC transendothelial migration.

4. CHAPTER THREE[6]

FACTORS INVOLVED IN REGULATION OF SPLENIC DC MIGRATION *IN VIVO*

In this chapter, we evaluate how the stage of maturation and route of administration of splenic DC affects their ability to traffic from the site of injection to secondary lymphoid tissues. Concomitantly, we examine the dependence of DC subsets on CCL19 and CCL21 for their directed migration *in vivo*. These findings offer insight into optimizing the delivery of DC for therapeutic applications, through manipulation of their state of maturation and route of administration, and possible exploitation of their chemotactic signals *in vivo*, as detailed in the Discussion and Chapter 4.

[6] Data from this chapter are excerpted from (48) where indicated.

4.1. ABSTRACT

Several groups have reported disparate findings regarding the ability of murine CD8α$^+$ DC to migrate *in vivo*. As these groups have employed diverse methods for monitoring CD8α$^+$ DC trafficking, and since our goal is to exploit their purported tolerogenic properties therapeutically, we evaluated the two most common routes of DC administration in an attempt to optimize delivery of these DC to T cell areas. To examine the *in vivo* significance of CCL19 and CCL21 in eliciting mDC migration, we utilized *p*aucity of *l*ymph node *T* cells (BALB/c *plt/plt*; H2^d) mice, which lack expression of CCL21-serine (ser) and have severely impaired expression of CCL19. Metrizamide-enriched, iDC and mDC subsets isolated from spleens of FL-treated B10 (H2^b) mice were separated by magnetic bead-sorting into CD8α$^+$ and CD8α$^-$ populations and labeled with chloromethylfluorescein diacetate (CMFDA). *In vivo* trafficking of the fluorochrome-labeled DC was assessed by immunohistochemistry (for *in situ* localization) and by rare event, flow cytometric analysis (for quantitation) of allogeneic recipient [control wt BALB/c ByJ (H2^d) and *plt*] draining lymph node (DLN) and spleen cells. As observed in our *in vitro* trafficking studies, labeled mDC were detected in draining secondary lymphoid tissues in greater numbers than iDC. Both mDC subsets were detected in greatest numbers in normal mice after iv injection, and considerably fewer CD8α$^+$ mDC were detected in DLN than CD8α$^-$ DC. Conversely, in the *plt* mouse, sc injection proved to be the more efficient route of administration, with CD8α$^-$ DC still trafficking the most competently. These data correlate well with the *in vitro* migration data presented in Chapter One. They also confirm the importance of CCL19 and CCL21 in regulating mDC trafficking to T cell areas of secondary lymphoid tissues and further suggest that CD8α$^+$ mDC may indeed respond to higher concentrations of chemotactic ligands than do CD8α$^-$ mDC.

4.2. INTRODUCTION

Four groups (52, 71, 86, 304) directly or indirectly have studied CD8α$^+$ DC migration *in vivo*. Each has reported disparate results regarding the ability of this DC subset to traffic to secondary lymphoid tissues. With careful consideration of the methods employed in each investigation (Table 7), it becomes clear that differences in experimental protocol for migration analysis *in vivo* are responsible for the disparate findings. Two groups have reported that spleen-derived, sc-injected CD8α$^+$ iDC fail to migrate to DLN (86, 304), whereas another has observed reduced (compared to CD8α$^-$ DC) CD8α$^+$ iDC migration to the spleen after their iv injection (304). O'Connell *et al* (52) found that *in vivo*-mobilized, liver-derived CD8α$^+$ mDC trafficked to DLN and spleen after sc injection, while Drake *et al* (71) detected *in vivo*-mobilized spleen-derived CD8α$^+$ mDC in DLN after their sc administration in a mixed population together with CD8α$^-$ mDC. Two of these studies (52, 71) used FL-expanded mDC (both groups reported migration to the DLN); two (86, 304) used non-FL-mobilized iDC (both reported no detection of iDC in DLN). The two groups (52, 304) that investigated DC migration to the spleen after injection both detected DC, although Ruedl and Bachman (304) used non-FL-treated spleen iDC and iv injection, and O'Connell *et al* (52) used FL-treated liver mDC and sc injection. Further, the groups differed in their methods to sort (or not) the subsets, in their technique for detection of the injected DC in secondary lymphoid tissues, and in the time points at which they excised the spleen or DLN. These inconsistencies in experimental protocol have not been previously compared directly, nor has the route or number of cells for delivery of DC subsets been evaluated and optimized, despite frequent discussion about their potential therapeutic use [e.g., (29, 253, 331)].

The *plt/plt* mouse lacks CCL21-ser (the lymphoid tissue-expressed gene of CCL21; the non-lymphoid tissue-expressed form [CCL21-leucine (leu)] is not impaired) and exhibit a severe reduction in CCL19 expression compared to wild-type mice (200, 256, 332, 333). Early reports of these mice showed that there was a drastic reduction in the number of DC and T cells in LN (200, 332). In the spleen, however, normal numbers of DC and T cells are found, but with poorly formed or virtually absent T cell area structures. The *plt* mouse can mount a delayed but enhanced response to contact sensitization and sc immunization to OVA (334) and can clear some viral infections (eg, Vesicular stomatitis-Indiana virus) (334, 335) but is highly susceptible to other viral infections [eg, mouse hepatitis virus (MHV)] (200). Its counterpart, the CCR7 KO mouse, is similarly deficient in T cell and DC numbers/T cell structure in secondary lymphoid tissues (257). These mice fail to mount primary T cell responses in contact sensitivity or DTH reactions, and their humoral responses are delayed but eventually as vigorous as wt (257). Thus, the natural mutation in chromosome 4 in *plt* mice that appears to be responsible for the absence of CCL19 and CCL21 (63) makes them ideal candidates for evaluating the importance of these two chemokines for regulating mDC trafficking *in vivo*.

Although all *in vitro* studies with mDC, regardless of species, subset, or tissue of origin/derivation have reported positive migration to CCL19 and/or CCL21, no studies previous to these have tested the importance of these chemokines for DC subsets *in vivo*. The natural mutation in chromosome 4 in *plt* mice that appears to be responsible for the absence of CCL19 and CCL21 (63) makes these mice ideal candidates for examining the importance of the two chemokines in DC subset *in vivo* trafficking (the CCR7 KO mouse would be an equally interesting model to investigate these issues, especially in parallel with the *plt* mouse, but our

efforts to obtain these mice from Germany for our studies was not successful). Both Gunn *et al* (200) and Förster *et al* (257) examined activated DC migration to T cell areas after skin contact sensitivity reactions (to FITC-painting) and found that a reduced number of DC trafficked to DLN of *plt* compared to wt mice. However, neither group injected normal DC into their mutant mice (or their mutant DC into normal mice) to confirm that the impaired migration was indeed due to lack of CCL19/CCL21 or CCR7 and not due to an inherent lack of ability of the DC in each model to migrate to secondary lymphoid tissues. Nor did they phenotype the DC that migrated to the DLN post FITC-painting to ascertain whether the DC subsets were differentially affected by the deficiency in chemotactic signaling.

In this chapter, we build on the studies of Chapters One and Two, investigating whether the failed migration of spleen iDC subsets *in vitro* is seen also *in vivo* [one possible explanation for some of the disparate findings between groups for $CD8\alpha^+$ DC migration (86, 304)]. We also examined to what degree DC migration is affected by route of administration (Table 7), and whether the differences in mDC subset migration *in vitro* carries over into *in vivo* trafficking. We further investigated how the drastic reduction in CCL19/CCL21 concentrations would affect $CD8\alpha^+$ mDC *in vivo* migration, given the poor *in vitro* chemotaxis responses and decreased dendritic extension exhibited by these DC in high concentrations of chemokine.

4.3. MATERIALS AND METHODS

4.3.1. Mice

Male B10 ($H2^b$) and BALB/c ByJ ($H2^d$) mice, 8-12 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). Breeding pairs of BALB/c *plt/plt* ($H2^d$) mice were obtained from Dr. Hideki Nakano at Duke University Medical Center, NC. They were maintained in the specific pathogen-free facility of the University of Pittsburgh Medical Center and fed Purina rodent chow (Ralston Purina, St. Louis, MO) and tap water *ad libitum*. Experiments were conducted in accordance with the National Institutes of Health guide for care and use of laboratory animals and under an Institutional Animal Care and Use Committee protocol.

4.3.2. DC isolation

Pooled spleens from 3-4 *plt* or normal BALB/c mice were digested with 100 U/ml collagenase, teased apart, and then further digested in 400 U/ml collagenase for two 30 min incubation periods in order to maximize DC recovery. Bulk spleen cell cultures were then enriched for mDC with 14.5% (w/v) nycodenz (Sigma) as with metrizamide as reported for cells from FL-treated mice (Chapter One; Materials and Methods). As 16% (w/v) nycodenz (as used with metrizamide) resulted in a substantial loss of recovery of DC, iDC were enriched over a 0.3055 g/ml (stock solution) nycodenz gradient (resulting in cell recovery similar to the enrichment experienced with 16% metrizamide). Too few cells were recovered to sort the subsets by FACS or immunomagnetic beads, so bulk populations were used in experiments with these cells. Pooled popliteal and inguinal LN from *plt* and BALB/c mice were gently mascerated over a nylon filter, and due to the extremely low number of cells in *plt* LN, no further enrichment was performed.

4.3.3. Flow cytometric analyses

For subset ratio determination of *plt* spleen and LN DC, cell surface phenotypic analysis was performed by flow cytometry, using an EPICS Elite ESP analyzer (Beckman Coulter). All flow cytometric assays were done at 4°C. Leukocytes were first blocked with 10% v/v normal goat serum for 10 min, then stained with mAb for 30 min. Cells stained with the appropriate isotype-matched Ig (BD PharMingen) were used as negative controls. Phenotypic analysis of cell surface markers was performed with the following Abs: FITC-CD11b, -CD31, -CD40, -CD54, -CD62L, -CD80, -CD86, and -IA^b, -$H2^k$; PE-CD11c; and Cy-Chrome-CD8α (mAbs, BD PharMingen). After staining, the cells were fixed with 4% v/v paraformaldehyde (PFA).

4.3.4. Labeling of DC for *ex vivo* detection

$CD8\alpha^+$ and $CD8\alpha^-$ (B10, $H2^b$) DC were cytoplasmically labeled with the green fluorescent dye chloromethylfluorescein diacetate (CMFDA; Molecular Probes, Leiden, The Netherlands) (15 μM; 45 min at 37° C) according to the manufacturer's instructions. After two washes, 2-4x10^6 $CD8\alpha^+$ or $CD8\alpha^-$ DC were injected either iv into the lateral tail vein or sc into one hind footpad of allogeneic recipients (BALB/c ByJ or BALB/c *plt/plt*; $H2^d$). Spleens or popliteal DLN were removed 24, 48, or 72 h after iv or sc injection.

4.3.5. Rare event, flow cytometric analysis

Quantification of *in vivo* DC migration was performed by enumerating the percentage of green fluorescent cells in 10^5 whole spleen/DLN cells as compared to control spleens/DLN from age-matched mice that had not been injected with DC.

4.3.6. Immunostaining of tissue sections

In situ localization of injected DC was ascertained by immunohistochemistry. Tissue samples were embedded in Tissue-Tek OCT (Miles Laboratories, Elkhart, IN), snap-frozen in isopentane (pre-chilled in liquid N_2), and stored at -80°C until use. Ten μm cryostat sections were mounted on slides pretreated with Vectabond (Vector Laboratories Inc, Burlingame, CA), air-dried, and fixed in cold 4% v/v PFA for 10 min. Sections were blocked with 5% v/v normal goat serum, followed by avidin blocking solution (Vector Laboratories Inc). Biotinylated anti-CD3ε (T cells), -CD11c (DC), and -CD19 (B cells) mAbs (BD PharMingen) and biotinylated F4/80 (red pulp macrophages), and MOMA-1 (marginal zone metallophillic macrophages) mAbs (Bachem Bioscience Inc, King of Prussia, PA) followed by Cy3-streptavidin (Jackson Laboratories) were used to localize, T cell, DC, B cell, red pulp, and marginal zone areas, respectively.

