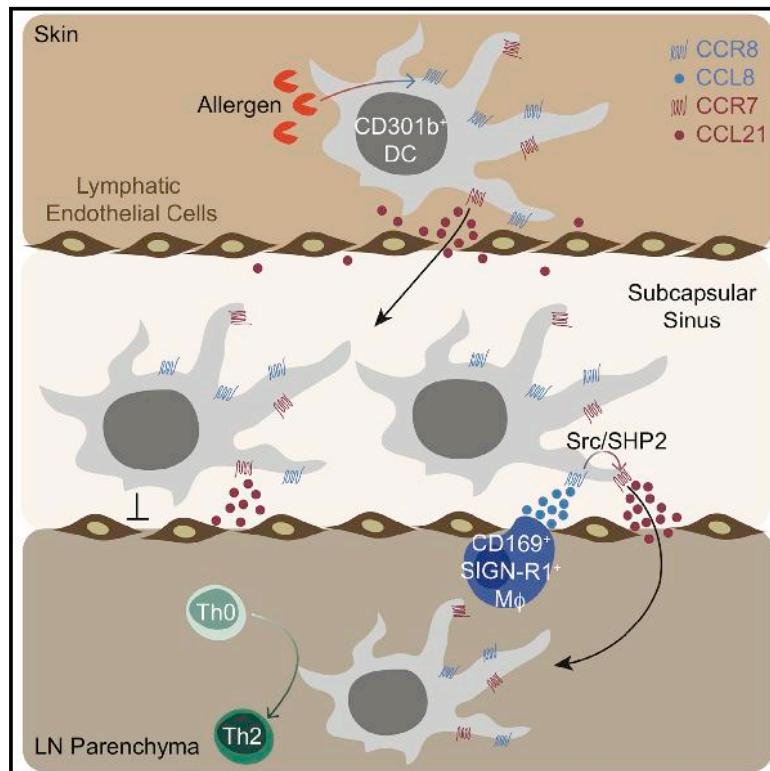


Immunity

The Chemokine Receptor CCR8 Promotes the Migration of Dendritic Cells into the Lymph Node Parenchyma to Initiate the Allergic Immune Response

Graphical Abstract



Authors

Caroline L. Sokol, Ryan B. Camire,
Michael C. Jones, Andrew D. Luster

Correspondence

aluster@mgh.harvard.edu

In Brief

Migration of dendritic cells (DCs) into the lymph node (LN) initiates adaptive immunity and is thought to be solely dependent on CCR7. Sokol et al. show that in a cutaneous type 2 immune response, CCR8 synergizes with CCR7 and is necessary to promote the entry of allergen-activated DCs into the LN parenchyma, which is required for Th2 differentiation.

Highlights

- Allergen exposure promotes CD301b⁺ DC LN migration without upregulating CCR7
- CD169⁺SIGN-R1⁺ macrophages produce CCL8 in the LN in response to allergen exposure
- CCL8 and CCL21 synergize in a Src-dependent manner to augment CD301b⁺ DC migration
- CD301b⁺ DCs require CCR8 expression to enter the LN and promote Th2 differentiation



The Chemokine Receptor CCR8 Promotes the Migration of Dendritic Cells into the Lymph Node Parenchyma to Initiate the Allergic Immune Response

Caroline L. Sokol,¹ Ryan B. Camire,¹ Michael C. Jones,^{1,2} and Andrew D. Luster^{1,3,*}

¹Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy, and Immunology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA

²Present address: Department of Pathology, University of Massachusetts Medical School, Worcester, MA 01605, USA

³Lead Contact

*Correspondence: aluster@mgh.harvard.edu

<https://doi.org/10.1016/j.immuni.2018.07.012>

SUMMARY

The migration of mature dendritic cells (DCs) into the draining lymph node (dLN) is thought to depend solely on the chemokine receptor CCR7. CD301b⁺ DCs migrate into the dLN after cutaneous allergen exposure and are required for T helper 2 (Th2) differentiation. We found that CD301b⁺ DCs poorly upregulated CCR7 expression after allergen exposure and required a second chemokine signal, mediated by CCR8 on CD301b⁺ DCs and its ligand CCL8, to exit the subcapsular sinus (SCS) and enter the lymph node (LN) parenchyma. After allergen exposure, CD169⁺SIGN-R1⁺ macrophages in interfollicular regions produced CCL8, which synergized with CCL21 in a Src-kinase-dependent manner to promote CD301b⁺ DC migration. In CCR8-deficient mice, CD301b⁺ DCs remained in the SCS and were unable to enter the LN parenchyma, resulting in defective Th2 differentiation. We have defined a CCR8-dependent stepwise mechanism of DC-subset-specific migration through which LN CD169⁺SIGN-R1⁺ macrophages control the polarization of the adaptive immune response.

INTRODUCTION

Dendritic cells (DCs) play a central role in linking innate sensing with the activation and skewing of the adaptive immune response. The activation of pattern-recognition receptors (PRRs) in DCs leads to a coordinated response known as DC maturation. DC maturation is classically characterized by a decrease in DC endocytosis, migration to the draining lymph nodes (dLNs), an increase in the expression of major histocompatibility type II (MHC II) and co-stimulatory molecules, and the production of skewing cytokines involved in CD4⁺ T helper (Th) cell differentiation and activation. By reducing endocytosis and upregulating MHC II presentation, DC maturation creates a snapshot of the antigens encountered by DCs at the time of PRR activation. Once in the dLN, DCs display antigens as well

as co-stimulatory molecules and skewing cytokines that are influenced by the inflammatory stimuli previously encountered. Thus, DCs can independently provide the precise co-stimulation and skewing cytokines required for promoting the ideal adaptive immune response.

