The chemokine CX3CL1 promotes trafficking of dendritic cells through inflamed lymphatics

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Summary

Tissue inflammation is characterized by increased trafficking of antigen-loaded dendritic cells (DC) from the periphery via afferent lymphatics to draining lymph nodes, with resulting stimulation of ongoing immune responses. Transmigration across lymphatic endothelium constitutes the first step in this process and is known to involve the chemokine CCL21 and its receptor CCR7. However, the precise details of DC transit remain obscure and it is likely that additional chemokine-receptor pairs have roles in lymphatic vessel entry.

Here, we report that the transmembrane chemokine CX3CL1 (fractalkine) is induced in inflamed lymphatic endothelium, both in vitro in TNF-α-treated human dermal lymphatic endothelial cells (HDLEC) and in vivo in a mouse model of skin hypersensitivity. However, unlike blood endothelial cells, which express predominantly transmembrane CX3CL1 as a leukocyte adhesion molecule, HDLEC shed virtually all CX3CL1 at their basolateral surface via matrix metalloproteinases. We show for the first time that both recombinant soluble CX3CL1 and endogenous secreted CX3CL1 promote basolateral-to-luminal migration of DC across HDLEC monolayers in vitro. Furthermore, we show in vivo that neutralizing antibodies against CX3CL1 dramatically reduce allergen-induced trafficking of cutaneous DC to draining lymph nodes as assessed by FITC skin painting in mice. Finally, we show that deletion of CX3CL1 receptor in CX3CR1−/− DC results in markedly delayed lymphatic trafficking in vivo and impaired translymphatic migration in vitro, thus establishing a previously unrecognized role for this atypical chemokine in regulating DC trafficking through the lymphatics.
Introduction

Trafficking of dendritic cells (DC) from tissues to draining lymph nodes is required to maintain normal immune surveillance and peripheral tolerance (Scheinecker et al., 2002; Steinman et al., 2003). Following infection or the induction of inflammation, bacterial endotoxins and pro-inflammatory cytokines induce maturation of DC from phagocytes to professional antigen-presenting cells and migration from the tissue into afferent lymphatic capillaries (Steinman et al., 2003). This transmigration across the lymphatic endothelium is one of the most critical steps in initiating an immune response. It is well-documented that the lymphatic endothelial-expressed chemokine CCL21 is required for chemotaxis of DC to lymphatic vessels during both resting conditions and in inflammation, through binding its cognate receptor CCR7 ( Förster et al., 1999; Gunn et al., 1999; Ohl et al., 2004; Saeki et al., 1999). Indeed, chemokine-directed, integrin-independent migration, a process termed amoeboid movement, has been proposed as the main mechanism controlling lymphatic entry of DC, at least in uninflamed tissue (Lammermann et al., 2008). However, upon the onset of inflammation, when increased production of CCL21 in activated lymphatic endothelium (Johnson and Jackson, 2010; Martín-Fontecha et al., 2003) is accompanied by induction of leukocyte adhesion molecules including ICAM-1 and VCAM-1 (Johnson et al., 2006), DC can transmigrate through conventional integrin-dependent mechanisms.

It is becoming increasingly clear that CCL21 is not the only chemokine that controls leukocyte trafficking in the lymphatics. Monocyte-derived DC have been shown to use CCR8 in addition to CCR7, during migration from the tissues to lymph nodes (Qu et al., 2004). In addition, a role for CXCR4 and its ligand CXCL12 (SDF-1) has been reported for lymphatic migration of both dermal DC and Langerhans cells (LC) in skin hypersensitivity responses (Kabashima et al., 2007). Interestingly, the chemotactic effects of CXCL12 and CCL21 were reported to be non-additive, indicating either a sequential mode of action or multiple chemokine redundancy. In this present study, we sought to identify further chemokines that might contribute to lymphatic entry, focusing primarily upon CX3CL1 (fractalkine).

Expressed in a number of cell types including neurons, intestinal epithelium and activated vascular endothelium (Bazan et al., 1997; Pan et al., 1997), CX3CL1 is structurally distinct from other chemokines in several respects. Unlike most chemokines, CX3CL1 is synthesized as a large, type I integral membrane protein of 373 amino acids, comprised of an
extracellular domain containing a novel C-X-X-X-C chemokine motif and an extended mucin-like stalk (Bazan et al., 1997). This membrane-anchored form of CX3CL1 was originally shown to induce tight, shear-resistant endothelial adhesion of leukocytes in a manner that was apparently independent of integrin and Gi protein activation (Bazan et al., 1997; Fong et al., 1998; Haskell et al., 1999; Imai et al., 1997; Viemann et al., 2004). Subsequently however, soluble forms of CX3CL1 generated by proteolytic cleavage with the disintegrin and metalloproteinases ADAM10 and ADAM17 were shown to promote conventional chemotaxis (Garton et al., 2001; Hundhausen et al., 2003). The functional differences between membrane-bound and soluble CX3CL1, and their respective roles in inflammation remain obscure (Garton et al., 2006; Goda et al., 2000; Umehara et al., 2001).

The sole receptor for CX3CL1, CX3CR1, is widely expressed by leukocytes, including CD14+ cells of the monocyte/macrophage/DC lineage and subsets of tissue resident DC and epidermal Langerhans cells (Geissmann et al.; Imai et al., 1997; Jung et al., 2000). In addition to supporting leukocyte extravasation from the blood (Rao et al., 2007), the CX3CL1/CX3CR1 axis has been implicated in pathogenesis of inflammatory diseases such as atherosclerosis, rheumatoid arthritis and renal fibrosis - seemingly through supporting monocyte recruitment and exacerbating tissue damage (Moatti et al., 2001; Tacke et al., 2007; White and Greaves, 2012). Curiously, CX3CL1 was also shown to promote extension of transepithelial dendrites by monocytes and/or DC within intestinal lamina propria, for the purpose of antigen sensing in the intestinal lumen (Muehlhoefer et al., 2000). However, the significance of the CX3CL1/CX3CR1 axis for control of DC migration in lymph has yet to be established. Although the original characterization of constitutive CX3CR1 knock-out mice concluded no apparent defects in immune function, the specific role of CX3CL1 and its receptor in regulating lymphatic trafficking was not explored in detail (Jung et al., 2000).