4.3.7. 2-photon confocal microscopic analysis

FACS-sorted, CMFDA-labeled $CD8\alpha^+$ and $CD8\alpha^-$ splenic mDC were injected either iv or sc into BALB/c recipients. 48 h later, the spleens and LN were removed and imaged using a multi-photon laser scanning confocal microscope system comprising a titanium-sapphire ultrafast tunable laser system (Coherent Mira model 900-f), Olympus Fluoview confocal scanning electronics, an Olympus IX70 inverted system microscope, custom-built input power attenuation, and external photomultiplier tube detection systems (Olympus, Melville, NY). Dual photon excitation was at 870 nm, with fluorescence emission detected using a HG510/50 steep passband emission filter (Chroma, Brattleboro, VT).

4.4. RESULTS

4.4.1. CD8α$^+$ and CD8α$^-$ iDC do not localize to the spleen after iv injection

Iv injection is not the most direct measurement of chemokine-elicited trafficking of leukocytes to secondary lymphoid tissues as it deposits cells directly into the bloodstream, thus eventually conveying the injected leukocytes through the spleen. However, this route of injection is appropriate for the study of the ability of leukocytes to extravasate through EC walled blood vessels into peripheral tissues. It also is a valuable method for determining the localization of leukocytes in lymphoid tissues, an activity that is regulated/organized (at least partly) by chemokines. It is also a common route for therapeutic delivery of DC [i.e., infectious disease (336), transplantation (47, 130, 131), autoimmunity (337, 338), and cancer (339, 340) studies].

We have reported previously that allogeneic liver-derived CD8α$^+$ mDC migrate to the DLN of recipients within 24 h of sc injection (52). However, two other groups have been unable to detect fluorochrome-labeled spleen CD8α$^+$ iDC administered subcutaneously to syngeneic recipients in DLN (86, 304). Differences in protocols between studies, coupled with the present findings that at least both subsets of mDC apparently express the cellular machinery necessary for *in vitro* migration, led us to investigate further the disparity in the reports regarding the ability of CD8α$^+$ DC to traffic *in vivo*. Since the two groups that reported CD8α$^+$ DC are incapable of migration used freshly-isolated DC, we first examined the migration of iDC to the spleen after iv injection. However, surprisingly [although perhaps in correlation of our *in vitro* data (Fig 11A)], neither CD8α$^+$ nor CD8α$^-$ splenic iDC could be detected in the T cell areas of spleens of normal recipients, 24 or 48 h after iv injection (Fig 26), despite the reported ability of these DC to prime

naïve T cell responses via this route of administration (71, 75, 86, 304, 307). These results were confirmed by rare-event, flow cytometric analysis (Fig 29). To determine whether the pattern of splenic iDC traffic was related to their origin [as in *in vitro* chemotaxis assays (Fig 11A)], iBMDC were injected iv as controls. We were unable to detect iBMDC in the spleen after 24 h, but these cells could be detected in the liver and lung 24, 48, and 72 h their after injection (Morelli, AE, personal communication).

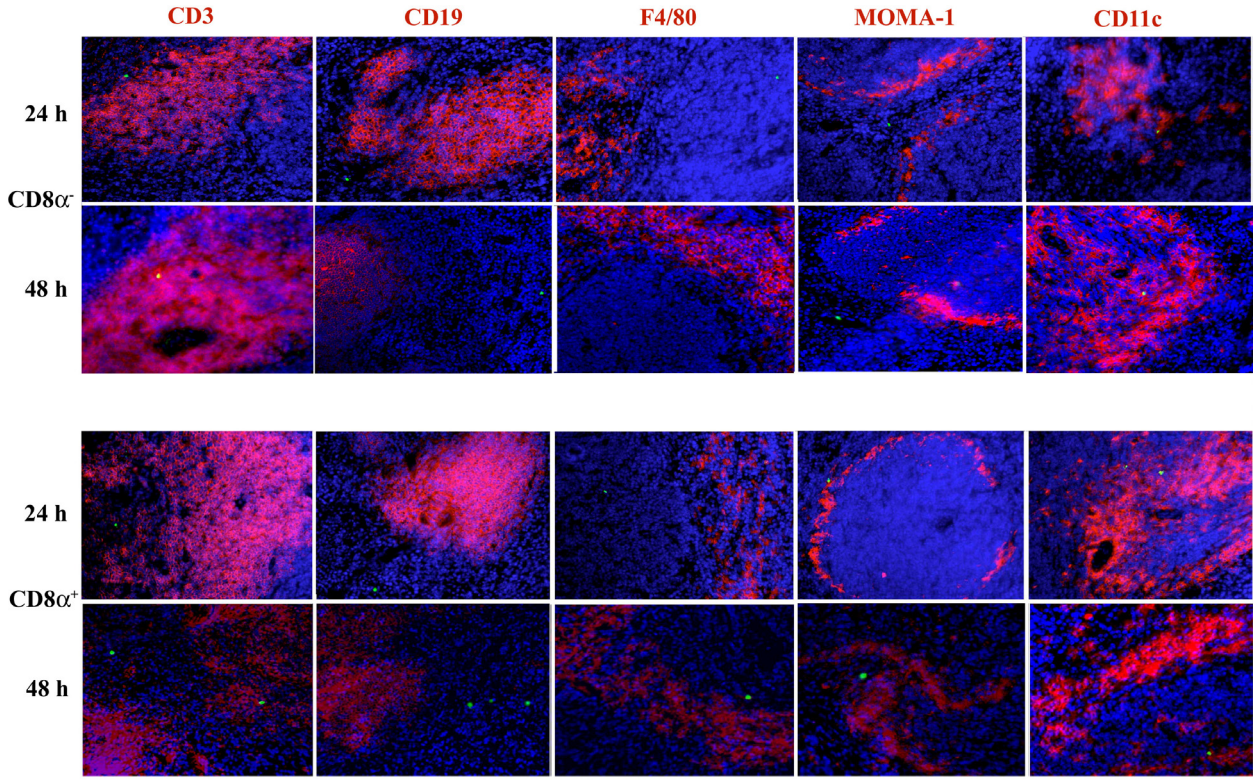


Fig 26. iDC are not detected in T cell areas of the spleen 24 or 48 h after iv injection.
Immunomagnetic bead-sorted, CMFDA-labeled (green) $CD8\alpha^-$ (upper panels) or $CD8\alpha^+$ (lower panels) B10 iDC (3-5 x 10^6) were injected iv into BALB/c mice. Twenty-four or 48 h later, spleen sections were stained with biotinylated mAb anti-CD3 (T cell), -CD19 (B cell), -F4/80 (red pulp Mϕ), -MOMA-1 (marginal zone Mϕ), -CD11c (DC), followed by Cy3-streptavidin (red). Neither subset appeared to migrate in significantly greater numbers. The near total absence of iDC at 24 and 48 h precluded further investigations at later time points (i.e., 72 h). DAPI (blue) indicates nuclear staining. x 200. [modified: (48)]

4.4.2. CD8α$^+$ and CD8α$^-$ iDC do not localize in DLN after sc injection

As some of the studies of CD8α$^+$ DC trafficking *in vivo* injected DC into the footpad (52, 71, 86), we also examined the ability of CD8α$^+$ and CD8α$^-$ mDC to reverse transmigrate out of peripheral tissues into lymph vessels in order to reach DLN. Despite data obtained in the rhesus macaque with blood iDC that suggest otherwise (43), iDC injected sc into the hind footpad of allogeneic recipient mice also did not localize to the DLN, 24 h or 48 after injection (Fig 27). Nor were the iDC detected after rare-event flow cytometric analysis (Fig 30) or in the footpad, as assessed by two-photon confocal microscopy (data not shown).

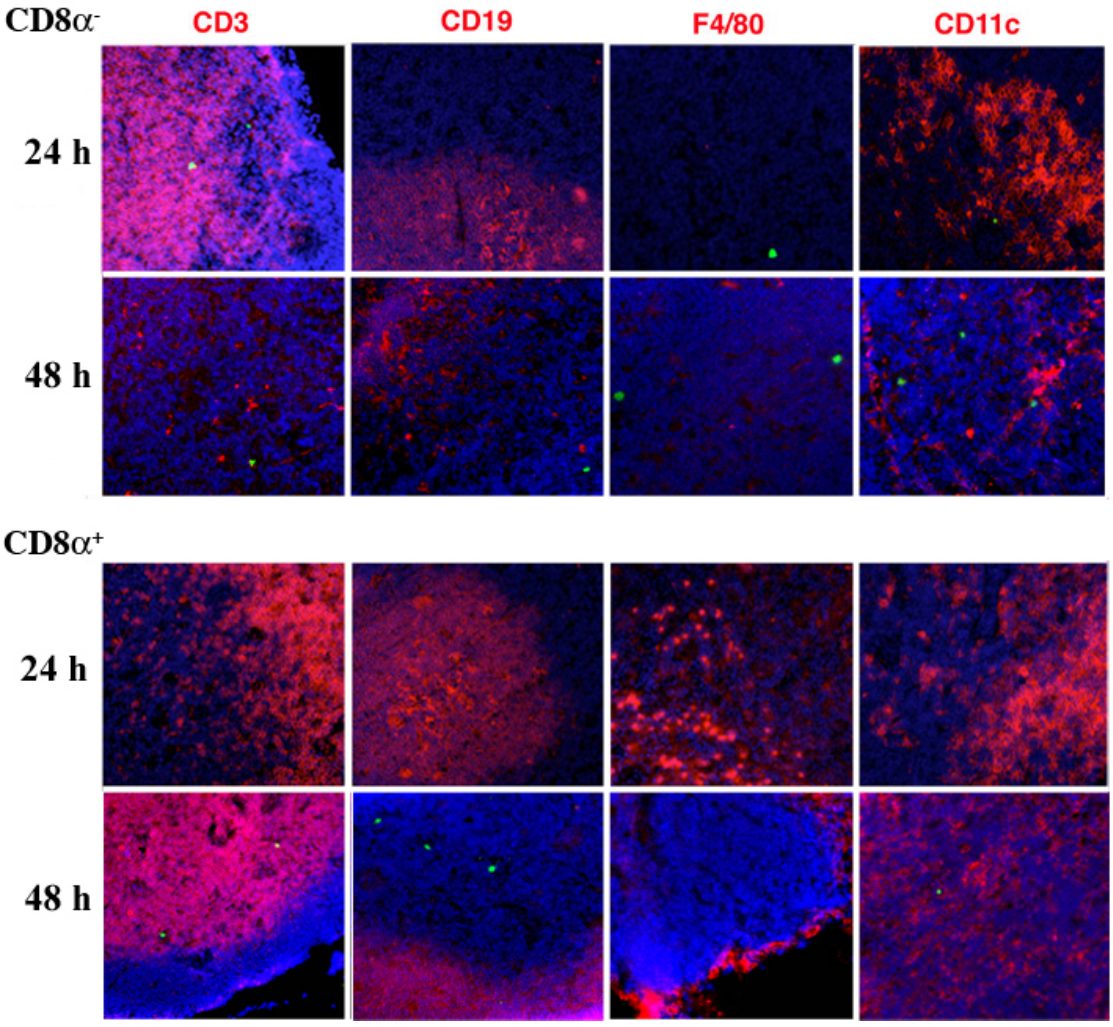


Fig 27. iDC do not colocalize with recipient DC and T cells after sc injection.

Immunomagnetic bead-sorted, CMFDA-labeled (green) $CD8\alpha^+$ or $CD8\alpha^-$ B10 iDC (3-5 x 10^6) were injected sc into one hind footpad of BALB/c mice. Twenty-four and 48 h later, DLN sections were stained with biotinylated mAb anti-CD3 (T cell), -CD19 (B cell), -F4/80 (red pulp Mϕ), -CD11c (DC) followed by Cy3-streptavidin (red). Few $CD8\alpha^-$ iDC localized to T cell areas after sc injection; no $CD8\alpha^+$ iDC were visualized. Blue: DAPI indicates nuclear staining. x 200. [modified: (48)]

4.4.3. CD8α$^+$ and CD8α$^-$ mDC colocalize with splenic T cells after iv injection

Unlike iDC, both mDC subsets migrated to T cell areas (CD3$^+$) and colocalized with (recipient) DC (CD11c$^+$) at 24, 48, and 72 h after iv injection (Fig 28). Administered mDC were not found in B cell follicles (CD19$^+$), red pulp (F4/80$^+$), or marginal zone (MOMA-1$^+$). In keeping with the quantitative rare-event data (data not shown), fewer DC were visualized over time. The comparatively inferior CD8α$^+$ to CD8α$^-$ DC migration seen in the *in vitro* chemotaxis studies, however, was not borne out in the *in vivo* experiments, as both DC subsets were detected in similar numbers in the spleen at 24, 48, and 72 h after injection (Fig 30 and data not shown).