Although all conventional DCs share a robust capacity for antigen uptake and presentation, DC subsets show functional heterogeneity. Multiple subsets of DCs have been described to have different skewing effects on Th differentiation (Iwasaki and Medzhitov, 2015). For example, CD11b[−]CD103⁺ DCs have been shown to preferentially induce Th1 differentiation while CD11b⁺CD103⁺ DCs preferentially induce Th17 differentiation (King et al., 2010; Lewis et al., 2011; Schlitzer et al., 2013). In contrast, the CD11b⁺CD301b⁺PDL2⁺ DC population has been shown to be required for Th2 differentiation after cutaneous exposure to Th2-skewing stimuli (Gao et al., 2013; Kumamoto et al., 2013; Murakami et al., 2013). Given that Th differentiation is dependent on the DC subset, the migration of DC subsets must be differentially regulated in order to effectively skew the adaptive immune response. But how is this subset-specific migration controlled? One mechanism would be for DC subsets to express distinct PRRs, thereby allowing only certain subsets to be activated by any specific stimulus. However, although some subset-specific PRR expression has been described, most PRRs are widely expressed across DC subsets (Iwasaki and Medzhitov, 2015). Therefore, we hypothesized that subset-specific DC migration could be controlled by a distinct mechanism.

The processes governing DC positioning and migration are largely dependent on chemokines. Immature DCs express a wide variety of chemokine receptors, including CCR1, CCR2, CCR5, CCR6, CCR8, CXCR1, CXCR2, and CXCR4, on their surface (Dieu et al., 1998; Griffith et al., 2014; Sallusto et al., 1998). The reason for this broad expression remains unclear, but it could be involved in the positioning of DC precursors in peripheral tissues as well as the migration of immature DCs to sites of inflammation. Once DCs are activated and undergo maturation, they upregulate CCR7 surface expression. Expression of CCR7 permits the maturing DCs to sense the CCR7 ligand CCL21, which is produced by the lymphatic endothelium (Worbs et al., 2017). This promotes their emigration from the peripheral tissues into the draining lymphatics, where they travel via lymphatic flow into the LN sinuses, including the subcapsular sinus (SCS) (Tal et al., 2011; Weber et al., 2013). Once in the LN sinuses, DCs



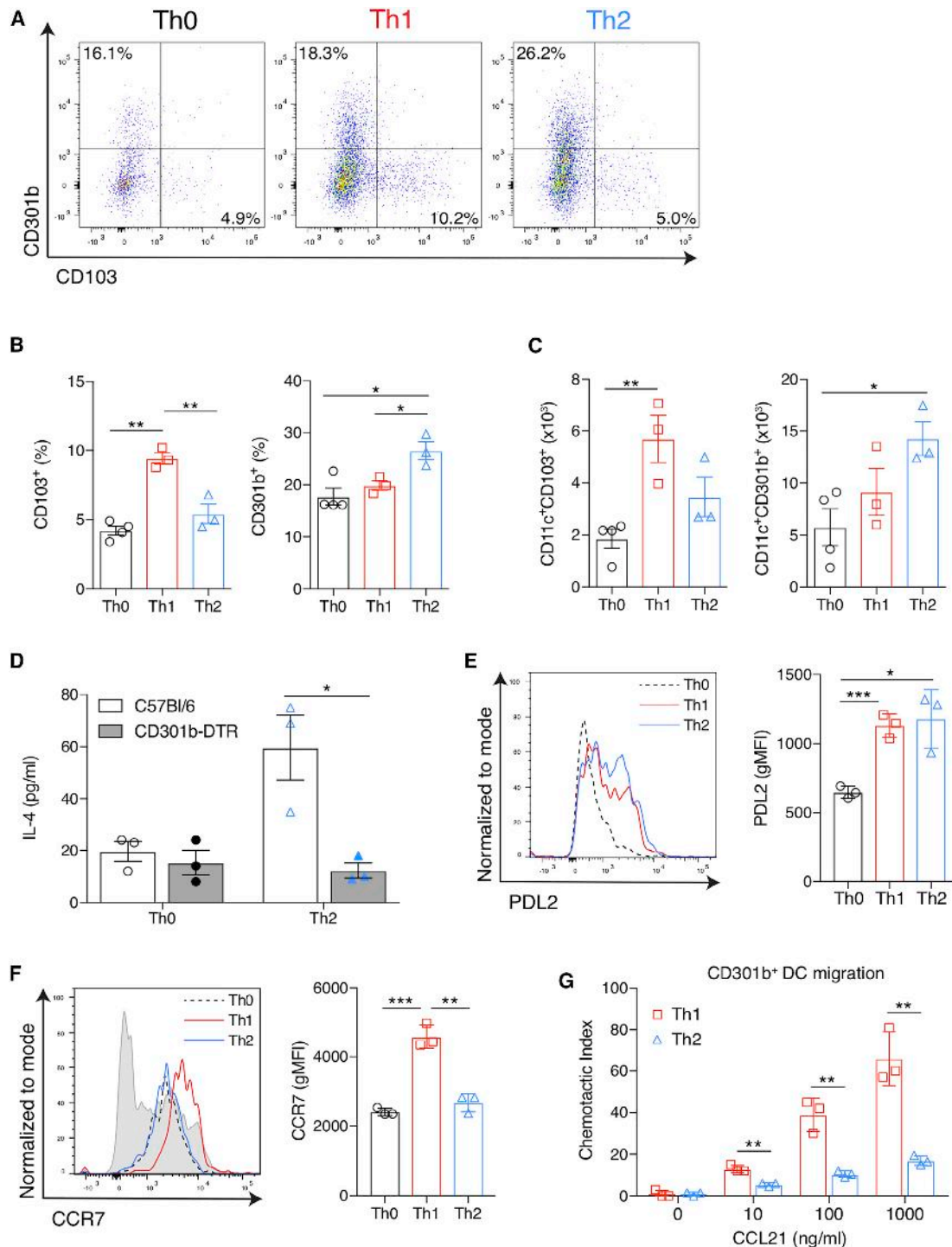


Figure 1. CD301b⁺ DCs Specifically Migrate in Response to Th2-Skewing Immunization