In this present study, we show for the first time that CX3CL1 is actively secreted by lymphatic endothelial cells in response to inflammation and that soluble rather than membrane-anchored chemokine attracts DC migration towards lymphatic vessels and lymphatic endothelial transmigration, both in vitro and in vivo.
**Results**

CX3CL1 is dramatically induced in HDLEC after stimulation with pro-inflammatory cytokines

To investigate expression of CX3CL1 in lymphatic endothelium, early passage human dermal lymphatic endothelial cells (HDLEC), supplementary figure 1, typically 3 passages from resection, were cultured in the presence or absence of a panel of pro-inflammatory cytokines and assayed by ELISA for soluble and cell-associated chemokine, in tissue culture supernatant and detergent lysates respectively. Resting HDLEC were found to secrete little if any CX3CL1 (Figure 1A-C) whereas stimulation with TNF-α induced a dramatic increase (typically 100 ng/ml). By comparison, IL-1α, IL-1β, IFN-γ and LPS induced more modest up-regulation (15-30 ng/ml) and IL-6 had no effect. Similar degrees of induction of cell-associated CX3CL1 were observed when assessed in detergent-lysed HDLEC (Figure 1C and data not shown). To characterize the mode of regulation, we compared CX3CL1 levels in HDLEC stimulated with TNF-α, either alone or in the presence of the RNA polymerase inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide. As shown in Figure 1B and C, both agents completely suppressed CX3CL1 production, consistent with transcriptional regulation as opposed to mobilization of pre-packaged chemokine. This was supported by reverse transcriptase PCR analyses, which detected abundant CX3CL1 message in RNA from TNF-α-treated but not control HDLEC, (Figure 1D).

Membrane-anchored CX3CL1 is immediately shed from the surface of activated HDLEC.

To further confirm induction of CX3CL1 in activated HDLEC, intact control and TNF-α-stimulated cells were imaged after staining with fluorescently labelled antibodies. Curiously, no CX3CL1 was detected on the cell surface, regardless of whether HDLEC were stained as adherent monolayers or detached, as single cell suspensions (Figure 2A, B). Significantly however, permeabilization with saponin revealed diffuse intracellular CX3CL1 staining in a proportion of TNF-α-stimulated cells (Figure 2A), indicating either that the protein is not targeted to the plasma membrane or that it traffics to the plasma membrane and is then shed rapidly. To distinguish between these possibilities, TNF-α-stimulation was repeated in the presence or absence of the broad-range matrix metalloproteinase inhibitor, Ilomastat (GM6001) to block sheddase activity. As shown in Figure 2A-C and Supplementary Figure 2,
the inclusion of inhibitor led to significant accumulation of CX3CL1 at the cell surface of individual HDLEC and also reduced the amount secreted into the culture media (Figure 2D). These results indicate that the soluble form of CX3CL1 is shed from the HDLEC surface almost immediately after synthesis.

**CX3CL1 shedding is mediated by ADAM10/17**

Next, we sought to identify the metalloproteinases that mediate CX3CL1 shedding. Previously, ADAM10, ADAM17 and MMP2 have been shown to mediate both constitutive and activation-induced shedding of CX3CL1 from vascular endothelial cells (HUVEC), transfected fibroblasts, and hepatic stellate cells (Bourd-Boittin et al., 2009; Garton et al., 2001; Goda et al., 2000; Hundhausen et al., 2003). Incubation of TNF-α-stimulated HDLEC monolayers with the ADAM10/17 inhibitors GI254023X and GW280264X revealed a concentration-dependent inhibition of CX3CL1 secretion (Figure 3A and Supplementary Figure 3). Importantly, neither inhibitor significantly affected HDLEC viability or secretion of the cleavage-independent chemokine CCL2 (MCP-1), which served as a control (Figure 3B). In addition, MMP2/9 inhibitor II and MMP8 inhibitor I also reduced CX3CL1 shedding although to a lesser extent, (approximately 25%), than GI254023X and GW280264X (Figure 3C), suggesting that ADAM10 and ADAM17 are the prime sheddases involved in endogenous secretion of CX3CL1 from HDLEC.

**CX3CL1 is shed predominantly from the basolateral surface of HDLEC monolayers.**

To determine whether secretion of CX3CL1 was polarized, HDLEC monolayers cultured on transwell inserts were stimulated with TNF-α and chemokine secretion measured from both luminal and basolateral surfaces (i.e. top and bottom chambers) by ELISA. Strikingly, CX3CL1 was secreted predominantly (> 70%) from the basolateral surface (Figure 4A). In contrast, secretion of the chemokines CCL5 (RANTES) and CCL2, both of which are induced in activated HDLEC (Johnson et al., 2006), was almost exclusively luminal, (> 90%), (Figure 4B, C). Furthermore, dual fluorescence staining of permeabilized HDLEC with the marker von Willebrand Factor (vWF) showed essentially no CX3CL1 within Weibel-Palade bodies - the organelles associated with luminal secretion of chemokines such as CXCL8 (IL-8) in blood endothelial cells (Wolff et al., 1998), (Figure 4D, E). These findings suggest that in vivo, LEC-derived CX3CL1 functions perivascularly, influencing events directly adjacent to rather than on the inner surface of lymphatic vessels. This would
distinguish CX3CL1 from chemokines such as CCL2 that are secreted in lymphatic vessels for remote functions in downstream lymphoid tissue (Palframan et al., 2001).

**CX3CL1 is expressed in inflamed lymphatic vessels in vivo.**

To assess CX3CL1 expression in intact lymphatic endothelium, we performed immunostaining of frozen sections from both freshly resected and TNF-α-treated human dermis after permeabilization (Figure 5). No CX3CL1 was detected within resting podoplanin+ lymphatics in normal skin. In contrast, high levels were detected in lymphatic vessel endothelium of TNF-α-treated skin, in intracellular patches that were visible only after permeabilization of the sections. Significantly, no CX3CL1 appeared to co-localise with podoplanin at the surface of lymphatic endothelium. Next, we investigated CX3CL1 induction in lymphatic endothelium within oxazolone contact hypersensitized skin from both BALB/c and C57Bl/6 mice, and in explanted TNF-α-treated mouse dermis. As shown in Figure 6A and Supplementary Figure 4, CX3CL1 staining was detected in podoplanin+ lymphatic vessels in both cases, but only when tissue had been permeabilized with Triton X-100 prior to staining. In contrast, CX3CL1 could be readily detected on podoplanin+ blood capillaries in non-permeabilized tissue (Figure 6B). Furthermore, CX3CL1 in inflamed lymphatic endothelium could be seen in a perinuclear pattern, although in some sections residual amounts appeared also to be present in the vessel lumen (Figure 6B, lower left panel). No significant amount could be detected near the subendothelial surfaces of lymphatic vessels, in contrast to reports of this location for CCL21 (Tal et al., 2011; Weber et al., 2013) and consistent with the fact that CX3CL1 does not bind heparin and acts as a fluid phase rather than an immobilized chemokine (Patel et al., 2001). In addition to CX3CL1, oxazolone sensitization also led to up-regulation of CCL5 and CCL20, which were concentrated around podoplanin+ lymphatic vessels, and CCL2, which had a more diffuse pattern within the tissue (Supplementary Figure 5). These results show for the first time that CX3CL1 is induced as part of a pleiotropic response by lymphatic endothelium to inflammation in both mice and humans, and confirm that the molecule is stored intracellularly in preparation for secretion, rather than retained at the cell surface, both *in vitro* and *in vivo*.