4.4.4. CD8α$^+$ and CD8α$^-$ mDC localize with DLN DC and T cells after sc injection

Unlike previous reports (86, 304), we detected both CMFDA-labeled CD8α$^+$ and CD8α$^-$ mDC in the popliteal DLN [no DC were detected in inguinal LN at any time point tested (data not shown)] 24 h after sc footpad injection (Fig 29). Immunohistochemical analysis of the DLN revealed DC localized in recipient DC T cell areas, as seen in the spleen, and again the DC did not colocalize with B cells (CD19$^+$) or macrophages (F4/80$^+$) [MOMA-1$^+$ cells were not found in the LN (data not shown)]. CD8α$^+$ DC were furthered identified at 24 h by their faint co-staining for PE, – residual from flow sorting on CD8α expression immediately before injection (Fig 30). Only CD8α$^-$ DC were detected in DLN at 48 h, and neither subset was detected 72 h after sc injection. Unlike rhesus macaque blood-derived mDC (43), neither murine mDC subset was detected in the injected footpad mice as assessed by 2-photon confocal microscopy (data not shown).

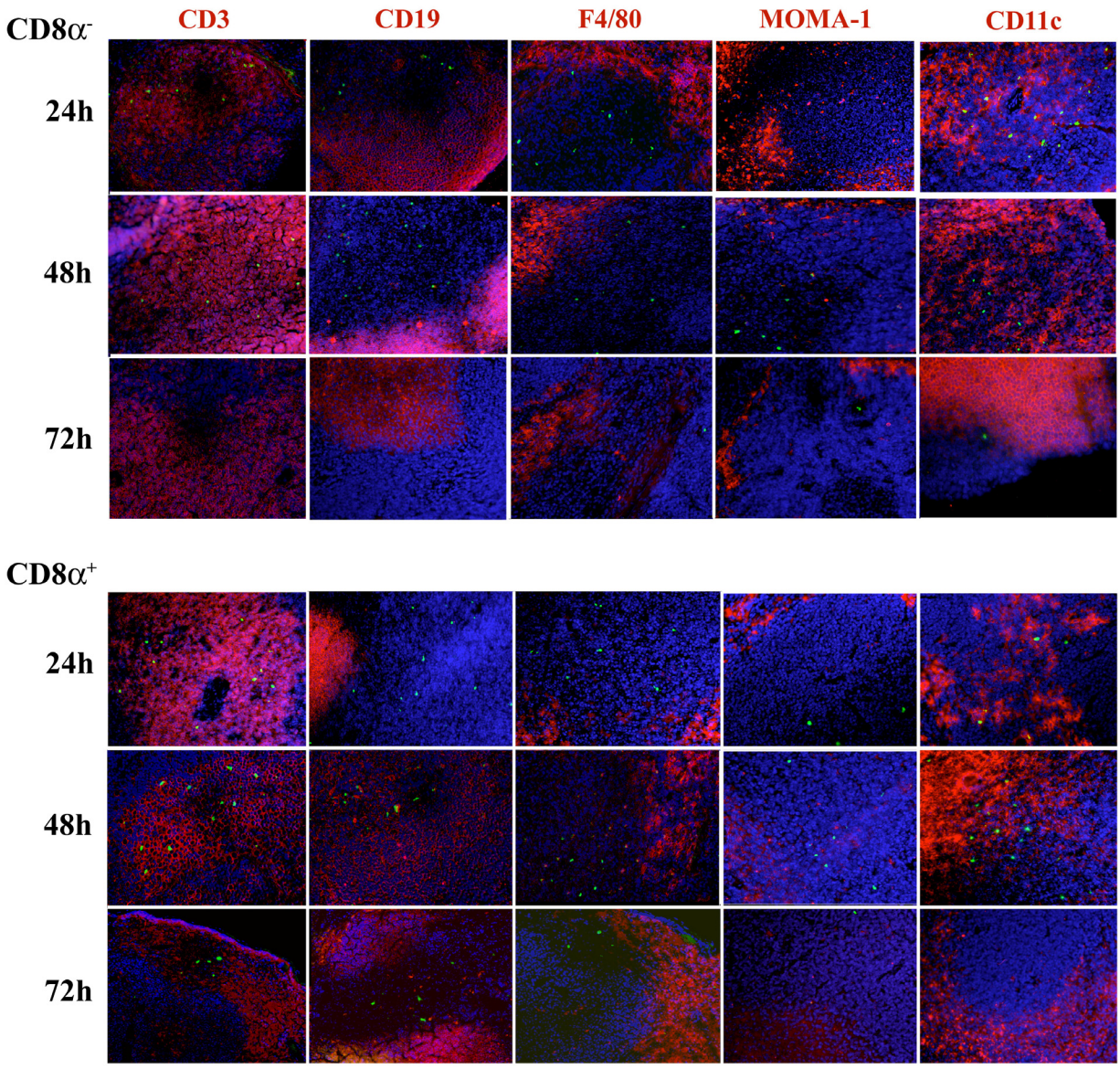


Fig 28. mDC of both subsets are found in T and DC areas of the spleen after their iv injection and at all time points investigated.

Immunomagnetic bead-sorted, CMFDA-labeled (green) $CD8\alpha^+$ or $CD8\alpha^-$ B10 mDC (3-5 x 10^6) were injected iv into BALB/c mice. Twenty-four, 48, or 72 h later, spleen sections were stained with biotinylated mAb anti-CD3 (T cell), -CD19 (B cell), -F4/80 (red pulp), -MOMA-1 (marginal zone), or -CD11c (DC) followed by Cy3-streptavidin (red). Unlike *in vitro* experiments, $CD8\alpha^-$ mDC (upper panels) did not appear to migrate with more efficiency than $CD8\alpha^+$ mDC (lower panels). DAPI (blue) indicates nuclear staining. x 200. [modified: (48)]

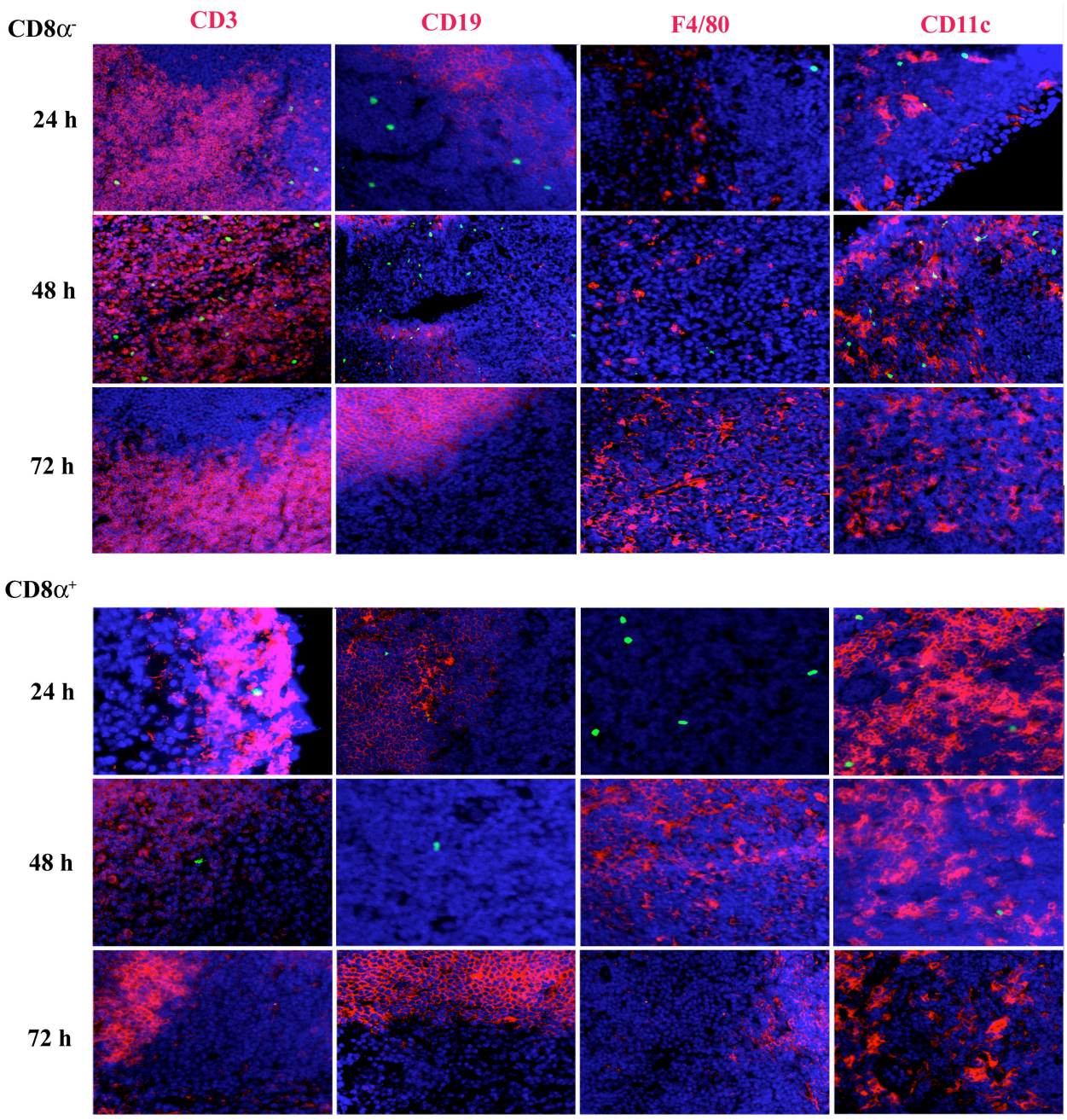


Fig 29. mDC colocalize with recipient DC and T cells 24 h after sc injection.

Immunomagnetic bead-sorted, CMFDA-labeled (green) $CD8\alpha^+$ or $CD8\alpha^-$ B10 mDC (3-5 x 10^6) were injected sc into one hind footpad of BALB/c mice. Twenty-four, 48, or 72 h later, DLN sections were stained with biotinylated mAb anti-CD3 (T cell), -CD19 (B cell), -F4/80 (red pulp), or -CD11c (DC) followed by Cy3-streptavidin (red). While both subsets of mDC were detected in T cell and DC areas 24 h after sc injection, $CD8\alpha^+$ mDC (lower panels) were found in fewer numbers than their $CD8\alpha^-$ counterparts (upper panels) at 48 h. Blue: DAPI indicates nuclear staining. x200. [modified: (48)]

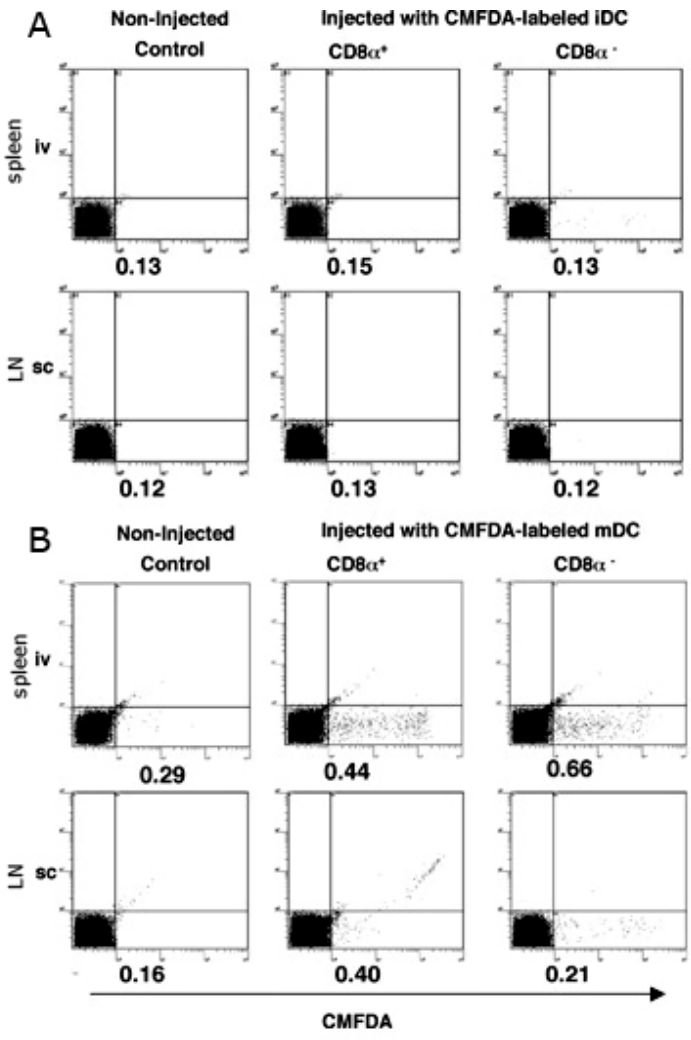


Fig 30. Efficient DC delivery is dependent both on the route of administration as well as the state of maturation of the DC. Flow-sorted, CMFDA-labeled DC (3-5 x 10^6) were injected iv or sc and spleens or DLN excised 24, 48, or 72 h later. Spleen DC-enriched suspensions (iv) and LN cell suspensions (sc) were analyzed by flow cytometry for CMFDA-positive cells. Only $CD8\alpha^+$ and $CD8\alpha^-$ mDC were detected in spleens after iv injection, with the greatest number of DC at 24 h of the 72 h (data not shown) follow-up period. Though fewer $CD8\alpha^+$ mDC were visualized by immunohistochemistry at 24 h, these DC were apparent by flow cytometric analysis, and in similar numbers to those for $CD8\alpha^-$ mDC. Non-injected spleens or lateral LN were used as controls. The data are from a single experiment representative of at least 3 performed. Numbers indicate the percentage of CMFDA positive cells. (48)