(A) Flow-cytometric analysis of viable CD11c⁺ cells from the popliteal LNs of mice 24 hr after footpad immunization with Th0-skewing (OVA), Th1-skewing (OVA, LPS, and poly I:C), or Th2-skewing (OVA and papain) immunizations. Numbers indicate percentages of CD11c⁺ cells in the indicated quadrant.

(B and C) Quantification of the percentage of CD103⁺ or CD301b⁺ cells out of the total viable CD11c⁺ cells (B) and the total CD11c⁺CD103⁺ or CD11c⁺CD301b⁺ cells from the dLN (C) as gated in (A) 24 hr after the indicated immunization.

(D) ELISA of IL-4 in the supernatant of LN cultures from WT (white bars) or CD301b-DTR (gray bars) mice after *in vitro* restimulation with OVA. All mice received DT and 2.5×10^5 CD4⁺ OTII cells before the indicated immunization.

(E and F) Surface expression of PDL2 (E) and CCR7 (F) from viable CD11c⁺CD301b⁺ cells taken from the dLN 24 hr after the indicated immunization and displayed in comparison with the total CD11c⁺ cells from a Th0-immunized mouse (gray shaded histogram).

(legend continued on next page)

again follow CCL21 gradients that in the SCS are established by the expression of the atypical chemokine scavenger receptor ACKR4 on the SCS ceiling (Ulvmar et al., 2014). After crossing the floor of the LN sinuses in the interfollicular regions (IFRs), DCs continue to follow gradients of the CCR7 ligands CCL19 and CCL21 to enter the T cell area where they can interact with naive T cells (Braun et al., 2011). However, CCR7 might not be sufficient for proper DC positioning within the LN. In a *Heligmosomoides polygyrus* infection model, CXCR5 expression on DCs was shown to be necessary for their localization in IFRs of the dLN and induction of follicular T helper (T_{fh}) and Th2 responses (León et al., 2012). Thus, other chemokines could play a role in DC positioning and adaptive immune outcomes.

We hypothesized that alternative, non-CCR7 chemokine networks could be involved in subset-specific DC migration and entry into the dLN. We found that the Th2-skewing stimulus papain induced robust migration of CD301b⁺ DCs into the dLN despite low surface expression of CCR7. Coinciding with CD301b⁺ DC entry, allergen immunization led to an upregulation in CCL8 expression from CD169⁺SIGN-R1⁺F4/80⁺ macrophages present within the IFRs of the dLN. CCL8 enhanced CCL21-mediated migration of CD301b⁺ DCs in a Src-dependent manner, and both CCR7 and CCR8 were required for the entry of CD301b⁺ DCs into the dLN after papain administration. CD301b⁺ DCs lacking CCR8 expression trafficked to the dLN but were retained in the SCS space, leading to a loss of Th2 differentiation in response to multiple Th2-skewing stimuli. These data reveal a chemokine-mediated mechanism by which LN macrophages control DC-subset migration and the outcome of the adaptive immune response.

RESULTS

The Migration of DC Subsets Is Context Dependent

To understand how the innate immune system might control the migration of DC subsets, we first analyzed the migration of cutaneous DC subsets after immunization with ovalbumin (OVA) alone (Th0 immunization), OVA plus lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (poly I:C) (Th1 immunization), or OVA plus papain (Th2 immunization). As others have shown, subcutaneous Th2 immunization promoted the specific migration of CD301b⁺ DCs into the dLN (Figures 1A–1C) (Kumamoto et al., 2013). Conversely, Th1 immunization specifically promoted the migration of CD103⁺ DCs into the dLN (Figures 1A–1C). Because female mice had a higher frequency of CD301b⁺ DCs in the dLN after both Th0 and Th2 immunization (Figure S1A), we used male mice for all DC migration experiments to control baseline DC migration. As previously shown, CD301b⁺ DCs were absolutely required for Th2 differentiation after papain immunization (Figure 1D) (Kumamoto et al., 2013). Thus, allergen immunization specifically promoted the migration of CD301b⁺ DCs, which are required for Th2 differentiation.

We next examined the phenotype of the CD301b⁺ DCs in the dLN 24 hr after Th0, Th1, or Th2 immunization. As expected, Th2

immunization led to upregulated expression of PDL2, an activation marker, on CD301b⁺ DCs (Figure 1E) (Gao et al., 2013; Kumamoto et al., 2013). This was not significantly different from that seen after a Th1 immunization, indicating that PDL2 upregulation is a common feature of CD301b⁺ DC activation by both allergens and PRRs (Figure 1E). Despite evidence of CD301b⁺ DC maturation and migration after Th2 immunization, CD301b⁺ DCs from the dLN of Th2-immunized mice did not upregulate CCR7 expression, whereas DCs from Th1-immunized mice did (Figure 1F). CCR7 expression on intracutaneous CD301b⁺ DCs after Th2 immunization was slightly higher than after Th0 immunization, but these levels did not approach those seen on CD301b⁺ DCs after Th1 immunization (Figures S1B and S1C). This lower level of CCR7 expression had functional consequences: CD301b⁺ DCs from the dLN of Th2-immunized mice exhibited less chemotaxis toward CCL21 at all concentrations tested than CD301b⁺ DCs isolated from the dLN of Th1-immunized mice (Figure 1G).