**CX3CL1 promotes polarized transmigration of DC across lymphatic endothelium.**

The observed induction and polarized secretion of CX3CL1 is clearly suggestive of a role in directing leukocyte chemotaxis and/or entry to lymphatic vessels. To explore this possibility, we focused on trafficking of DC, the major migratory leukocyte population in afferent lymph,
using as a model human blood monocyte-derived DC (MDDC) induced to mature by
treatment with LPS. Both immature and mature MDDC express significant levels of CX3CL1
receptor, CX3CR1, assessed by quantitative Western analysis and flow cytometry (Figure
7A, B), in addition to the critical lymphoid chemokine receptor CCR7 (Figure 7C). We first
measured the capacity of exogenously added CX3CL1 to stimulate basolateral-to-luminal
migration of fluorescently-labelled MDDC across monolayers of HDLEC plated on the
under-surface of transwell FluoroBlok™ membranes (Johnson et al., 2006). As shown in
Figure 7D, addition of CX3CL1 to the upper chamber significantly increased both the
number of transmigrating MDDC and the rate of transmigration compared with
unsupplemented medium. Next, to assess the contribution of endogenous, activation-induced
CX3CL1 to DC transmigration, we carried out assays using TNF-α-stimulated HDLEC, in
the presence of either a CX3CL1 neutralizing antibody or irrelevant rabbit IgG added to the
upper chamber. As shown in Figure 7E, neutralizing antibody reduced DC transmigration by
approximately 50%, confirming the proposed role for HDLEC-expressed CX3CL1 in
mediating inflammatory MDDC transit in vitro. Notably, addition of CX3CL1 neutralizing
antibodies to the upper chamber did not inhibit migration of MDDC in the reverse luminal-to-
basolateral direction (Figure 7F), consistent with the observed polarity of CX3CL1 secretion
from the basolateral face of activated HDLEC (Figure 4A). Importantly, blockade of
CX3CL1 shedding by treatment of HDLEC with ADAM10/17 inhibitors, or Ilomastat, led to
the same reduction in MDDC transmigration as the CX3CL1 neutralizing antibody (Figure
6J). Hence, we conclude it is the secreted rather than the membrane bound form of CX3CL1
that is active in mediating DC transmigration.

Given that activated HDLEC also secrete CCL21 and that neutralizing antibodies to
CCL21 partially block DC transmigration across TNFα-treated HDLEC in vitro (Johnson and
Jackson, 2010), we investigated the consequences of imposing dual blockade with CCL21
and CX3CL1 neutralizing antibodies in the upper chamber of the transwell. The results
(Figure 7K) show that the effect of the antibodies was not additive, yielding less than
complete inhibition of DC transmigration. Hence, it is possible that CX3CL1 and CCL21
may act sequentially in activated lymphatic endothelium and that additional as yet
unidentified chemokines contribute to transmigration. Furthermore, our previous studies have
shown that when G-protein-coupled chemokine receptors are inhibited by pertussis toxin, 15-
20% of input DC can still transmigrate across lymphatic endothelium, i.e. in a chemokine-
independent manner (Johnson and Jackson, 2010).
Lastly, we evaluated whether the mode of CX3CL1 action in DC transmigration involves activation of β2 integrin, similar to that documented for CCL21 (Johnson and Jackson, 2010). Accordingly, we employed mAb24, an antibody that selectively binds an active conformation of the β2 integrin subunit (Dransfield et al., 1992) and compared reactivity with MDDC exposed to either CX3CL1 or CCL21. CX3CL1 exposure increased mAb24 reactivity of both immature and mature MDDC, to levels that were comparable to those of CCL21 (Figure 7H-I). However, CX3CL1 had no significant effect on ICAM-1 or VCAM-1 levels in HDLEC within the 4 h time-frame of the in vitro transmigration assays (Supplementary Figure 6). These results demonstrate for the first time that soluble CX3CL1 secreted from TNFα-stimulated lymphatic endothelium can activate β2 integrin adhesion in DC and direct their basolateral-to-luminal transmigration.

**CX3CL1 promotes lymphatic trafficking of DC in a mouse model of skin inflammation in vivo**

Having established inflammation-induced expression of CX3CL1 in primary LEC and a role in transmigration of DC in vitro, we next assessed the role of CX3CL1 in lymphatic trafficking of endogenous DC in vivo, using the well-characterized oxazolone-induced skin hypersensitivity model mentioned above (figure 6). Oxazolone-treated mice were injected with neutralizing antibodies to CX3CL1 and migration of cutaneous DC following FITC skin painting was measured by flow cytometry of disrupted skin-draining lymph nodes. The results showed that after 24 h, CX3CL1 blockade suppressed lymph node trafficking of FITC+/CD11c+ DC by > 70% when compared with rabbit IgG controls (Figure 8A, B). As expected, skin-derived DC recovered from draining lymph nodes of all mice were found to express both CCR7 and CX3CR1 (data not shown).

**Genetic deletion of CX3CR1 impairs lymphatic migration of DC in vivo**

Cutaneous DC migrate to lymph nodes almost exclusively via afferent lymphatics, rather than by entering the blood circulation. Hence, CX3CL1 and its receptor must play a role in either the initial entry to or subsequent migration within lymphatics. To address this question, we compared the migration of bone marrow-derived DC (BMDC) from wild-type and CX3CR1−/− mice, after co-injection into the inflamed skin of topical oxazolone-hypersensitized wild-type mice. To allow discrimination between the cell types, wild-type BMDC were labelled with Q-tracker®655 and CX3CR1−/− BMDC were labelled with Q-tracker®525, immediately prior to adoptive transfer. Importantly, before injection both cell populations showed similar
expression of MHC class II, the DC selective β2 integrin CD11c, the co-stimulatory molecule CD86 (B7.2), and the lymph migratory dermal DC marker EpCAM (Supplementary Figure 7). As shown in Figure 9A however, the excised cervical lymph nodes revealed marked differences in the migratory properties of wild-type and CX3CR1−/− BMDC. The majority of wild-type BMDC had migrated within 24 h but largely dispersed by 48 h, either having exited in efferent lymph or undergone apoptosis in situ. In contrast, the CX3CR1−/− BMDC population was significantly slower to migrate, with the majority taking 48 h to reach the same lymph nodes, (0.094% wild-type BMDC, SEM+/− 0.018, versus 0.048% CX3CR1−/− BMDC, SEM+/− 0.001, at 24 h, and 0.011% wild-type BMDC, SEM+/− 0.003, versus 0.040% CX3CR1−/− BMDC, SEM+/− 0.006 at 48 h). Importantly, the total numbers of wild-type and knock-out BMDC recovered from the cervical lymph nodes after 24 h and 48 h were almost identical, indicating that CX3CR1-deficient BMDC experience a delay in migration via afferent lymphatic vessels, rather than undergoing apoptosis. Similar results were also obtained when wild-type and CX3CR1−/− BMDC were injected into separate animals, rather than co-injected (data not shown). These findings indicate for the first time that CX3CL1 functions as an accessory chemokine for DC mobilization via lymph in vivo.