4.4.5. 2-photon confocal microscopic analysis of mDC *in vivo* trafficking to secondary lymphoid tissues

Originally, we planned to quantify each individual migrated DC in the spleen and non-migrating DC retained in the footpad using 2-photon confocal microscopy. Unfortunately, due to technical difficulties this method did not prove as useful as we had hoped. The thickness of the tissue prevented full penetration by the laser, and there appeared to be hair on the footpad, which also prevented penetration. These few studies did provide, however, further morphological evidence that the labeled cells detected by immunohistochemistry and rare-event analysis were in fact DC (Fig 31).

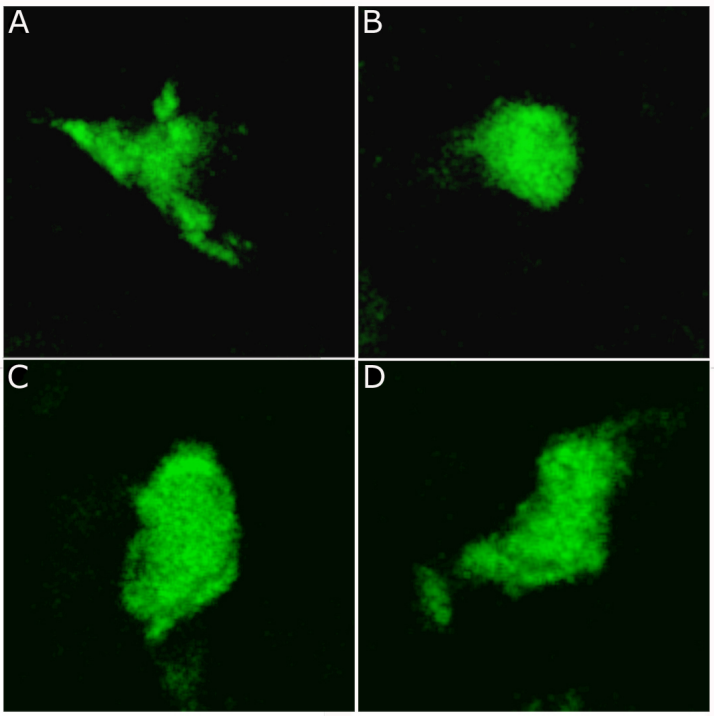


Fig 31. CMFDA-labeled cells detected in spleens after iv or sc transfer are morphologically DC.
Nycodenz-enriched, magnetic bead-sorted CD8α$^-$ (A and B) and CD8α$^+$ (C and D) spleen mDC from B10 mice were injected iv or sc (data not shown) into the lateral tail vein or footpad of normal BALB/c recipients. 48 h later, the secondary lymphoid tissues were excised, fixed, and CMFDA-labeled (green) cells were detected via 2-photon confocal microscopy. CMFDA-labeled cells with distinct mDC morphology were identified in both the spleen (A-D) and DLN (data not shown). x100.

4.4.6. Dependence on CCL19 and CCL21 for mDC trafficking *in vivo* is affected by both subset and route of delivery

Though in some sections, fewer CD8α$^+$ than CD8α$^-$ DC were detected by fluorescence microscopy [Figs 28 (48 h) and 29], quantitative rare-event flow cytometric analyses revealed both subsets of mDC migrated in similar numbers to the appropriate secondary lymphoid tissue. Their overall proficiency was dependent on the route of administration (Fig 30). This finding is somewhat unexpected considering the consistent pattern of lower CD8α$^+$ mDC migration to CCL19 and CCL21 *in vitro*. Therefore we utilized the *plt* mouse to determine whether these CC chemokines were key factors in directing CD8α$^+$ and CD8α$^-$ mDC migration *in vivo* (as iDC did not migrate efficiently *in vitro* or *in vivo*, we did not pursue further migration studies with DC at this stage of differentiation).

4.4.7. CD8α$^-$ mDC are detected in greater numbers in DLN than CD8α$^+$ mDC after sc injection in *plt* recipients

Gunn et al (200) painted the skin of *plt* mice in order to determine whether there was normal DC (LC) trafficking to DLN during inflammation, as in the steady state, *plt* mice are severely deficient in LNDC. The experiment did not verify, however, whether the lack (vs wt mice) of FITC$^+$ DC in the DLN in *plt* mice was due to an inherent inability of *plt* DC to migrate, or whether the poor migration was due directly to the lack of CCL19 and CCL21. Nor did they determine whether DC subset migration to the DLN was differentially affected. Though *plt* mice lack CCL19 and CCL21-ser in DLN, there is normal expression of CCL21-leu in lymphatic vessels. This suggests that mDC should be able at least to enter the lymph from peripheral tissues, such as the skin. This, in turn, should result in accumulation of normal numbers of DC in DLN during inflammation (although likely within poorly-formed T cell areas as seen in the spleen). As this was not the case in the FITC-painting experiments (200, 257), it may be that the lack of CCL19 and CCL21 in secondary lymphoid tissues may not be the only factor influencing the decreased number of leukocytes in these tissues. Thus, we injected sc CMFDA-labeled, normal B10 DC [which we have shown migrate normally to T cell areas of secondary lymphoid tissues (Figs 28-30)] into BALB/c *plt/plt* footpads and examined their migration to DLN (Fig 32).

As predicted, mDC were able to reach the DLN after sc injection, but did not localize exclusively with T cells. Interestingly, while both subsets migrated with equal efficiency to DLN after sc injection into the footpads of normal BALB/c, CD8α$^+$ mDC were detected in much fewer numbers compared to CD8α$^-$ mDC in *plt* mice (Fig 32), somewhat similar to CD8α$^+$ mDC *in*

vitro migration. Nor did DC of either subset localize exclusively with recipient DC and T cells, but were found dispersed throughout the tissue.

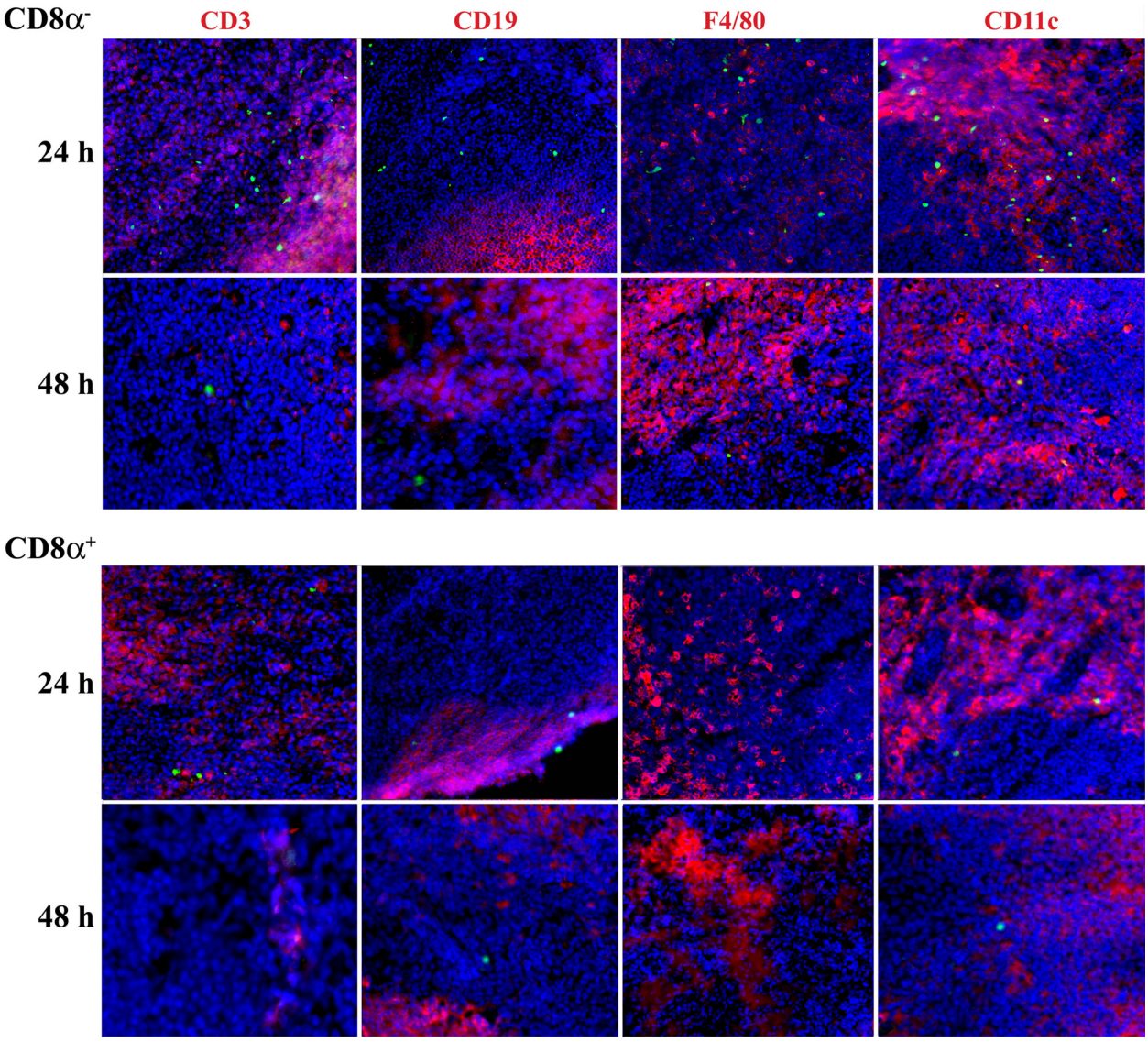


Fig 32. CD8α$^-$ mDC are less impaired in their ability to reach the DLN 24 h after sc injection. Immunomagnetic bead-sorted, CMFDA-labeled (green) CD8α$^+$ or CD8α$^-$ B10 mDC (3-5 x 10^6) were injected sc into one hind footpad of BALB/c *plt*/*plt* mice. Twenty-four or 48 h later, DLN sections were stained with biotinylated mAb anti-CD3 (T cell), -CD19 (B cell), -F4/80 (red pulp), or -CD11c (DC) followed by Cy3-streptavidin (red). While both mDC subsets were found in T cell areas of normal BALB/c mice 24 h after injection, only CD8α$^-$ mDC (upper panels; lower panels: CD8α$^+$ mDC) were detected in DLN. As expected, the mDC were found in DC and T cell areas, but were also found dispersed throughout B cell (CD19$^+$) and red pulp (F4/80$^+$) areas. x 200.

4.4.8. Few mDC of either subset remain in/localize with T cells in spleens of *plt* mice after iv administration

As CD8α$^+$ mDC migrated to the T cell areas of the spleen with equal, even superior, efficiency as CD8α$^-$ mDC in normal mice (Figs 28 and 30), we wanted to ascertain whether the reduced CD8α$^+$ mDC migration after sc injection in *plt* mice would be reproduced after iv injection. Therefore, we injected CD8α$^+$ or CD8α$^-$ mDC into the lateral tail vein of *plt* mice to compare the ability of the subsets to enter the spleen and colocalize with host DC or T cells in the absence of CCL19 and CCL21.