Th2-Skewing Immunization Promotes a Specific Chemokine Signature in the dLN

Given that Th2 immunization promoted robust CD301b⁺ DC migration without concurrently inducing optimal CCR7 expression, we hypothesized that additional allergen-induced chemokines could be involved in promoting CD301b⁺ DC migration from the periphery into the dLN. We focused on two potential sites of chemokine-mediated control: the point of DC entry into lymphatics (i.e., the skin) and the point of LN entry from the lymphatics. We focused our examination at 24 hr after immunization, which corresponded with DC entry into the dLN (Sokol et al., 2008). In the skin, both Th1 and Th2 immunizations promoted upregulation of the CCR2 chemokine ligands *Ccl2*, *Ccl7*, and *Ccl12* (Figure 2A). Th2 immunization induced the CCR8 ligand *Ccl8*, but this occurred past the window of DC emigration, making it unlikely to play a role in CD301b⁺ DC migration (Figure 2A). Therefore, we turned our attention to the dLN. Within 24 hr of footpad immunization, Th1 immunization led to upregulation of the CCR2 ligands, whereas Th2 immunization specifically promoted upregulation of *Ccl8* (Figure 2B). *Ccl8* upregulation was specific to Th2 immunization; Th1 immunization reduced the expression of both CCR8 ligands *Ccl1* and *Ccl8* (Figure 2B).

After leaving the afferent lymphatics, DCs enter the SCS space and the LN sinuses, which are lined by lymphatic endothelium and LN sinus macrophages, which must be traversed for DCs to enter the LN parenchyma (Girard et al., 2012; Qi et al., 2014). Using *Ccl8*^{−/−}*Ccl12*^{−/−} mice as a negative control, we examined the localization of CCL8 within dLN sections. In Th0-immunized wild-type (WT) mice, we detected CCL8 in two locations (Figure S2): adjacent to gp38⁺ lymphatic endothelium in the medullary region (Figure 2Ci) and within SCS regions, specifically where the SCS met the medullary region (Figure 2Cii). Concordant with the qPCR data, the number of discrete CCL8-expressing cells significantly increased after Th2 immunization in WT mice but not in *Ccl8*^{−/−}*Ccl12*^{−/−} mice (Figures 2C and 2D). In

(G) Transwell chemotaxis to CCL21 of CD11c⁺CD301b⁺ cells sorted from the dLN 24 hr after Th2-skewing immunization. The chemotactic index reflects the number of cells migrating through the Transwell at the indicated concentration over that of media alone. In all experiments, bars indicate mean values of the individual data points presented. Error bars indicate SEM. Data are representative experiments from three independent experiments with three to four mice (A–F) or three replicates (G) each. *p < 0.05, **p < 0.01, ***p < 0.001, Student's t test. See also Figure S1.