To further investigate the role of CX3CL1 in DC trafficking, oxazolone-hypersensitized mice were injected intradermally with equal mixtures of wild-type and CX3CR1−/− BMDC labelled with Cell Tracker™-red and Cell Tracker™-green respectively, to allow fluorescent microscopic imaging of both the draining lymph nodes (Figure 9B) and the skin injection site (Figure 9C) at 24 h and 48 h after adoptive transfer. This confirmed the delay in trafficking of CX3CR1−/− BMDC in lymph nodes observed by quantitative flow cytometry. Analysis of DC that had trafficked to lymph nodes revealed no obvious differences between localization of wild-type and CX3CR1−/− populations (Figure 9B). In contrast, imaging of the skin injection site revealed that CX3CR1−/− BMDC were heavily predominant at 24 h post injection, visible either as discrete cells or larger clusters (Figure 9C), despite similar numbers of CX3CR1−/− and wild-type BMDC within the dermis at early time points (2 h). Significantly, few if any CX3CR1−/− BMDC were seen to accumulate at the basolateral surface of lymphatic vessels in these areas at 24 h, suggesting that CX3CL1/CX3CR1 influence DC migration towards endothelium rather than endothelial adhesion. By 48 h, BMDC were rarely observed within the dermis, the majority of cells having exited the tissue, most likely via the afferent lymphatics.
To verify that the delay in lymphatic trafficking of CX3CR1<sup>−/−</sup> BMDC in vivo reflects an impairment in transendothelial migration, we compared the kinetics of basolateral-to-luminal migration for wild-type and receptor-null DC in vitro using TNF-α-activated mouse dermal lymphatic endothelial cell (MDLEC) monolayers (Supplementary Figure 8), plated on the underside of transwell inserts. Over the course of 24 h, strikingly fewer BMDC from CX3CR1-deficient mice migrated across the lymphatic endothelium than their wild-type counterparts, and at a consistently slower rate (Figure 9D). Hence, we conclude that CX3CL1 regulates both migration of DC towards lymphatic capillaries and also the subsequent transendothelial transit of DC into the vessels.

**Discussion**

In this manuscript we have presented the first evidence that CX3CL1, the sole member of the atypical transmembrane CX3C chemokine family, is synthesized de novo and shed in soluble form by inflamed human and murine lymphatic endothelial cells (LEC). Additionally, we have confirmed induction of CX3CL1 in intact lymphatic vessels of TNF-α-treated mouse and human dermis, and skin sections from contact hypersensitized mice. Moreover, we have demonstrated that secreted CX3CL1 promotes migration of MDDC across activated lymphatic endothelium in vitro and that neutralizing antibodies against CX3CL1 inhibit lymphatic trafficking of cutaneous DC to draining lymph nodes in vivo. In further corroboration of these findings, we showed that targeted disruption of the CX3CL1 axis in BMDC by deletion of the receptor CX3CR1 delays trafficking from inflamed skin to draining lymph nodes. These results identify a significant new player in the regulation of cell trafficking in the lymphatics during inflammation and attest further to the complexity of what has been considered in the past as merely a passive process.

CX3CL1 is particularly unusual insofar as it can occur both as an integral membrane adhesion protein and a conventional chemokine, generated through controlled cleavage by the disintegrin and metalloprotease enzymes ADAM17 and ADAM10 (Bazan et al., 1997; Garton et al., 2001; Hundhausen et al., 2003; Pan et al., 1997). Although the precise functional differences between the two forms are still obscure, it is clear that interaction of membrane-anchored CX3CL1 with its receptor CX3CR1 on leukocytes confers shear resistant binding to blood vascular endothelium in a mechanism independent of integrin and Gi protein involvement (Fong et al., 1998; Haskell et al., 1999). In contrast, the secreted form of CX3CL1 has been shown to promote chemotaxis of CX3CR1<sup>+</sup> monocytes, T cells and NK
cells by a conventional mechanism of integrin-mediated adhesion (Goda et al., 2000; Imai et al., 1997; Yoshikawa et al., 2004). Notably however, it is the membrane-anchored form that predominates in most cells and hence the function of CX3CL1 has been regarded as one of cell adhesion as much as chemotaxis.

An intriguing finding from the studies in this present manuscript was that both cultured LEC and lymphatic endothelium in vivo generate almost exclusively the soluble form of CX3CL1, through the action of an ADAM-like sheddase activity, and retain little if any of the membrane-anchored form. Previous reports indicate that in other cell types, CX3CL1 can evade proteolytic cleavage by a constitutive clathrin-mediated endocytosis pathway (Huang et al., 2009; Liu et al., 2005). It may be that this pathway operates less efficiently in LEC, or alternatively that they exhibit a greater level of CX3CL1 sheddase activity. Moreover, as the interstitial migration of leukocytes into lymphatic vessels does not have to combat the shear flow that restricts their exit from blood capillaries, there may be a lesser requirement for CX3CL1 as an adhesion receptor, and, in contrast with the blood vascular endothelium, the soluble chemokine form of the molecule may be favoured. We noted heterogeneity of CX3CL1 expression within TNFα-stimulated HDLEC, and suspect that this reflects the subtle variability in HDLEC subtype, as these cells are isolated from skin by immunoselection with LYVE-1 and thus derive from a mixture of both initial and precollector lymphatics. Indeed, we have previously observed similar heterogeneity in expression of VCAM-1 (Johnson et al., 2006).

Another particularly important finding was that CX3CL1 is secreted almost exclusively from the basolateral surface of lymphatic endothelium, where it selectively promotes basolateral-to-luminal transmigration of DC. This makes CX3CL1 virtually unique within the large repertoire of CC and CXC chemokines synthesized by resting and inflamed LEC including CCL21, CXCL12 (SDF-1), CCL2 (MCP-1), CCL5 (RANTES), CCL20 (MIP-3α), CXCL2 (GROβ), CXCL5 (ENA78) and CXCL8 (IL-8) that influence DC, monocyte, T cell and neutrophil trafficking (Gunn et al., 1999; Johnson et al., 2006; Johnson and Jackson, 2010; Kabashima et al., 2007; Kriehuber et al., 2001; Saeki et al., 1999), (and data not shown), most or all of which are secreted predominantly from the luminal surface. Even CCL21, which is well-documented as being critical for lymphatic entry, is biased mainly (approximately 70 %) towards luminal secretion (Kriehuber et al., 2001). The fate of luminally secreted CCL21 is at present unclear, although it may be conveyed away from the
initial lymphatics via afferent lymph flow and play a role downstream in the lymph node, as has been shown for CCL2 (Palframan et al., 2001). In contrast, basolaterally secreted CCL21 is sequestered on the basement membrane of lymphatic vessels, where it is complexed with collagen IV at “entry portals”, facilitating the docking and transmigration of DC (Tal et al., 2011). Based upon its highly polarized secretion, we propose that CX3CL1 preferentially influences events in the subendothelial zone. For example, during inflammation when lymph flow is retarded, the accumulation of CX3CL1 may establish local chemokine gradients that attract DC towards the basolateral surface of the lymphatic vessel, prior to CCL21 guidance.