Unlike in normal BALB/c mice, iv injection was a less efficient route of injection than sc injection. mDC frequently were found near blood vessels (Fig 33, arrowheads), but they were neither localized with DC nor with T cells [as seen in wt mice (Fig 27)] nor dispersed throughout the tissue [as seen in the DLN after sc injection of *plt* mice (Fig 32)]. Nor did there appear to be a differential ability in mDC migration, as neither subset was detected in substantial numbers (< 1 DC/tissue section, on average).

As few iv-injected DC were detected in the spleen of *plt* recipients, and as only CD8α$^-$ DC were detected in DLN after sc injection, we considered whether endogenous CD8α$^-$ and CD8α$^+$ DC subsets were present in a normal ratio in *plt* secondary lymphoid tissues. Despite their near absence in LN and their poor distribution in spleens, there were no significant phenotypic differences in DC isolated from LN or spleens of *plt* and wt mice (data not shown). DC expressed comparable levels of CD11b, CD31, CD40, CD54, CD62L, CD80, CD86, MHC I, and MHC II. Nor was there a drastic difference in the ratio of CD8α$^-$ to CD8α$^+$ DC (an average of

1:1, accounting for inter-experimental variance) in the spleen (too few DC were isolated from the LN to determine their expression of CD8α).

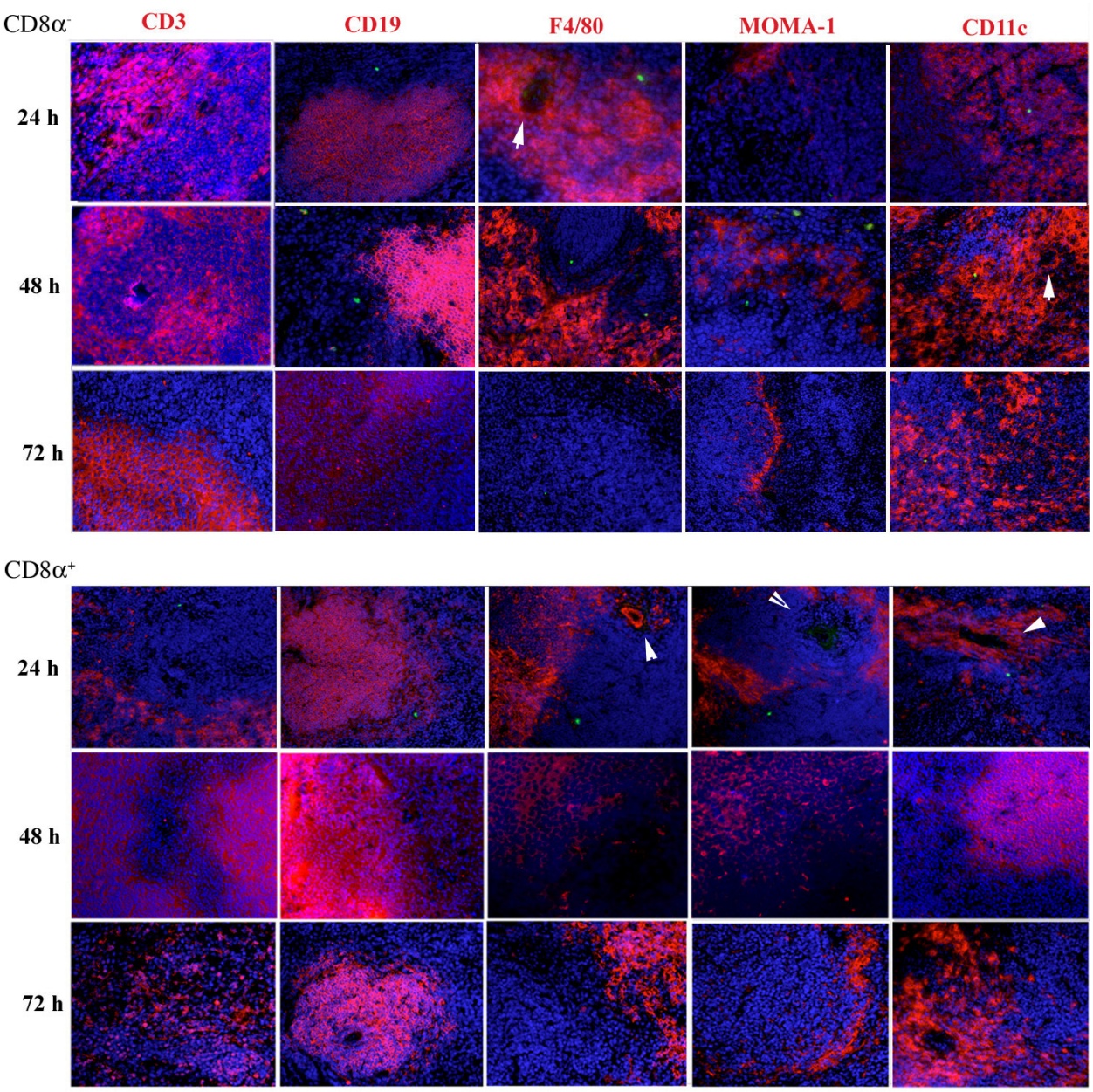


Fig 33. CD8α$^-$ and CD8α$^+$ CMFDA-labeled mDC are difficult to detect in spleens of *plt* mice after iv injection.

Immunomagnetic bead-sorted, CMFDA-labeled (green) CD8α$^+$ or CD8α$^-$ B10 mDC (3-5 x 10^6) were injected iv into the lateral tail vein of BALB/c *plt/plt* mice. Twenty-four, 48, or 72 h later, DLN sections were stained with biotinylated mAb anti-CD3 (T cell), -CD19 (B cell), -F4/80 (red pulp), MOMA-1 (marginal zone), or -CD11c (DC) followed by Cy3-streptavidin (red). While both mDC subsets were found in T cell areas of normal BALB/c mice at all time points 24 h after injection, almost no CD8α$^-$ (upper panels) or CD8α$^+$ (lower panels) mDC were detected at any time point, and they did not specifically co-localize with either DC or T cells. Arrowheads indicate blood vessels near injected DC. x 200.

4.5. DISCUSSION

The lack of *in vitro* migration of freshly-isolated splenic iDC to CCL19 and CCL21 and their low expression of CCR7 mRNA are consistent with what has been reported for *in vitro*-generated (cultured) iDC. This unresponsiveness to constitutive lymphoid CC chemokines is further supported by the lack of retention of iDC by the spleen after iv injection, presumably because of the inability of these DC to respond to CCR7 ligands. The lack of functional CCR7 on the cell surface of DC before injection, then, appears to preclude directed migration to secondary lymphoid tissue, which may also explain the absence of iDC in DLN 24 h after sc injection. Interestingly, rhesus macaque blood iDC do not express CCR7, but after their id injection, they apparently upregulate the molecule as they can be detected in the DLN with a concurrent upregulation of expression of surface CD83 and CD86 (341). Thus a possible explanation for the failure of murine iDC to do the same is that id injection may induce a greater degree of inflammation than sc injection, resulting in a level of rhesus iDC activation sufficient for their subsequent activation and emigration from the tissue that does not occur after sc injection in mouse recipients. After iv injection, i(BM)DC become dispersed throughout the lung and liver (Morelli, AE, personal communication). It is not clear whether these circulating iDC are capable of entering secondary lymphoid tissues after their activation by foreign Ag/inflammatory signals, or if they are fated to recirculate until they die. Splenic mDC, on the other hand, exhibit the migratory patterns of other mDC reported thus far. In the present study, upregulation of CCR7 mRNA expression in mDC correlated with the ability to respond to CCL19 and CCL21 *in vitro*. This responsiveness was further borne out by not only the retention of mDC in the spleen (up to 72 h after injection; Fig 28) but by their mobilization to T cell areas.

Comparatively fewer mDC reached the T cell areas of DLN after sc infusion and their persistence appeared more transient, with only CD8α$^-$ DC evident up to 48 h.

In the mature state, CD8α$^+$ DC and to a greater extent CD8α$^-$ DC, responded to CCL19 and CCL21 *in vitro*. This disparity seen with *in vitro* migration studies did not correlate with the level of *in vivo* traffic to the spleen and apparently is not necessarily an index of a cell's ability to mobilize to secondary lymphoid tissue. *In vivo* migration requires interaction with adhesion molecules and extravasation through lymphatic or blood vessel endothelia, and may be affected by other as yet unknown *in vivo* factors. Comparison of the two DC subsets clearly underlines the superior ability of CD8α$^-$ mDC to traffic *in vivo*, as strikingly fewer CD8α$^+$ than CD8α$^-$ mDC were apparent in the DLN of *plt* recipients at all time points tested. In Chapter One, we attempted to investigate whether the inferior ability of CD8α$^+$ mDC to migrate *in vitro* and *in vivo* compared to CD8α$^-$ mDC correlated with surface expression/function of CCR7 (Figs 12, 13, and 15). Conceivably, lower expression/function of CCR7 would impair the ability of CD8α$^+$ mDC to migrate from peripheral to secondary lymphoid tissues. As blocking of CD11b on CD8α$^-$ mDC did not reduce their migration to the level of CD8α$^+$ mDC (Fig 22), it is unlikely the low expression of this molecule on CD8α$^+$ mDC detrimentally affects their migration. Nor does it appear that any of the other adhesion molecules evaluated plays a role in their differential migration, as both blocking CD54 inhibited mDC subset migration to the same degree (Fig 24). Thus, chemokine-induced dendrite prolongation data (Fig 15) coupled with the *in vivo* migration data in *plt* mice (Fig 32) implicate a requirement for both high expression of CCR7 ligand(s) as well as another as yet unknown endogenous factor (apparently absent in our *in vitro* systems) for elicitation of efficient traffic by CD8α$^+$ mDC.

In normal BALB/c recipients, both mDC subsets exhibited equal ability to migrate to secondary lymphoid tissues, although their overall efficiency was dependent on their route of administration (Figs 28-30). While few mDC were detected in the DLN after sc injection (Figs 28 and 30), a comparatively large number of mDC were identified in the spleen after iv injection (Figs 29 and 30). Both $CD8\alpha^+$ and $CD8\alpha^-$ mDC also clustered almost exclusively with host DC or T cells (Figs 28 and 29), with only a few $CD8\alpha^+$ mDC dispersed in areas other than T cell after sc injection (Fig 29 and data not shown). On the contrary, after iv injection into *plt* recipients, few, if any, mDC were found in spleen tissue sections, and consistent with the lack of normal T cell areas in *plt* mice (200), the DC did not appear to be localized specifically with any particular cell type. Interesting, what few DC were found were located frequently close to blood vessels, suggesting recent immigration. However, unlike in normal CCL19/CCL21-expressing recipients, there were no chemotactic "roadmaps" to direct the mDC to their "proper" areas of residency. As iv-administered mDC are carried to the spleen shortly after injection [at least as early as 6 h after injection (31)], and as we have established that mDC from normal B10 mice are capable of extravasation from the bloodstream into the spleen, it seems likely that mDC enter the spleen normally, but the deficit in CCL19 and CCL21 expression prevents their retention in the spleen. This proffered explanation correlates well with the failed retention of iDC [which purportedly do not express CCR7, as they do not respond to its ligands *in vitro* (Table 8 and Figs 11A and 14)] after iv injection (Figs 29 and 30).