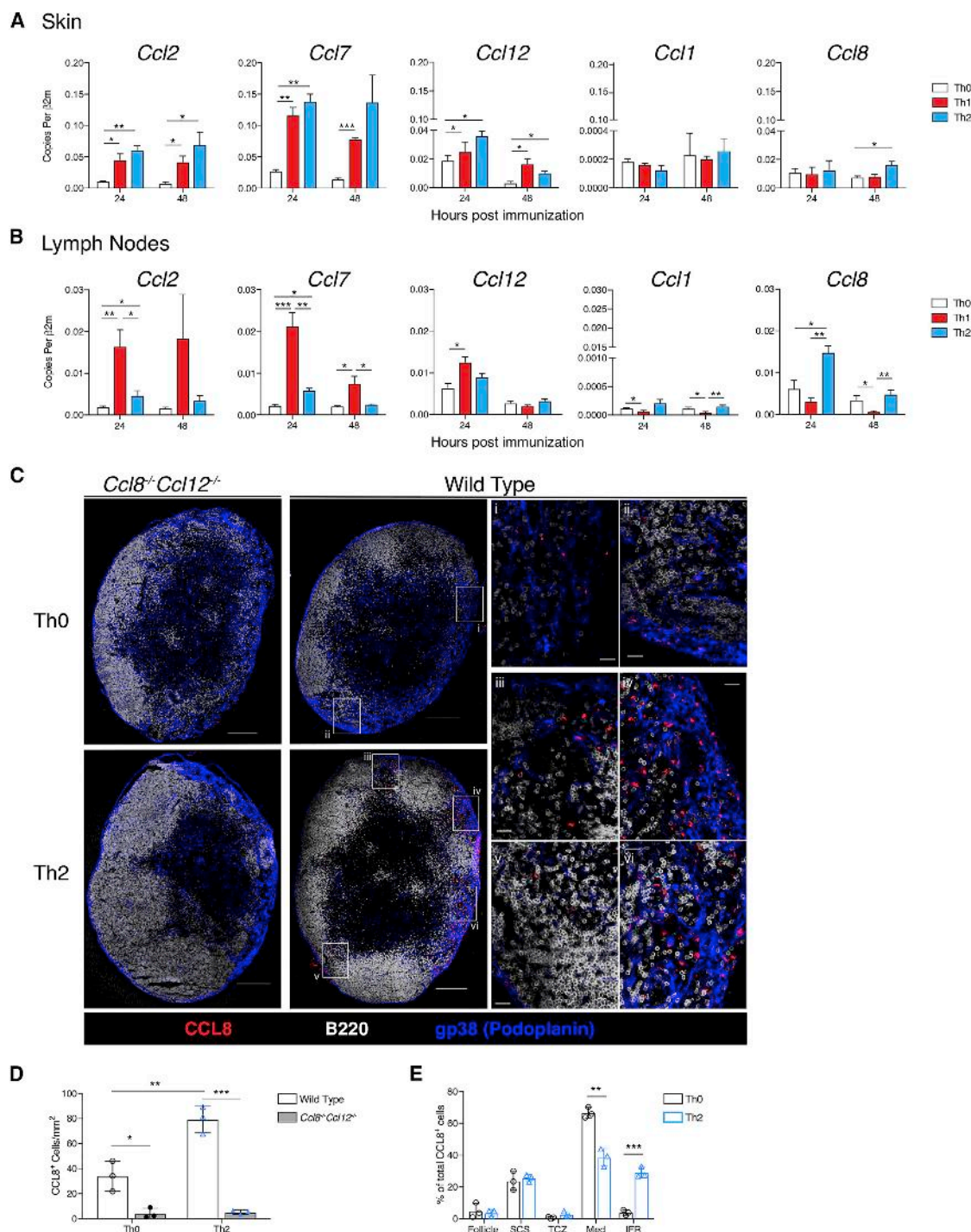


Figure 2. Allergen Immunization Promotes a Specific Chemokine Signature in the dLN

(A and B) qPCR analysis of the footpad skin immunization site (A) and popliteal LN (B) 24 and 48 hr after immunization with Th0-, Th1-, or Th2-skewing immunization. Data are expressed as cDNA copies of the indicated gene per copies of $\beta 2$ m. Each bar represents the mean of 3–6 mice from a representative of four independent experiments. Error bars indicate SEM

(C) CCL8 staining in whole LNs. Popliteal LNs of WT or *Ccl8*^{−/−}*Ccl12*^{−/−} mice were harvested 24 hr after immunization. Confocal immunofluorescence of whole LNs shows CCL8 (red), B220 (white), and gp38 (blue). Insets show the medullary region (i) and the medullary-follicular border (ii) in a Th0-immunized WT mouse. In a Th2-immunized WT mouse, insets show interfollicular regions (IFRs) (iii, iv, and v) and the medullary region (vi). Scale bars indicate 200 μ m (main panels) and 20 μ m (insets).

(legend continued on next page)

addition to this absolute increase in CCL8⁺ cells, we found that Th2 immunization specifically promoted CCL8 expression within the IFRs of the dLN (Figures 2Ciii, 2Civ, 2Cv, and 2E). CCL8⁺ cells were still readily appreciated within the medullary region after Th2 immunization (Figure 2Cvi). However, given observations by others that IFRs are the site of DC entry, we hypothesized that CCL8 produced in the IFRs could play a role in the allergen-induced movement of CD301b⁺ DCs across LN sinuses and into the LN parenchyma (Braun et al., 2011; Woodruff et al., 2014).

CD301b⁺ DC Entry into the dLN Requires CCR8 Signals

CCL8 and its functional human analog CCL18 bind specifically to the CCR8 receptor (Islam et al., 2011, 2013). We hypothesized that CD301b⁺ DCs express CCR8 and that this chemokine system promotes CD301b⁺ DC trafficking into the dLN in response to Th2-skewing stimuli. *Ccr8*^{-/-} mice showed decreased migration of mature, PDL2⁺CD301b⁺ DCs into the dLN at baseline (i.e., Th0 immunization) and after Th2 immunization (Figure 3A). This was not due to defective exit from or a decreased amount of CD301b⁺ DCs within the skin given that there was no significant difference in the frequency of CD301b⁺ DCs in the skin of *Ccr8*^{-/-} mice (Figure S3A). Perhaps as a result of the loss of CD301b⁺ DC migration, CD103⁺ DCs made a larger percentage of the total DC population in the dLN of *Ccr8*^{-/-} mice but were not present in significantly greater numbers (Figure 3B). In order to directly assess the migration of CD301b⁺ DCs from the skin to the dLN, we utilized Kaede transgenic mice, which express a photoconvertible protein (Kaede) that can be converted from green to red fluorescence (Kaede^{red}) (Bromley et al., 2013; Tomura et al., 2008). As expected, we found that migration of CD301b⁺ DCs from the skin to the dLN (i.e., Kaede^{red} cells) was completely dependent on CCR7 (Figure 3C). However, although some baseline migration of CD301b⁺ DCs was detected in *Ccr8*^{-/-} Kaede⁺ mice, CCR8 was required for the allergen-induced DC emigration from the skin to the dLN (Figure 3D).