A role for CX3CL1 in lymphatic trafficking of endogenous DC was demonstrated through measuring FITC$^+$/CD11c$^+$ cells in draining lymph nodes 24h following FITC skin painting. CX3CL1 blockade by neutralizing antibodies suppressed lymph node trafficking of FITC$^+$/CD11c$^+$ cells by > 70% when compared with IgG controls. In addition to impairing DC entry to lymphatics, it is also possible that CX3CL1 neutralizing antibodies might have blocked recruitment of monocytes from the blood in these experiments, thus reducing the numbers of monocyte-derived DC entering the dermis. However, at 24 h from the onset of inflammation, the major population trafficking to the lymph nodes is more likely to be dermal DC rather than newly recruited and differentiated monocyte-derived DC.

Most significantly, we observed in our adoptive transfer experiments that genetic deletion of CX3CR1 in DC impeded trafficking from the inflamed dermis of oxazolone hypersensitized mice, delaying their transit to skin draining cervical lymph nodes by approximately 24 h compared with wild-type cells. Indeed, the transient nature of this inhibition might also explain how the influence of CX3CL1 on DC trafficking was overlooked during the original characterization of global CX3CR1$^{-/-}$ knock-out mice (Jung et al., 2000). Also, the authors of the previous study made indirect measurements of DC migration rather than quantitating the kinetics of trafficking using fluorescently labelled DC, as we have done here. Importantly, in our experiments total numbers of wild-type and CX3CR1$^{-/-}$ BMDC recovered from the cervical lymph nodes after 24 h and 48 h were almost identical. Hence, the effects of disrupting CX3CR1/CX3CL1 cannot be explained by an increase in DC apoptosis.

The delayed trafficking of CX3CR1-deficient DC also contrasts with the sustained reduction in lymphatic entry and lymph node migration of DC that has been reported after disruption of the key CCL21/CCR7 axis in CCR7$^{-/-}$ mice (Ohl et al., 2004) or
following inhibition of the CXCL12/CXCR4 axis (Kabashima et al., 2007). This most likely reflects differences in the sites of action of each chemokine, as well as their relative positions in the functional hierarchy. CCL21 has several actions, promoting not only interstitial chemotaxis of DC by amoeboid movement but most notably transendothelial migration via β2-integrin activation and intraluminal crawling under both resting and inflammatory conditions (Johnson and Jackson, 2010; Tal et al., 2011). Such control over the rate-limiting step in DC transmigration therefore places the CCL21/CCR7 axis in a dominant position in the hierarchy of “lymphatic” chemokines and may explain why CX3CL1/CX3CR1 is insufficient to maintain DC trafficking in the absence of CCL21/CCR7. We propose that CX3CL1 and CCL21 act at sequential stages during the migratory cascade, consistent with our finding that both CX3CL1 and CCL21 neutralizing antibodies inhibit DC transmigration when added singly, but fail to yield additive effects on blockade when combined together. CX3CL1 does not contain a basic GAG binding tract as is found in other matrix-sequestered chemokines such as CCL21 and CCL5 and consequently is not sequestered in tissue extracellular matrix (Patel et al., 2001). Thus, CX3CL1 could be expected to play a role in fluid phase chemotaxis, rather than haptotaxis, and provide initial guidance to DC towards the lymphatic capillaries, prior to the stage at which immobilized CCL21 aids in docking and transmigration (Tal et al., 2011). Moreover, since CX3CL1 is induced strictly in response to inflammation, we conclude that the CX3CL1/CX3CR1 axis, unlike CCL21/CCR7, guides DC trafficking under inflammatory conditions only.

Overall, our findings demonstrate induction of CX3CL1 secretion in inflamed lymphatic endothelium and a novel role for this atypical chemokine in regulating trafficking of DC from inflamed skin through afferent lymphatic vessels. It is now clear that this complex process is far from passive, and CX3CL1 is induced as part of a wider pleiotropic response by lymphatic endothelium to inflammation.

**Materials and Methods**

**Human and animal studies**

All studies using human tissue were approved by the Oxford Regional Ethics Committee. All animal studies were performed with appropriate UK Home Office licences according to established institutional guidelines.
Cytokines, Chemokines and Growth Factors

Recombinant human and mouse proteins were from R&D Systems and used at the following concentrations: IL-1α and IL-1β, 1 ng/ml; IL-6, 20 ng/ml; IFN γ, 100 ng/ml; human TNFα, 2 ng/ml; mouse TNFα, 100 ng/ml; human IL-4, 10 ng/ml; human GM-CSF, 50 ng/ml; mouse IL-4, 20 ng/ml; mouse GM-CSF, 20 ng/ml; and human CX3CL1 (chemokine domain), 25 µg/ml. Lipopolysaccharide (LPS), 1 µg/ml from Salmonella Abortus was from Sigma-Aldrich.

Antibodies

Neutralizing antibodies rabbit anti-CX3CL1 and goat anti-CCL21 were from AMS Biotechnology and R&D Systems respectively. Other antibodies used were mouse anti-human CX3CL1 (R&D Systems), rat anti-CX3CR1 (AMS Biotechnology), rabbit anti-human podoplanin (Fitzgerald Industries International, Inc.), rabbit anti-mouse podoplanin, clone 8.1.1 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), mouse anti-human ICAM-1 (Chemicon), mouse anti-human VCAM-1 (BD Pharmingen), mouse anti-human E-selectin (Developmental Studies Hybridoma Bank), sheep anti-human von Willebrand factor (vWF), (SeroTec) and rat anti-mouse EpCAM (eBioscience). Goat anti-mouse CCL2, goat anti-mouse CCL5 and goat anti-mouse CCL20 were from R&D Systems. Directly conjugated anti-mouse CD86-PE, CD11c-APC and CCR7-PE were from BD Pharmingen. All anti-LYVE-1 antibodies were generated previously (Banerji et al., 1999; Johnson et al., 2006; Prevo et al., 2001). Isotype-matched antibodies were from Sigma-Aldrich or R and D Systems.

Cells

Primary human dermal lymphatic endothelial cells (HDLEC) and mouse dermal lymphatic endothelial cells (MDLEC) were prepared from freshly resected skin samples by immunoselection with LYVE-1 mAb and MACS® beads (Miltenyi Biotec), and cultured as previously described (Johnson et al., 2006).

Monocyte-derived DC (MDDC) were obtained from healthy donors and generated as previously described (Johnson et al., 2006). MDDC were fluorescently labelled using Cell Tracker™ Green (Invitrogen), following the manufacturer’s protocol.

Bone marrow-derived DC (BMDC) were extracted from tibia and fibula bone marrows of euthanized C57Bl/6 and CX3CR1−/− mice, passed through a 70-µm cell strainer (BD Biosciences) and cultured for 7 days in RPMI-10% FCS supplemented with GM-CSF.
and IL-4. Non-adherent cells were selected for MHC class II expression by MACS beads
(Miltenyi Biotec) and then either labelled with Q-tracker655® or Q-tracker525® (Molecular
Probes, Invitrogen), or with Cell Tracker™ Green or Cell Tracker™ Red (Invitrogen),
following the manufacturer’s protocol.