Surprisingly, sc injection proved to be the more efficient route of administration of DC in *plt* recipients (Fig 32) as compared to iv injection (Fig 33), whereas the opposite was true in normal BALB/c recipients (Figs 28 and 29). Perhaps even more interestingly, while both mDC subsets

migrated (on average) in equal numbers to the DLN after sc injection in normal mice (Figs 29 and 30), almost no CD8α$^+$ mDC were detected in *plt* recipients by the same route of administration (Fig 32). Unlike iv injection (which deposits injected DC directly into the bloodstream), sc injection deposits injected DC into the tissue of the footpad. Thus the DC must traverse the peripheral tissue, reverse transmigrate lymphatic vessel walls, enter the LN, and then locate the T cell area. Though HEV express normal levels of CCL21 in *plt* mice (256), in the absence of concomitant expression of CCL19 and CCL21 in LN, this level may not be high enough to attract CD8α$^+$ mDC out of peripheral tissues to the DLN. Another possibility is that CD8α$^+$ mDC may be able reach lymphatic vessels, but again, the lack of CCL19 and CCL21 expression by DLN may prohibit retention of CD8α$^+$ mDC. A third possibility is that murine spleen mDC exhibit a differential expression of CXCR4. CXCR4 reportedly is expressed on mature monocyte-derived mDC (194, 315, 322) and on human blood-isolated CD11c$^+$ and CD123$^+$ iDC (193), but while neither CD11c$^+$ nor CD123$^+$ *ex vivo*-matured blood DC express CXCR4, CD11c$^+$ DC still exhibit migration to CXCL12, while CD123$^+$ DC do not. Thus suggesting a possible selective recruitment of CD8α$^-$ mDC to CXCL12-expressing HEV and DLN (342). Finally, if CD8α$^+$ mDC require two stimuli (chemokine and another unknown *in vivo* factor) to fully extend their dendrites, the low expression of CC19 and CCL21 may an impediment to their prolongation.

Finally, we investigated whether the number of DC injected affected their ability to be detected *in vivo*. Most of the groups injected 1 x 10^6 or *less* (52, 86, 304). We found that injecting less than 3 x 10^6 DC, regardless of route of administration, state of maturation, or subset, made detection of DC by immunohistochemistry or rare event analysis extremely difficult (data not

shown). Thus, it is possible that in experiments where CD8α$^+$ DC Ag presentation was evident, but the DC themselves were not detected in secondary lymphoid tissues (86, 304), simply may be due to the fact that the number of DC injected were below the threshold of detection of the method used.

To summarize, these data indicate that CD8α$^+$ mDC can traffic to secondary lymphoid tissues *in vivo*, and reveal that previous reports that they are incapable of doing so (86, 304) likely are due to a combination of lower number of DC injected and their state of maturation (iDC), as we have shown iDC migrate with poor efficiency regardless of subset (Figs 27 and 28). However, we also report for the first time that there is differential migration of mDC subsets in *plt* mice, possibly due to differential prolongation of dendritic extension.

5. CHAPTER FOUR[7]

THE ROLE OF DC RECRUITMENT TO SECONDARY LYMPHOID TISSUES IN THE OUTCOME OF ORGAN TRANSPLANTATION

In this chapter, we exploited the CCL19/CCL21-deficient *plt* mouse to assess the necessity for DC recruitment to secondary lymphoid tissue for (initiation of) graft rejection. As the absence of these two chemokines delayed, but did not prevent, (cardiac) allograft rejection, we also investigated whether administration of a blocking Ab directed against a T cell-specific chemokine (CXCL9) would have an additive or synergistic effect on graft survival. As considered in the Discussion, differences in mean survival times between different types of allografts may be related to the migration patterns seen for mDC in the *in vivo* trafficking studies summarized in Chapter 3. The results also raise questions about both the number of mDC required to activate a naïve T cell response against alloAg and the necessity for these interactions to occur in classic secondary lymphoid tissue T cell areas.

[7] Data are excerpted from (361) where indicated.

5.1. ABSTRACT

The spleen is the draining lymphoid tissue of heterotypically-transplanted intra-abdominal cardiac allografts (343). $CD8\alpha^+$ and $CD8\alpha^-$ mDC are poorly retained in the spleens of BALB/c *plt/plt* ($H2^d$) mice after iv injection, and those DC that do remain are not detected in T cell areas with high frequency. Dorsal skin transplants, however, drain to local peripheral LN as well as spleen (343). While $CD8\alpha^+$ mDC exhibit poor migration to DLN after sc injection in *plt* mice, $CD8\alpha^-$ mDC show no significant impairment (although they do not localize exclusively in T cell areas, as in wt recipients). As mDC/naïve T cell interactions are believed to be necessary for the initiation of allograft rejection, we employed these mice as the recipients of either heart or skin allografts from B10 ($H2^b$) donors to investigate the influence of impairment of mDC migration *in vivo* on allograft survival. Whereas heart grafts in *plt* recipients survived significantly longer as compared with those in wt control recipients, skin transplants were rejected with the same rapidity on *plt* as on wt. Anti-CXCL9 (monokine induced by INFγ; Mig) antiserum was then used to determine whether simultaneous impairment of activated T cell migration could further prolong cardiac allograft survival. Graft survival in *plt* mice after anti-CXCL9 treatment was extended significantly compared to that in untreated *plt* mice, as well as wt controls, but this strategy did not elicit indefinite allograft survival. It is likely that therapies involving chemokine blockade may be most effective in combination with other strategies.

5.2. INTRODUCTION

As discussed previously, chemokines are essential for the regulation of innate and adaptive immune responses (159, 344-347). The release of specific chemokines in response to "danger" signals, such as TNFα and IL-1, by injured endothelia (for example) results in the recruitment of specific immune effector cells to the site of inflammation. The expression of certain inflammatory chemokines by endothelial and immune (both innate and adaptive) effector cells has been linked to allograft rejection in various rodent models and humans (165, 222, 223, 231, 232, 234, 237, 249, 348-354). The CXC chemokine CXCL9, which specifically recruits activated T cells and is released in response to IFNγ expression, has been shown to be especially important in the rejection in murine heart and skin allografts (224, 225, 355). Treatment with anti-CXCL9 polyAb, but not anti-CXCL10 (IFNγ-inducible protein of 10 kDa; IP-10) [another T cell-recruiting, inflammatory chemokine thought to be important in acute rejection (165, 225, 228, 234, 355)], significantly prolongs skin (355) and heart graft (248) survival in MHC class II-disparate recipients.

CCL21 and CCL19 are CC chemokines expressed constitutively by HEV and lymphatic vessels (184) and by the endothelia of secondary lymphoid tissues (183, 208), respectively. These chemokines in particular aid in the regulation of immune responses through recruitment of both mDC from the site of inflammation and naïve and memory T cells from the blood to T cell areas of secondary lymphoid tissues for the initiation of adaptive immune responses (159, 184, 200, 311, 344, 346, 356-358).

We have determined that iv- or sc-injected CD8α$^+$ mDC are not retained in the T cell areas of either the spleen or DLN of *plt* mice (Figs 31 and 32). CD8α$^-$ mDC, on the other hand, while also not detected in the spleen after iv-injection into *plt* mice, appear to reach the DLN in normal numbers (by qualitative analysis; compare Figs 32 and 29) within 24 h after sc injection. Unlike in CCL19/CCL21-expressing recipients, these DC do not localize exclusively with T cells and were not observed 48 h after injection (Fig 31). We hypothesized that the impaired expression of CCL19 and CCL21, coupled with the absence of T cell areas in the spleen and the near total lack of DC and T cells in LN, would drastically reduce the number of alloAg-specific T cells that could reach the allograft post-transplant, allowing prolongation of allograft survival.

Here we show that while heart graft rejection in *plt* mice is significantly delayed in an MHC-mismatched model, the absence of CCL19 and CCL21 does not affect the rejection of skin allografts. Administration of anti-CXCL9 (polyAb) antiserum to *plt* heart allograft recipients further significantly prolonged graft survival, indicating that rejection is at least partially T cell-mediated, but this combination strategy was not sufficient to induce indefinite graft survival/tolerance.

5.3. MATERIALS & METHODS

5.3.1. Immunostaining of tissue sections

Tissue samples were embedded in Tissue-Tek OCT (Miles Laboratories, Elkhart, IN), snap-frozen in isopentane (pre-chilled in liquid N_2), and stored at -80°C until use. Eight μm cryostat sections were mounted on slides pretreated with Vectabond (Vector Laboratories Inc, Burlingame, CA), air-dried, and fixed in cold 4% PFA for 10 min. Sections were blocked with 5% v/v normal goat serum, followed by avidin blocking solution (Vector Laboratories Inc). Biotinylated anti-Ia^b (donor MHC II^+ cells) mAb (BD PharMingen) followed by Cy2-streptavidin (Jackson Laboratories) were used to detect donor DC. DAPI labeling was performed to nuclei identification.

5.3.2. Heterotopic heart transplantation

Surgical procedures were performed under inhalation anesthesia using methoxyflurane (Pitman-Moore, Atlanta, GA). Vascularized heterotopic cardiac transplants to an abdominal site were performed as described (359). Contraction of the donor heart was monitored daily by abdominal palpation. Total cessation of cardiac contraction was defined as rejection, and confirmed by histological analysis.

5.3.3. Skin transplantation

Skin grafting was performed as described (360). Briefly, square full-thickness skin grafts (1 cm^2) were prepared from the tail skin of donors. Graft beds (1 cm^2) were prepared on the right lateral thoracic wall of the recipient mice. The graft was fixed to the graft bed with eight interrupted sutures of 5-0 silk thread and covered with protective tape. The first inspection was conducted 7

days post grafting, followed by daily inspection. Grafts were considered as rejected at the time of 70% necrosis. Survival was expressed as the mean survival time ± 1 SD.

5.3.4. Anti-CXCL9 treatment

To test the role of CXCL9 in heart graft rejection in *plt/plt* mice, graft recipients received 0.5 ml aliquots of rabbit anti-CXCL9 antiserum (a kind gift from Dr. Robert Fairchild, Cleveland Clinic, OH) or normal rabbit serum (NRS) (Cedarlane Labs Limited, Hornby, Ontario, Canada) (as a control), ip. every other day from day 0 to day 9 and every 3 days from day 9 to day 14 post-transplantation, as previously described (248).

5.3.5. Statistical analysis

Graft survival data were compared by Kaplan-Meier analysis and the log-rank test. A "*P*" value < 0.05 was considered to be significant.

5.4. RESULTS

5.4.1. Heart, but not skin, allograft survival, is prolonged significantly in CCL19/CCL21-deficient recipients

Plt ($H2^b$) or wt control BALB/c ($H2^b$) mice received heterotopic heart transplants from B10 ($H2^d$) donors. Transplants in wt mice were rejected normally, with a mean survival time of 9.2 days. *Plt* recipients, however, displayed a modest but significant ($P < 0.05$) extension of graft survival, with a mean of 13.8 days (Fig 34A).

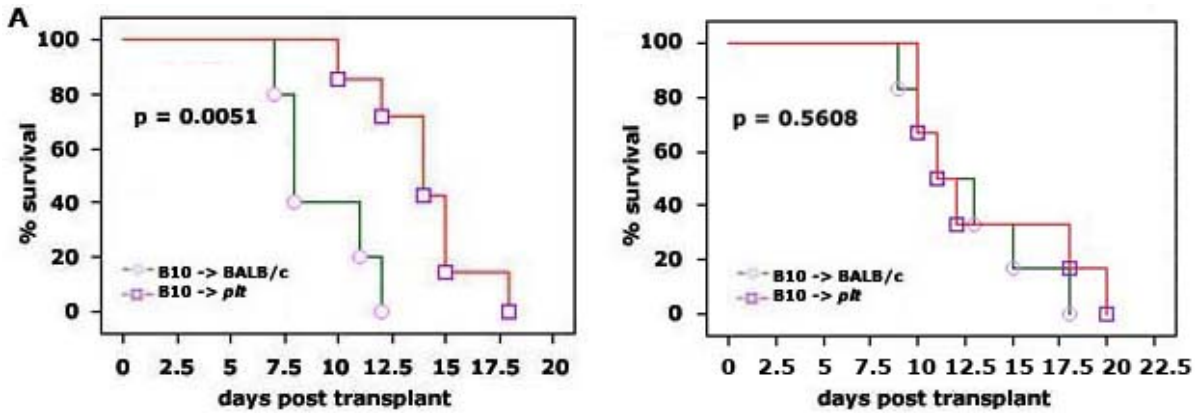


Fig 34. *Plt* recipients show significantly prolonged heart, but not skin, allograft survival.
(A) Heart allografts in *plt* recipients (squares) had a modest but significant increase in mean survival time as compared to wt BALB/c recipients (circles). (B) Skin grafts were rejected with equal rapidity by both *plt* (squares) and wt BALB/c (circles) recipients (361).

Plt and wt mice were then transplanted with trunk skin from B10 mice to ascertain whether this prolongation of graft survival could be extended to non-vascularized tissues. Skin graft survival was monitored daily, with 70% necrosis considered rejection. Skin transplants on experimental and wt recipients were rejected with nearly equal rapidity **(**12.3 days : wt vs 13.3 days : *plt*) (Fig 34B).