Our data suggest that CD301b⁺ DCs specifically require CCR8 for entry into the dLN after Th2 immunization. However, by immunofluorescence of the dLN after Th2 immunization, we found no significant difference in the total number of CD301b⁺ DCs between WT and *Ccr8*^{-/-} mice (Figures 3E and 3F). Instead, whereas CD301b⁺ DCs generally localized to the T-B interface of the dLN in WT mice after Th2 immunization, CD301b⁺ DCs in Th2-immunized *Ccr8*^{-/-} mice appeared to be excluded from entering the dLN and were retained at the periphery of the LN (Figure 3E). Closer inspection after Th2 immunization revealed an accumulation of CD301b⁺ DCs in the SCS space of *Ccr8*^{-/-} mice (Figures 3G and 3H). Because standard LN digestion protocols do not adequately release SCS stroma, these SCS CD301b⁺ DCs were unintentionally excluded in flow-cytometric experiments, leading to a decrease in CD301b⁺ DC numbers by flow cytometry but not immunofluorescence (Fletcher et al.,

2011; Gray et al., 2012). We were able to confirm this by using enhanced digestion methods designed to release LN stromal cells (Fletcher et al., 2011). Although standard digestion methods showed a decreased percentage of Kaede^{red} skin emigrant CD301b⁺ DCs in *Ccr8*^{-/-} mice (Figure 3D), no such defect was seen after enhanced digestion, indicating that enhanced digestion released CD301b⁺ DCs from the SCS (Figure S3B). Together, these data indicate that whereas CCR7 is essential for CD301b⁺ DC entry into the draining lymphatics, CCR8 plays an important role in promoting their migration across the floor of the LN sinus and into the dLN after Th2 immunization.

CD169⁺SIGN-R1⁺ Macrophages Produce *Ccl8*, which Is Required for CD301b⁺ DC Entry

Our initial CCL8 staining showed it to be closely associated with the lymphatic endothelium of the dLN, but we were unable to detect CCL8 or *Ccl8* RNA in Lyve-1⁺ or gp38⁺ lymphatic endothelium (data not shown). We next hypothesized that LN sinus macrophages found in association with lymphatic endothelium were the source of CCL8. LN sinus macrophages include CD169⁺SIGN-R1⁻F4/80⁻ SCS macrophages and F4/80⁺ medullary macrophages, which have been divided into CD169⁻F4/80⁺ medullary cord macrophages and CD169⁺SIGN-R1⁺F4/80⁺ medullary sinus macrophages (Gray and Cyster, 2012). In accordance with their name, medullary cord macrophages are present in the LN medullary region, and SCS macrophages make up the floor of the SCS overlying B cell follicles. However, CD169⁺SIGN-R1⁺ medullary sinus macrophages can be found along the lymphatic endothelium of the medullary region as well as IFRs (Gray and Cyster, 2012). CD169⁺ macrophages in the lamina propria have been shown to produce CCL8 in murine colitis models (Asano et al., 2015). Similarly, we detected CCL8 within CD169⁺F4/80⁺ macrophages after Th2-skewing immunization and visualized these cells in apposition with CD301b⁺ DCs (Figures 4A and S4A). Given their localization in serial sections, CCL8⁺CD169⁺ cells also expressed SIGN-R1, consistent with the F4/80⁺CD169⁺SIGN-R1⁺ macrophage population (Figure S4B). CD169⁺SIGN-R1⁻ SCS macrophages were specifically localized in the SCS floor adjacent to B cell follicles (Figure 4B, SCS), whereas CD169⁺SIGN-R1⁺ macrophages were present in the medullary region (Figure 4B, med) as well as in IFRs (Figure 4B, IFR). To confirm that CD169⁺SIGN-R1⁺ macrophages were the source of *Ccl8*, we utilized enhanced enzymatic digestion techniques to free LN-resident macrophages for further analysis (Fletcher et al., 2011). Using SIGN-R1⁺ selection by magnetic beads, we found that *Ccl8* and *Siglec1* (CD169) were both enriched in the SIGN-R1⁺ population (Figure 4C). We next performed flow-cytometric sorting of SIGN-R1⁺ cells, which identified the CD169⁺SIGN-R1⁺CD11c⁺CD11b⁺ macrophages as the source of *Ccl8* (Figure 4D). We confirmed this enrichment of *Ccl8* expression by qPCR by using an alternative sorting scheme to identify the CCL8-producing macrophages on the basis of CD11c⁺CD103⁺CD11b^{high}CD169⁺F4/80⁺

(D) Total CCL8⁺ cells per mm² of LN section from WT and *Ccl8*^{-/-} *Ccl12*^{-/-} mice 24 hr after Th0 or Th2 immunization.

(E) Percentage of CCL8⁺ cells in each indicated region out of the total CCL8⁺ cells per LN section in WT Th0- or Th2-immunized mice. Abbreviations are as follows: Follicle, B cell follicle; SCS, subcapsular sinus; TCZ, T cell zone; Med, medullary region; and IFR, interfollicular region.

Data represent three mice from each of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, Student's t test. See also Figure S2.