**Inhibitors**

Ilomastat (GM6001) was purchased from Calbiochem and used at 2.5 µM. ADAM
10/17 inhibitors GW280264X and GI254023X were a kind gift from Dr. Andreas Ludwig,
Aachen University, Germany). MMP2/MMP9 inhibitor II and MMP8 inhibitor I were
purchased from Calbiochem and used at 1 µM. Actinomycin D and Cycloheximide were
purchased from Sigma-Aldrich and used at 10 µg/ml and 20 µg/ml respectively.

**Immunofluorescence antibody staining of cells and tissues**

Cultured HDLEC and MDLEC were fixed in PFA (1% w/v in PBS, pH 7.4);
incubated with the appropriate primary antibodies in PBS-FCS 10% (v/v), BSA 1% (w/v),
with or without prior cell permeabilization in saponin 0.2%, for 45 min at room temperature.
AlexaFluor®488, AlexaFluor®568, AlexaFluor®647 conjugates and either TOPRO-3 or
DAPI nuclei stain (Molecular Probes, Invitrogen), were used for secondary detection before
fixing in PFA-PBS, mounting in Vectashield-DAPI (Vector Laboratories) and viewing under
BioRad Radiance 2000 or Zeiss LSM780 confocal microscopes, using either a Plan-
Apochromat 10X/0.3 DIC M27 (total magnification: 100 X) or Plan-Apochromat 63X/1.4Oil
(total magnification: 630 X, resolution: 0.24 µm). Whole-mount tissue staining was carried
out as described previously (Johnson et al., 2006), either permeabilizing tissue with Triton-X-
100 0.1 % (v/v) in PBS or washing in PBS alone, to observe non-permeabilized tissue.
Frozen sections of lymph node were prepared by cryostat, fixed for 5 min in ice-cold acetone
then incubated with rat anti-mouse LYVE-1 (clone C1/8, (Johnson et al., 2007)) directly
conjugated with AlexaFluor647 and biotinylated MECA79 in PBS-FCS 10% (v/v), BSA 1%
(w/v). Streptavidin-Pacific Blue (Molecular Probes, Invitrogen) was used for secondary
detection, and nuclei counterstained with TOPRO-3.

**Chemokine ELISA**

Supernatants and cell lysates from triplicate wells of confluent primary HDLEC
(either cultured in 6-well dishes or on transwell inserts (0.4 µm pore size; BD Biosciences),
were assayed for CX3CL1, CCL2 and CCL5 using commercial antigen capture ELISA kits
(DuoSet; R&D Systems), in accordance with the manufacturer’s instructions. Supernatants were either applied directly to the ELISA or concentrated using Amicon® Ultra 0.5-ml centrifugation tubes, 5 kDa MW cut-off (Millipore). Adherent cells were lysed in lysis buffer [50 mM Tris, pH 7.4, 100 mM NaCl, 1% NP-40 (v/v), 1 mM EDTA and protease inhibitor cocktail (Roche)] and then applied to the ELISA.

**Western blotting**

MDDC were lysed in NuPAGE LDS sample buffer (Invitrogen) and electrophoresed on Bis-Tris 4–12% polyacrylamide SDS gels (NuPAGE; Invitrogen) with MES buffer, alongside SeeBlue standard (Invitrogen). Protein was transferred to Immobilon™ membranes (Millipore) and incubated with mouse anti-β-actin and rabbit anti-CX3CR1 antibodies, 0.2 µg/ml (AMS Biotechnology) overnight, followed by goat anti-mouse IRDye®700 and goat anti-rabbit IRDye®800 (Odyssey) in Odyssey® blocking buffer. Blots were visualized using the LiCor Biosciences Odyssey® imaging system.

**Reverse transcriptase-polymerase chain reaction**

Total cellular RNA was isolated (RNeasy, Qiagen) from HDLEC cultured for 24 h in EGM-2 MV medium, either alone or supplemented with 2 ng/ml TNF-α. First-strand cDNA synthesis was carried out by Oligo dT priming (Invitrogen) using AMV reverse transcriptase (New England Biolabs), following the manufacturer’s instructions. CX3CL1 transcripts were amplified using the primer pair CX3CL1Fwd2 (5-ATGGCTCCGATATCTCTGTCGT-3) and CX3CL1Rev2 (5-AAAAGCTCCGTGCCCACA-3), and β-actin with the primer pair ActinFwd (5-AGGCATCCTCACCCTGAAGTAC-3) and ActinRev (5-TTGCCAATGGGTACCTGACCTCC-3). Products were electrophoresed on 1.8% agarose-Tris-Borate-EDTA gels, alongside 100bp DNA ladders (New England Biolabs).

**Flow cytometry**

MDDC, BMDC and HDLEC were fixed in PFA (1% w/v in PBS, pH 7.4), washed in PBS-FCS 10% (v/v), then incubated with appropriate primary antibodies for 30 min at 4°C then washed again in PBS-FCS 5% (v/v) followed by incubation with goat anti-rat AlexaFluor®488.

For analysis of surface expression of CX3CL1 in HDLEC, cells were cultured for 24 h in either EGM-2MV medium alone or with human TNF-α and/or Ilomastat, then lifted with
Accutase® in the presence of Ilomastat, to prevent subsequent shedding of CX3CL1. Cells were fixed in PFA and stained as for DC.

Measurement of MDDC β2 integrin activation was carried out as described previously (Johnson and Jackson, 2010). Cells were analysed by flow cytometry (CyAn, Dako Cytomation) using Flow Jo software.

**In vitro lymphatic endothelial transmigration assays**

Primary HDLEC and MDLEC were seeded onto the underside (unless otherwise stated) of gelatin-coated FluoroBlok™ cell culture inserts (3 µm pore size; BD Biosciences), cultured until confluent and transmigration assays performed as detailed previously (Johnson et al., 2006; Johnson and Jackson, 2010). Where indicated, cells were stimulated with TNF-α for 24 h prior to use and CX3CL1 was added 2 h before addition of MDDC. Rabbit anti-human CX3CL1-neutralizing antibodies (50 µg/ml, AMS Biotechnology) and goat anti-CCL21-neutralizing antibodies (50 µg/ml, R&D Systems) were applied 30 min before the addition of MDDC and maintained throughout the course of the experiment. To each well, 0.5 x 10⁶ fluorescently labelled MDDC were applied at the start of the assay and numbers of MDDC transmigrating through the filter and monolayer into the lower chamber were recorded on a fluorescent plate reader (Synergy HT; Bio-Tek) at 37°C using KC4 software (Biotech). Fluorescence emission was calibrated against a standard curve, and transmigration was expressed as the number of MDDC in the lower chamber.

To compare migration of BMDC from wild-type mice with those from CX3CR1−/− mice, Green Tracker Dye-labelled, MHC class II+ DC from age-matched donors (described above) were applied to MDLEC monolayers in parallel wells and fluorescence emission was calibrated against standard curves for wild-type and CX3CR1−/− cells.