5.4.2. MHC II^+ donor cell migration to the spleen is impaired in *plt* heart, but not skin, allograft recipients

Migration of both mDC subsets to the spleen after their iv injection was impaired significantly in *plt* mice, but $CD8\alpha^-$ mDC appeared to traffic normally to DLN after sc injection (Figs 32 and 33). Thus it is conceivable that alloAg-laden (donor) DC from skin grafts on *plt* recipients traffic to DLN in numbers comparable to those emigrating from skin allografts on wt BALB/c recipients, while fewer DC overall may be successful in trafficking to the spleen from hearts transplanted in *plt* mice (as compared to wt control recipients). Therefore, we considered whether numbers of donor DC reaching the lymphoid tissue might be a factor in the difference in rejection time between skin and heart transplants. We excised the spleens and DLN of skin and heart recipient *plt* mice 48 h after transplant. In *plt* recipients of heart transplants, there were qualitatively fewer donor MHC II^+ cells in both the spleen and DLN compared to wt BALB/c recipients (Fig 35A and B). In both *plt* and wt skin transplant recipients, however, similar numbers of donor MHC II^+ cells were detected in the spleen (Fig 35C) and DLN (Fig 35D), and more donor (MHC II^+) DC were detected in *plt* skin than in heart recipient secondary lymphoid tissues (Fig 35).

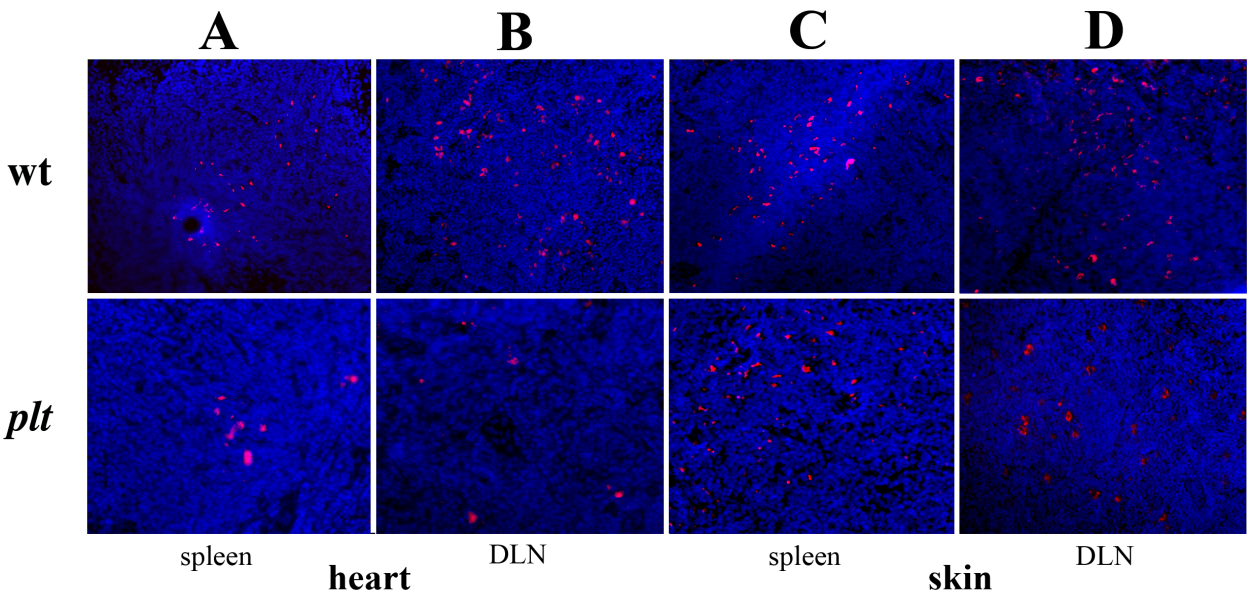


Fig 35. MHC II$^+$ cells in *plt* and wt BALB/c recipients of heart and skin allografts.
Spleens (A and C) and DLN (B and D) of heart (A and B) or skin (C and D) allograft recipients were removed 48 h after transplant, snap frozen, sectioned, and stained for donor MHC II (IA^b; red) and nuclei (DAPI; blue). Considerably fewer MHC II$^+$ cells were detected in draining lymphoid tissues of *plt* heart allograft recipients as compared to wt (A and B). In contrast, a drastic increase in number of MHC II$^+$ cells were detected in secondary lymphoid tissues of *plt* skin allograft recipients, with comparable MHC II$^+$ cells visualized in both the spleen and DLN of wt recipients. Hearts were still beating and skin allografts showed no signs of necropsy in all/any of the recipients at the time of sacrifice for donor MHC II$^+$ cell detection. 200x (361).

5.4.3. Impairment of activated T cell migration is effective in prolongation of heart allograft survival only in combination with deficient DC trafficking

CXCL9 has been reported by several groups to be expressed during the rejection of skin and heart transplants. CXCL9 is secreted by allograft endothelial cells and recipient graft-infiltrating Mϕ and neutrophils as early as two days posttransplant in response to IFNγ secretion (220, 225, 248, 355). Koga *et al* (220, 355) have reported that administration of anti-CXCL9 antiserum to skin transplant recipients significantly extends allograft survival in MHC class II disparate models. Further, other studies by the same group have revealed that the same therapy is equally effective in graft survival prolongation in a heterotopic heart transplant model (248). Since, in

the present study, graft survival was not indefinite in *plt* heart allograft recipients, we thus evaluated whether the additional impairment of effector T cell recruitment to the allograft could further enhance the delay in heart allograft rejection in *plt* recipients. Anti-CXCL9 antiserum was administered to heart allograft recipients on the day of transplant, every other day for the first week, and then every third day for the second week. *Plt* mice received either anti-CXCL9 antiserum or normal rabbit serum (NRS) as a control. Additionally, as studies with the antiserum originally were conducted with a different strain combination [A/J ($H2^a$) cardiac grafts into C57BL/6 ($H2^b$); performed by Miura *et al* (248)], wt BALB/c heart allograft recipients also received either anti-CXCL9 antiserum or NRS to control for the effect that inhibition of activated T cell migration alone would have on allograft survival (Fig 36).

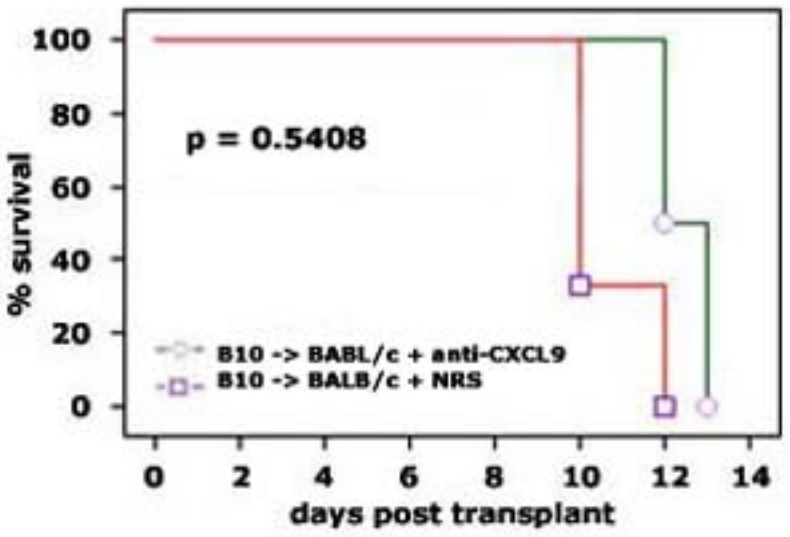


Fig 36. Impairment of activated T cell migration alone does not delay heart allograft survival in wt BALB/c recipients.
Heart allografts in BALB/c recipients administered anti-CXCL9 had a minimal increase in mean survival time as compared to control antiserum (normal rabbit serum; NRS)-treated recipients (361).

Anti-CXCL9 antiserum did not extend heart graft survival in wt BALB/c recipients significantly compared to untreated wt BALB/c recipients. Dual inhibition of activated T cell (anti-CXCL9 antiserum) and mDC (*plt* recipients) migration further enhanced the survival of donor hearts, but again the grafts were not accepted permanently (*plt* alone mean 13 days vs *plt* + anti-CXCL9

mean 21 days). Mice that received NRS treatment rejected with the same time course as mice that received no treatment (Fig 37).

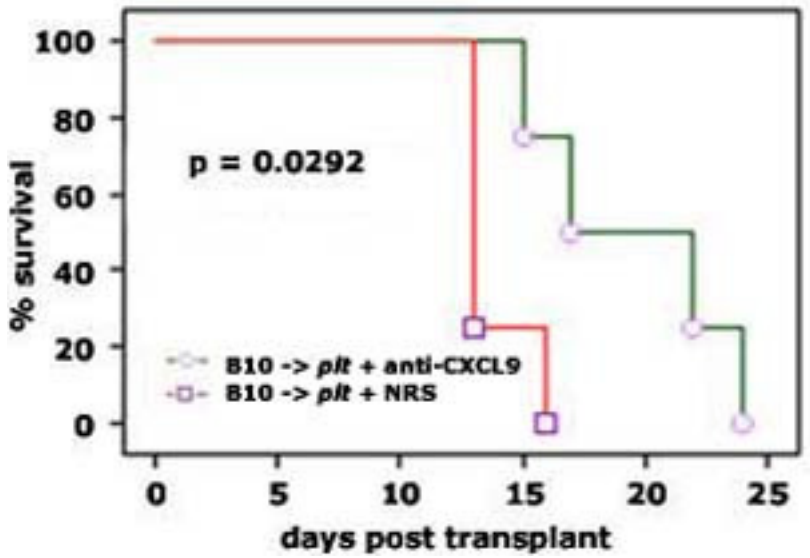


Fig 37. Dual impairment of mDC and effector T cell migration further significantly prolongs heart allograft survival in *plt* recipients.
Heart allografts in *plt* recipients administered anti-CXCL9 had a significant increase in mean survival time as compared to *plt* recipients treated with control polyAb (normal rabbit serum; NRS) (361).

5.5. DISCUSSION

We have investigated the roles of specific chemokines in promoting the immune response against vascularized and non-vascularized allografts. CCL19 and CCL21 are key to the recruitment of mature DC to secondary lymphoid tissues and are important in localizing Ag-laden DC in T cell areas. CXCL9 is expressed early in the rejection process, eliciting activated T cell migration to the graft. Here we have shown that the near-absence of CCL19 and the total lack of CCL21 in secondary lymphoid tissues (*plt* mice) is enough to prolong allograft survival, but does not completely attenuate the rejection process (Fig 34). Although impaired mDC migration in *plt* mice did not result in indefinite graft survival, blocking of activated T cell migration alone (CXCL9 antagonist) had no effect whatsoever on graft survival (Fig 36). However, combining inhibition of activated T cell recruitment with reduced mDC migration (as occurs in *plt* mice) further prolonged transplant survival, although this too was not enough to prevent eventual rejection (Fig 37).

Plt mice infected with MHV succumb to the virus at an LD_{50} that is 300-fold lower than that for wt BALB/c mice (LD_{50} of 5.67 pfu for *plt* mice vs LD_{50} of 1840 pfu for BALB/c) (200). However, when *plt* mice are immunized sc, they have a delayed but hyper response to the viral Ag (334). In these studies, DC and T cells were unable to colocalize in conventional T cell areas, but instead came together in the superficial cortex of LN and in splenic bridging channels (334). It is possible, then, that the recruitment of mDC by CCL21-leu expressed by lymphatic vessels allows co-localization of alloAg-bearing mDC with naïve alloAg-specific T cells outside "classical" T cell areas, thus initiating allograft rejection with equal rapidity to wt recipients.

Another possibility as to why heart graft rejection in *plt* recipients is delayed and skin allografts are rejected with normal speed may have to do with the number of cells required to initiate and mediate graft rejection. While only a few DC bearing alloAg may reach naïve spleen T cells capable of recognizing alloMHC, the fact that T cells in *plt* mice have been reported to have delayed but enhanced proliferation in response to specific Ags (334) suggests that only a few DC may be required to initiate the process. The number of dermal and epidermal "passenger" DC may simply be more than enough to initiate rejection. Heart allografts may have a lower incidence of heart "passenger leukocytes", and therefore, their rejection may be mediated by recipient DC. This also could account, at least partially, for the delay in rejection, as DC would first have to be recruited to the graft, mature, and then "find" alloAg-specific naïve T cells without the aid of "classic" T cell areas. The lower incidence of donor (MHC II^+) DC detected in secondary lymphoid tissues of *plt* heart allograft recipients (Fig 35A and B) versus the apparently normal numbers detected in wt recipients (Fig 35A and B) appears to be consistent with this theory. In addition, comparable numbers of MHC II^+ donor cells were detected in the DLN of both wt and *plt* skin allograft recipients (Fig 35D), suggesting equal ability of donor DC to emigrate out of skin transplants, enter lymphatics, and reach T cell areas [consistent with our *in vivo* migration data for $CD8\alpha^-$ mDC migration after sc injection (Fig 32)].