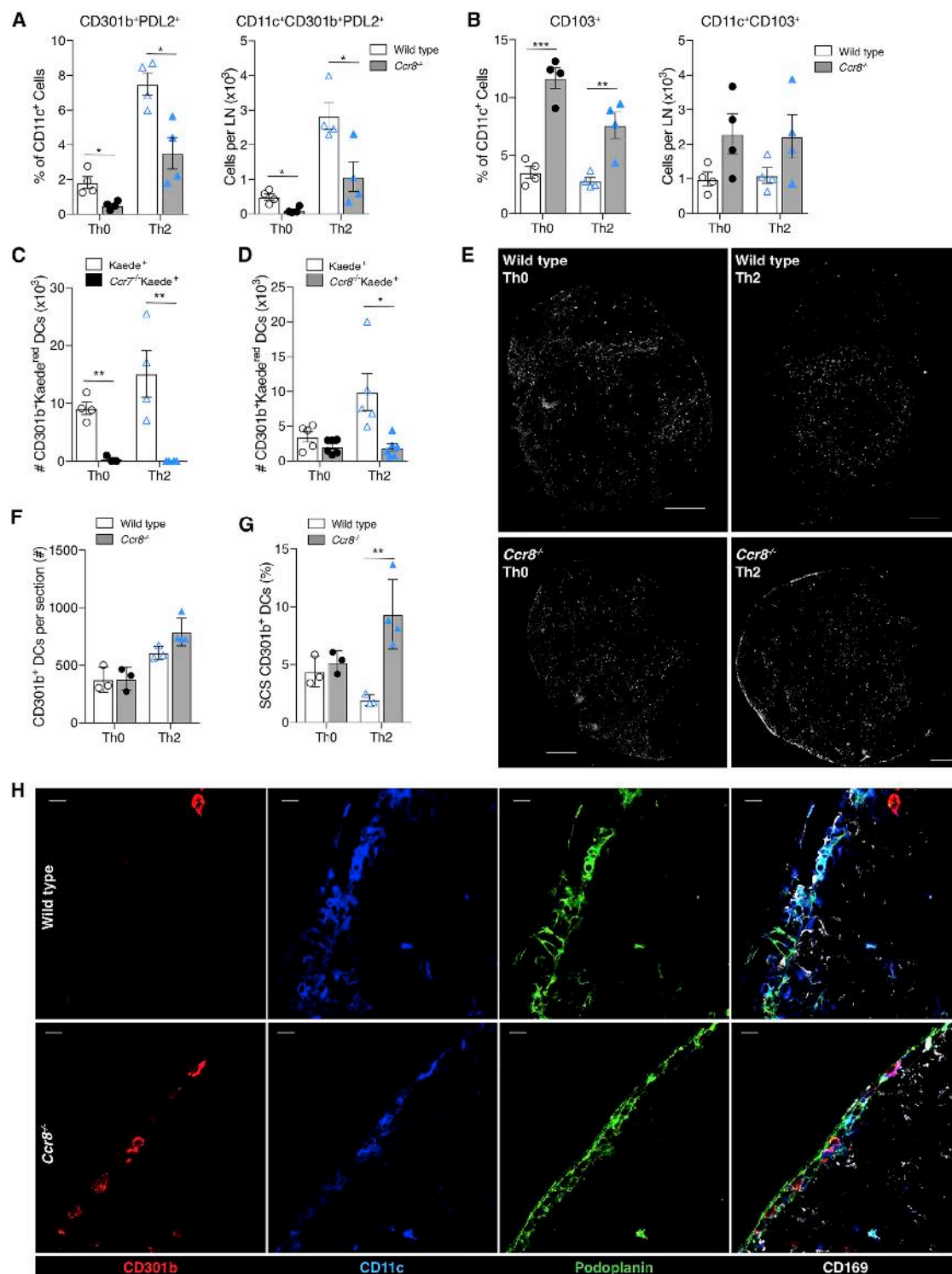


Figure 3. CCR8 Is Required for CD301b⁺ DC Entry into the dLN

(A and B) Quantification of CD301b⁺PDL2⁺ DCs (A) and CD103⁺ DCs (B) in the dLN of Th0- or Th2-immunized WT (clear bars) or *Ccr8*^{-/-} (gray bars) mice as determined by flow cytometry. Percentages reflect CD301b⁺PDL2⁺ or CD103⁺ cells out of the total CD11c⁺ cells, and numbers reflect total cells per popliteal LN. (C and D) Flow cytometry of inguinal LNs was performed 24 hr after immunization of photoconverted flank skin of Kaede⁺ transgenic mice. Total CD11c⁺CD301b⁺ Kaede^{red} (i.e., photoconverted) cells per inguinal LN are shown in comparison with (C) *Ccr7*^{-/-}Kaede⁺ and (D) *Ccr8*^{-/-}Kaede⁺ groups. (E) CD301b staining (white) of whole popliteal LNs of WT or *Ccr8*^{-/-} mice 24 hr after Th0 or Th2 immunization. Scale bars indicate 200 μ m.

(legend continued on next page)

expression (Figures S5A and S5B). Finally, to confirm our cell-sorting data, we depleted CD169⁺SIGN-R1⁺F4/80⁺ macrophages by using clodronate-mediated depletion (Figure S5A). Clodronate treatment completely abolished *Siglec1* and *Ccl8* expression within the dLN (Figure 4E). Together, these data indicate that CD169⁺SIGN-R1⁺ macrophages, located in the IFRs and LN medullary area, are the source of CCL8 in the dLN.

CCR8 has two ligands in mice: CCL1 and CCL8 (Griffith et al., 2014). CCL1 has been shown to be produced by the lymphatic endothelium on the floor of the SCS and to promote entry of monocyte-derived DCs into the dLN (Qu et al., 2004). We found that Th2-skewing immunization did not promote *Ccl1* production in the dLN (Figure 2B). However, to ensure that the defect in CD301b⁺ DC migration was due to CCL8 and not CCL1, we examined CD301b⁺ DC migration in *Ccl8*^{-/-} mice. Because of the genes' proximity, *Ccl8*^{-/-} mice are only currently available as *Ccl8*^{-/-}*Ccl12*^{-/-} dual-knockout mice (Tsou et al., 2007). Consistent with our data from *Ccr8*^{-/-} mice with standard enzymatic digestion, we found that *Ccl8*^{-/-}*Ccl12*^{-/-} mice exhibited defective CD301b⁺ DC migration by flow cytometry in both total frequency and number after Th2-skewing immunization (Figure 4F). Therefore, we conclude that CCL8 production from CD169⁺SIGN-R1⁺ macrophages is required for CD301b⁺ DC entry into the dLN after Th2-skewing immunization.