**In vivo assays for CX3CL1-mediated DC trafficking in inflamed mouse skin**

Male BALB/c and C57Bl/6 mice aged 8–10 weeks were sensitized and challenged by topical application of oxazolone (4-ethoxymethylene-2 phenyl-2-oxazoline-5-one; Sigma–Aldrich) as previously described (Johnson et al., 2006). For FITC painting experiments, oxazolone-sensitized BALB/c mice were intraperitoneally injected with 0.4 mg of either rabbit anti-CX3CL1 neutralizing antibodies (AMS Biotechnology) or rabbit IgG (R and D Systems), then challenged with oxazolone (0.8 % w/v) + FITC (1.5 mg/ml) in 95 % ethanol (v/v in water) after 24 h and sacrificed after a further 24 h. The draining cervical lymph nodes were
removed and tissue was disrupted, passed through a 70-µm cell strainer (BD Biosciences), and analysed by flow cytometry (CyAn, DAKO).

For adoptive transfer experiments, C57Bl/6 male mice were sensitized to oxazolone and subsequently 5 days later, both ears were challenged by topical application of 0.8% oxazolone and 10⁶ Q-tracker 655® (Molecular Probes, Invitrogen)-labelled BMDC from littermates + 10⁶ Q-tracker 525®-labelled BMDC from age-matched CX3CR1−/− mice (described above) were dermally injected. After a further 24 h or 48 h period, animals were sacrificed and lymph nodes processed as detailed above.

For visualizing DC post-injection, BMDC were pre-labelled with either Red or Green Tracker™ Dyes (Invitrogen), mixed in equal numbers and 10⁵ cells injected. Dermis was then stained in whole-mount for LYVE-1⁺ lymphatics and frozen sections prepared of lymph nodes.

Statistical Analyses

The Mann-Whitney U test was used to compare data sets throughout this study. A p-value < 0.05 was considered significant. For transmigration assays, statistical analyses were performed on data from the 8 h time point.

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Authorship

Contribution: L.A.J. designed and performed research, analysed data and wrote the manuscript; D.G.J. designed research and wrote the manuscript.

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References


**Figure Legends**

**Figure 1.** Induction of CX3CL1 expression in activated lymphatic endothelial cells. Levels of CX3CL1 secreted from HDLEC stimulated for 24 h with individual pro-inflammatory cytokines (A) and in the supernatant (B) and detergent lysates (C) of HDLEC stimulated for 24 h with TNF-α in the presence of Actinomycin D (ActD) or Cycloheximide (CHX), assessed by ELISA (mean +/- standard error, n = 3). Comparison of CX3CL1 mRNA levels in resting and 24 h TNF-α-treated HDLEC by RT-PCR with β-actin as a control, D. Representative data from one experiment of three are shown in each case, *p < 0.03 in comparison with CX3CL1 from unstimulated cells.

**Figure 2.** Rapid shedding of CX3CL1 from the surface of activated HDLEC. A. Dual immunofluorescence staining of control and 24 h TNF-α-stimulated HDLEC for CX3CL1 (green) and podoplanin (red) to determine the effect of Ilomastat on intracellular and cell surface CX3CL1 in saponin-permeabilized and non-permeabilized HDLEC monolayers respectively. Nuclei were counterstained with DAPI, magnification: 630 X. FACS histograms (B) and derived mean fluorescence values (C) for cell surface CX3CL1 levels in control HDLEC and HDLEC stimulated with TNF-α in the presence or absence of Ilomastat. Levels of CX3CL1 in supernatants from cells in panels B and C were also assessed by ELISA (D). Representative data are the mean +/- standard error (n = 3) from one experiment of three.

**Figure 3.** Shedding of CX3CL1 involves cleavage by ADAM10/17. Amounts of CX3CL1 (A) and CCL2 (B) shed from HDLEC cultured in medium alone (control) or in the presence of TNF-α for 24 h, with either Ilomastat or the ADAM10/17 inhibitors GI254023X and GW280264X, assessed by ELISA. Amounts of CX3CL1 shed from HDLEC under similar conditions to A and B but with inhibitors to either MMP8 and MMP2/9 are shown in C. Data are the mean +/- standard error (n = 3) from one representative experiment of three, *p < 0.03.

**Figure 4.** CX3CL1 is selectively secreted from the basolateral surface of activated HDLEC. Amounts of CX3CL1 (A), CCL5 (B) and CCL2 (C) secreted from the basolateral and luminal surfaces of control and TNF-α stimulated HDLEC monolayers cultured for 24 h on Fluoroblok™ transwell inserts, as assessed by ELISA. Data are the mean +/- standard error (n = 3) from one representative experiment of three, *p < 0.03. Dual immunofluorescence staining for CX3CL1 (green) and vWF (red) in saponin-permeabilized TNF-α-treated HDLEC indicates lack of co-localization with Weibel-Palade bodies (D).
Nuclei were counterstained with TOPRO-3, (scale bar = 10 μm). Inset (E) shows digital zoom on indicated cell.

Figure 5. Visualization of CX3CL1 in lymphatic vessels of TNFα-activated human dermis. Dual immunofluorescence staining for CX3CL1 (green) and podoplanin (red) in permeabilized frozen sections (< 4 μm) of human dermis, either freshly resected (control) or cultured for 24 h in presence of TNF-α. Nuclei were counterstained with TOPRO-3, (scale bar = 10 μm); arrow indicates typically intracellular location of CX3CL1.

Figure 6. Visualization of CX3CL1 in dermal lymphatics of contact hypersensitized mouse skin. Whole-mount sections of (control) contralateral uninfamed ear skin and inflamed ear skin from mice subjected to topical oxazolone stimulated skin hypersensitivity, permeabilized and stained for CX3CL1 (red) and podoplanin (green), magnification: 100 X, (scale bar = 100 μm), A. Representative images from four mice are shown. Whole mount sections of inflamed ear skin were prepared in either the absence or presence of Triton X-100 (non-permeabilized and permeabilized, respectively), immunostained as above and imaged by confocal microscopy as a Z-series. Single planes of view are shown in left-hand panels; orthogonal views of different vessels in right-hand panels. Magnification: 630 X.

Figure 7. CX3CL1 promotes DC transmigration across resting and activated HDLEC in vitro. Levels of CX3CR1 expression in immature and LPS matured MDDC as assessed by Western blotting, (A), (with inclusion of β-actin as loading control) and flow cytometry (B). Expression of CCR7 also detected (C) and maturation assessed by expression of CD83 (G). Numbers of fluorescently-labelled MDDC transmigrating resting HDLEC incubated either alone or with recombinant human CX3CL1 (D) and TNF-α-stimulated HDLEC incubated either with chemokine neutralizing antibodies or irrelevant IgG controls (E, F, K) or ADAM and metalloproteinase inhibitors (J). Activation of β2 integrin was assessed through binding of mAb24 to immature (H) and mature (I) MDDC, *p < 0.03, **p < 0.015. Mouse IgG1 was included as an isotype-matched control. EDTA chelates divalent cations and inhibits integrin activation, thus indicating specificity of mAb24 binding. All data represent the mean +/- standard error (n = 4) from one representative experiment that was repeated 2-5 times.