Lakkis *et al* (343), have reported that murine cardiac allografts can be rejected with either intact spleen or LN as the draining lymphoid tissue(s), while skin allografts rely solely on T cells from DLN (in the absence of LN, spleen T cells alone cannot reject skin allografts). Alymphoplastic mice have been reported to have a slight delay in cardiac allograft rejection compared to asplenic mice (343), perhaps similar to *plt* recipients which exhibited lower numbers of donor (MHC II^+)

DC in both their spleen and LN (Fig 35), but had significantly delayed rejection (compared to wt recipients) (Fig 34). Conversely, asplenic skin allograft recipients reject their grafts normally, but alymphoplastic do not (343). That *plt* recipients have similar numbers of detectable donor (MHC II^+) DC in their LN (as compared to wt) (Fig 35) and reject their skin allografts with equal rapidity to wt controls also is perhaps similar in mechanism to the Lakkis data.

In skin allograft recipients, we detected similar numbers of donor MHC II^+ cells in the spleen of *plt* and wt mice. This appears to be inconsistent with our *in vivo* trafficking experiments (Fig 33), as neither subset of mDC efficiently migrated to the spleen after iv injection. As we did not examine the spleen for CMFDA-labeled DC after sc injection into either *plt* or wt recipients, we cannot completely rule out the possibility that sc-injected/skin allograft donor mDC traffic to both the spleen and DLN 48 h after injection/transplant. In our experiments with hepatic mDC (52), we found that DC, though few, were detected in the spleen after sc injection.

It is possible that the donor MHC II (Ia^b) detected in the spleen and DLN of the allograft recipients (Fig 35) are not themselves donor DC, but only donor MHC II taken up and transported to secondary lymphoid tissues by recipient DC (Ia^d). However, as allografts are rejected by both the direct (donor DC alloAg presentation) and indirect (recipient DC alloAg presentation) pathways, that fewer MHC II-alloAg-bearing recipient DC are found in *plt* heart allograft recipients at 48 h than wt or *plt* skin allograft recipients still does not exclude the possibility that the delayed heart rejection of *plt* recipients is at least partly mediated by a delay in presentation of alloAg to recipient alloreactive T cells.

As DC and T cells were found to assemble in "non-classical" T cell areas in MHV-infected *plt* mice (334), it would have been informative to localize MHC II^+ donor cells with recipient B and T cell, Mϕ, and DC as we have done previously in this work. However, due to the limits in available primary and secondary Abs, as well as the animals from which the antibodies were derived, we were unable to coordinate a combination that would allow dual-staining of both Ia^b and localizing Abs.

It may be that impairment of mature DC migration from the graft is useful during the early "danger" phases of rejection, and that combination with other therapies such as suppression of T cell proliferation (e.g., rapamycin) may allow the inflammatory phase to pass, permitting any presentation of alloAg after inflammation to be treated as other "self" Ag, rendering the immune system tolerant to the foreign tissue. The extended graft survival seen coupling blockade of T cell migration with deficient DC (and naïve T cell) migration further confirms that regulation of leukocyte migration may be a valuable approach to preventing rejection. Further experimentation in this area likely also will reveal that effective DC/T cell migration targeting strategies will require allograft specificity, as we have shown leukocytes likely have unique trafficking patterns to and from different allografts (Fig 35). Thus, it is likely that DC- and T cell-targeted therapies will work best in combination, and may need to be regulated according to allograft model as well as in conjunction with conventional anti-rejection treatment.

6. SUMMARY

DC are the only APC to date reported to upregulate CCR7 expression in response to inflammatory signals, and they are the only APC capable of activating naïve T cells. Thus, the migration of Ag-bearing DC from the periphery to secondary lymphoid tissues is critical for both the initiation of an immune response and the maintenance of tolerance to self Ags. The CD8α-expressing DC subset originally was purported to exhibit tolerogenic effects through Fas-mediated ($CD4^+$ T cells) (83) and IL-2-deprivation-mediated ($CD8^+$ T cells) (84) apoptotic cell death. While these observations were not borne out by subsequent experiments by other investigators, the expression of IDO by $CD8\alpha^+$ DC (305), their ability to prolong allograft survival even when mature (47), and their superior ability (compared to $CD8\alpha^-$ DC) to cross present Ag from apoptotic bodies for induction of T reg cells (32) suggests that $CD8\alpha^+$ DC do possess some tolerogenic abilities, although the mechanisms by which they exert them are yet unknown (and maybe be specific to each system). These studies provide ample material with which to devise methods to manipulate $CD8\alpha^+$ DC migration, in addition to having established a protocol for future studies with other DC of interest (i.e., the plasmacytoid or regulatory DC, the migratory patterns of which are understudied).

We have determined herein that while $CD8\alpha^+$ mDC exhibit a decreased ability to migrate to CCR7 ligands *in vitro* compared to $CD8\alpha^-$ mDC, both subsets migrate with equal agility to T cell areas of secondary lymphoid tissues *in vivo* (in normal wt recipients), regardless of route of injection. On the contrary, neither spleen iDC subset migrated in response to any of the CC chemokines tested (despite mRNA expression and protein production for several CCR) either *in*

vitro or *in vivo*. Thus it is indeed likely disparate reports regarding the ability of CD8α$^+$ DC to migrate *in vivo* are linked to their state of maturation rather than their phenotype.

Interestingly, we found that although mDC traffic with equal competence in normal wt BALB/c recipients, when mice deficient in lymphoid tissue CCL19 and CCL21 expression are used as recipients, the difference seen in DC subset migration *in vitro* is apparent *in vivo*. We found that while both mDC subsets migrated to/remained in T cell areas of the spleen in low numbers after iv injection, only CD8α$^+$ mDC were impaired in their ability to reach/be retained within lymph nodes draining the footpad of *plt* mice. These data, coupled with the poor prolongation of dendritic extensions of CD8α$^+$ mDC in response to CCL19 and CCL21, suggest that these DC may require both ligation of CCL19 and/or CCL21 by CCR7 as well as other as yet unknown factors found only *in vivo* to elicit full extension of their dendrites for complete facilitation of transmigration. As 100 nM of chemokine is a physiologically high concentration, we did not carry out our *in vitro* chemotaxis or Ca^{++} studies with concentrations of 1000 nM or above. Had we done so, we may have seen an improved chemotactic/morphologic response in CD8α$^+$ mDC. The lack of a reliable anti-mouse CCR7 mAb also prevented any conclusive data regarding the expression of CCR7 by these DC. The advent of this, as well as mAb against the other mouse CR, will provide valuable insight into the trafficking patterns of i and mDC from the various mouse tissues from which they may be isolated.

Despite the low expression of CD11b on CD8α$^+$ DC and its reported importance in facilitating DC adhesion to (and eventual transmigration through) EC in humans (278, 283), blocking of this adhesion molecule on CD8α$^-$ DC did not appear to effect their transendothelial migration *in*

vitro. Nor did any of the adhesion molecules blocked in our experiments show a selective importance for murine spleen DC subset migration [blocking of CD54 elicited in a similar inhibition of $CD8\alpha^-$ and $CD8\alpha^+$ mDC transendothelial migration (Fig 24)], in contrast to the differences reported (205) in human blood-isolated DC subsets. However, in those reports, the DC subsets were "myeloid" ($CD11c^+CD123^-$) and plasmacytoid ($CD11c^-CD123^+$) DC – it is possible murine plasmacytoid spleen DC will have different reliance on adhesion molecules than their $CD8\alpha^+$ and $CD8\alpha^-$ counterparts. It is also possible [and more likely, given that murine spleen pDC have similar chemotactic abilities to murine $CD8\alpha^-$ and $CD8\alpha^+$ DC as reported by Abe *et al* (54)] that more extensive investigations of the ability of murine blood i and mDC to migrate through EC will show different adhesion molecule requirements than do murine spleen DC, and these may be more similar to human blood DC. Indeed, with more investigations of DC from various tissues, it is probable that DC migratory requirements (i.e., chemokines which elicit their migration and essential adhesion molecules) will be found to be linked to their method of generation/tissue of isolation.

That $CD8\alpha^-$ DC showed no impairment in their ability to migrate to DLN after sc injection into the footpads of *plt* recipients may in part explain why skin transplants on CCL19/CCL21 deficient mice rejected their allografts with the same rapidity as normal BALB/c recipients, as to date $CD8\alpha^+$ DC have not been identified in peripheral tissues [without addition of an exogenous growth factor (52, 190)]. Additionally, the increased number of donor (MHC II^+) DC present in secondary lymphoid tissues of *plt* skin versus heart recipients (the number of donor DC detected in *plt* skin recipients is qualitatively comparable to the numbers detected in wt skin recipients) is consistent with this theory. The delayed cardiac allograft rejection witnessed in *plt* recipients was

further prolonged with the addition of an activated T cell migratory blocking antiserum (e.g. anti-CXCL9 Ab), suggesting that impairment of both mDC as well as activated T cell trafficking may act synergistically to the benefit of the patient, especially in combination with conventional pharmacologic immunosuppressants.

These studies have brought to light several novel findings that will further our understanding of DC immunobiology: (i) unlike *in vitro*-derived and blood human and mouse iDC populations, freshly-isolated mouse spleen iDC do not respond to inflammatory CC chemokines, as determined both by chemotaxis and Ca^{++} flux assays; (ii) unlike human monocyte-derived and blood DC, mouse splenic mDC utilize the adhesion molecule CD54, but not CD11b, CD31, or CD62L, for their transendothelial migration; (iii) thus, the reduced migration of $CD8\alpha^{+}$ DC observed both *in vitro* and *in vivo* does not appear to be due to their low expression of CD11b, but may be related to the expression of functional CCR7/poor ability to lengthen their dendritic extensions; (iv) DC trafficking to T cell areas *in vivo* is dependent on the state of maturation, the route of administration, and the number of the injected DC; (v) mDC subset migration to T cells of secondary lymphoid tissues is directed, at least in part, by CCL19 and CCL21, as evidenced by their reduced detection in these areas in spleen and DLN of *plt* mice; (vi) despite their inability to form proper T cell areas, endogenous DC subsets are found in the normal ratio in the spleen and LN of *plt* mice; (vii) an impaired ability of alloAg-bearing DC to reach T cell areas of secondary lymphoid tissues post transplant can delay, but not prevent mouse cardiac allograft rejection; (viii) the lack of significant impairment of donor MHC II^{+} cells to the spleen and DLN in skin allograft transplantation offers one explanation as to why skin allografts on *plt* recipients are rejected with the same rapidity on normal recipients; (ix) the conflicting reports regarding the

ability of CD8α$^+$ DC to migrate *in vivo* indeed are likely to be reflective of differences in protocols between groups, as (with the exception of migration to DLN after sc injection into *plt* mice) in our hands, CD8α$^+$ and CD8α$^-$ DC appear to have similar trafficking ability *in vivo*.

Thus the experiments discussed herein have provided insight into the field of DC/chemokine immunobiology. These studies have provided the impetus for investigations in our own laboratory of the migration of (CD8α$^-$, CD8α$^+$, and B220$^+$) DC subsets directly isolated from the liver (54), kidney (42, 190), and blood (Yuk Yuen Lan, unpublished observations). Our discovery that tissue-isolated iDC do not exhibit as pluripotent an ability to respond to inflammatory CC chemokines as previously reported *in vitro*-generated iDC, as well as the preliminary investigations of the essential adhesion molecules necessary for mDC transendothelial migration, will aid future studies regarding the manipulation of specific i and mDC subsets. Further, our investigations regarding the importance of CCL19 and CCL21 in facilitating mDC migration *in vivo* and the impact that the lack of ability of mDC to migrate properly in the absence of their expression has on allograft survival, commence the first steps in understanding DC/chemokine immunobiology in the field of transplantation.

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