Figure 8. In vivo trafficking of cutaneous DC via afferent lymphatics is dependent upon CX3CL1. Recoveries of FITC⁺CD11c⁺ skin DC in the draining lymph nodes, 24 h after FITC skin painting of oxazolone-sensitized mice that received prior injection of neutralizing antibodies to CX3CL1 or control rabbit IgG. Data represent the mean recoveries +/- standard
error (n = 5) from one experiment of two, (A), with representative flow cytometry contour plots (B).

**Figure 9. CX3CR1 is required for efficient DC trafficking via inflamed skin lymphatics.**
Numbers of wild-type (Q-tracker525® labelled) and CX3CR1−/− (Q-tracker655®) BMDC arriving in draining lymph nodes 24 or 48 h after co-injection of a 50:50 mixture into the skin of topical oxazolone-sensitized C57BL/6 mice (A). Data represent the mean +/- standard error (n = 3) from one representative experiment of three, *p < 0.001, **p < 0.0079. Fluorescent imaging of wild-type (red tracker labelled) and CX3CR1−/− (green tracker labelled) BMDC in draining lymph nodes at 24 h and 48 h after injection. Frozen sections of lymph nodes were stained with anti-LYVE-1 (yellow) and MECA79 (blue), to label lymphatic vessels/subcapsular sinus and HEVs, respectively. Nuclei were counterstained with TOPRO-3, yellow. Arrows indicate examples of BMDC in the subcapsular sinus, arrowheads indicate BMDC in paracortex of lymph nodes, magnification: 100X (B). Whole mount skin sections with LYVE-1+ lymphatic vessels (blue) comparing numbers of wild-type (red) and CX3CR1−/− (green) adoptively transferred BMDC remaining at 2 h, 24 h and 48 h post co-injection, (C), scale bar = 50 µm. Arrow indicates one of a very few CX3CR1−/− BMDC which remained at 48 h. Representative images from six mice are shown. Numbers of fluorescently-labelled wild-type and CX3CR1−/− DC transmigrating across TNF-α-stimulated MDLEC monolayers plated on the underside of FluoroBlok™ inserts, (D). Data represent the mean +/- standard error (n = 4) from one representative experiment of three.

**Supplementary Figure 1. Phenotype of primary HDLEC.** Cells isolated from human dermis by LYVE-1 immunomagnetic bead selection are shown after immunofluorescence staining for lymphatic and blood vascular-specific markers. Confluent monolayers were found to express the pan-endothelial cell marker CD31, the lymphatic-endothelial specific transcription factor Prox-1 and the lymphatic markers podoplanin and LYVE-1. The heterogeneity in LYVE-1 expression reflects that observed in normal tissue lymphatics. HDLEC did not express the blood vascular endothelial cell marker PAL-E antigen, or CD44. Nuclei were counterstained with DAPI. Magnification: 100X. FACS histograms of HDLEC stained for podoplanin, LYVE-1 and CD31 (black) show that a pure population of HDLEC was obtained. Isotype controls are shown in grey.
Supplementary Figure 2. Expression of CX3CL1 in TNFα-stimulated HDLEC. Split panels of the merged images shown in figure 2 are shown, following dual immunofluorescence staining of 24 h TNF-α-stimulated HDLEC for CX3CL1 (green) and podoplanin (red) in saponin-permeabilized and non-permeabilized HDLEC monolayers respectively. Nuclei were counterstained with DAPI, magnification: 6300 X.

Supplementary Figure 3. Effects of metalloproteinase inhibitors on CX3CL1 shedding from activated HDLEC. HDLEC monolayers were stimulated with TNF-α in the presence of varying concentrations of either Ilomastat or the ADAM10/17 inhibitors GI254023X or GW280264X. Shedding of CX3CL1 into the supernatant was measured by ELISA, n = 3.

Supplementary Figure 4. Expression of CX3CL1 in lymphatic endothelium ex vivo. Whole-mount staining was performed on freshly resected (control) mouse ears and following TNF-α stimulation. Tissue was stained for podoplanin (green) and CX3CL1 (red). Representative images from four mice are shown, (bars, top panel = 100 µm, lower panels = 50 µm).

Supplementary Figure 5. Induction of CX3CL1 and inflammatory CC chemokines in inflamed mouse dermal lymphatics in vivo. Whole-mount staining for podoplanin (green), LYVE-1 (blue) and either CCL2, CCL5 or CCL20 as indicated (red) in ipsilateral inflamed ear dermis from mice subjected to topical oxazolone induced skin hypersensitivity, compared with the non-inflamed contralateral ear. Representative images from four mice are shown, (bar = 100 µm).

Supplementary Figure 6. Exposure of HDLEC to exogenous CX3CL1 does not affect expression of CAMs. Flow cytometry to measure expression of E-selectin, VCAM-1 and ICAM-1 was carried out on HDLEC following incubation for 4 h in either the presence (grey line) or absence (black line) of recombinant human CX3CL1. Irrelevant isotype-matched controls are shown in pale grey, one representative histogram of three shown in each case.

Supplementary Figure 7. Phenotype of BMDC from wild-type and CX3CR1−/− mice. Flow cytometry to show expression of cell surface markers on MHC class II+ BMDC from wild-type (grey lines) and CX3CR1−/− mice (black lines), with isotype-matched controls depicted as filled grey histograms.
Supplementary Figure 8. Expression of CX3CL1 in TNFα-stimulated MDLEC. Staining for CX3CL1 (red), podoplanin (green) and LYVE-1 (blue) in control, non-permeabilized and saponin-permeabilized cells. Magnification: 630 X.
Figure 5

Control

CX3CL1

Podoplanin

Merge

TNFα

Merge

TNFα
Figure 6

A

CX3CL1  Podoplanin  Merge

Control

Oxazolone

B

Non-permeabilized

blood capillary

lymphatic vessel

50 μm

Non-permeabilized

Permeabilized

Permeabilized

20 μm
Figure 7

A

Immature DC
Mature DC
CX3CR1
β-actin

B

% of Max
Immature (IgG)
Mature (IgG)
Mature (CX3CR1)
Immature (CX3CR1)

C

% of Max
IgG
CCR7

D

$\rho < 0.03$

E

DC migration ($\times 10^6$)

F

DC migration ($\times 10^6$)

G

CD83 expression

H

β2 integrin activation
Immature MDDC

I

β2 integrin activation
Mature MDDC

J

DC migration ($\times 10^6$)

K

DC migration ($\times 10^6$)
Figure 8

A

\[ p = 0.0079 \]

% CD11c+FITC+cells

Rabbit IgG

anti-CX3CL1

B

Rabbit IgG

anti-CX3CL1

FITC

CD11c-APC