Analysis of *Ccl1*-*Ccl8* Dual-Reporter Transgenic Mice Confirms *Ccl8* Production by CD169⁺SIGN-R1⁺ Macrophages

In order to verify our CCL8 expression data, we generated a transgenic mouse, called REC8 (reporting the expression of CCR8 ligands), that reports the expression of both CCL1 and CCL8. To allow for detection of *Ccl1* and *Ccl8* expression, we inserted mCherry at the start codon of *Ccl1* and eGFP at the start codon of *Ccl8* in a bacterial artificial chromosome (BAC) that contained *Ccl1* and *Ccl8* (Figure 5A). Consistent with RNA data from dLNs (Figure 2B), *Ccl1*-mCherry expression was unaffected, whereas *Ccl8*-eGFP expression was induced by Th2 immunization (Figure 5B). CCL1 has been described to be produced by CD3⁺ T cells as well as the lymphatic endothelium (Miller and Krangel, 1992; Qu et al., 2004). We were unable to detect *Ccl1*-mCherry expression in lymphatic endothelial cells, blood endothelial cells, or fibroblastic reticular cells of the dLN (Figure S5C). Instead, we found that *Ccl1*-mCherry⁺ cells were uniformly CD3⁺, whereas *Ccl8*-eGFP⁺ cells were CD11b⁺ (Figure 5C). Accurate *Ccl1* and *Ccl8* reporting by REC8 mice was confirmed by enrichment of *Ccl1* and *Ccl8* RNA within the mCherry⁺ and eGFP⁺ populations, respectively (Figure 5D). The expression of CD11b on *Ccl8*-eGFP⁺ cells suggested that *Ccl8*-eGFP marked CD169⁺SIGN-R1⁺ macrophages. Confirming this, CD169⁺SIGN-R1⁺ macrophages exhibited *Ccl8*-eGFP expression, which was induced by Th2 immunization, but not *Ccl1*-mCherry expression

(Figure 5E). These data indicate that Th2 immunization specifically induces *Ccl8* expression by CD169⁺SIGN-R1⁺ macrophages.

CCR8 Promotes CCR7-Mediated Migration of CD301b⁺ DCs

Our data suggest that whereas both CCR7 and CCR8 are necessary for CD301b⁺ DC migration, CCR8 plays a specific role in promoting CD301b⁺ DC migration across the SCS and into the LN parenchyma. *Ccr8* mRNA has been detected in dermal DC populations, but the detection of CCR8 has been hampered by the lack of suitable reagents (Jakubzick et al., 2006; Qu et al., 2004; Sallusto et al., 1998; Yabe et al., 2015). Flow-cytometric staining using commercially available anti-mouse CCR8 antibodies consistently showed staining of DCs from *Ccr8*^{-/-} mice (data not shown). Therefore, to determine the expression of CCR8 on CD301b⁺ DCs, we utilized a fluorescently labeled ligand of CCR8, human CCL1 (hCCL1). hCCL1 binds to the murine CCR8 receptor, inducing calcium (Ca²⁺) flux and chemotaxis in transfected cell lines (Islam et al., 2011). Using this reagent to fluorescently "label" CCR8, we found that, compared with DCs from *Ccr8*^{-/-} mice, CD11c⁺CD11b⁺CD301b⁻ DCs (CD11c⁺ DCs) did not express detectable CCR8 but that CD11c⁺CD11b⁺CD301b⁺ DCs (i.e., CD301b⁺ DCs) from the dLNs of Th0-immunized mice expressed detectable CCR8 that increased after Th2 immunization (Figures 6A and S6A). We found no significant difference in CCR7 expression between WT and *Ccr8*^{-/-} DCs after Th0 or Th2 immunization (Figure 6B). As expected, *ex vivo* CCL21 stimulation of CD301b⁺ DCs promoted CCR7 internalization, but this was unaffected by co-stimulation with CCL8 (Figure S6B). Likewise, CCL21 stimulation had no effect on CCR8 expression (Figure S6B). These data suggest that CCR8 is expressed by CD301b⁺ DCs and that CCR8 expression does not affect CCR7 expression.

We next performed *in vitro* chemotaxis assays to determine what role CCR8 and its ligand, CCL8, play in CD301b⁺ DC migration. CCL8 had no effect on migration of the CCR8⁻CD11c⁺ DCs (Figure 6C). Despite expression of CCR8, CD301b⁺ DCs showed no migration in response to CCL8 alone (Figure 6D). However, the combination of CCL8 and the CCR7 ligand CCL21 was synergistic in CD301b⁺ DCs, leading to an almost doubling of migration at the physiologic 100 ng/mL concentration of these chemokines (Figure 6D). This synergy was also seen with the other CCR8 ligand, CCL1 (Figure S6C). *In vitro* assays with μ M chemokine concentrations have shown that human CCL1, CCL2, CCL7, CCL8, and CCL11 synergize with CCL21 by forming heteromeric chemokine complexes that promote the migration of mature human DCs (Paoletti et al., 2005). Therefore, we examined whether this synergistic effect on CCR7-mediated CD301b⁺ DC migration is similarly indiscriminate but found no effect of murine CCL2, CCL7, or CCL11 on CD301b⁺ DC migration in the presence or absence of CCL21 (Figures S6D–S6F).

(F and G) Quantification of the total number CD11c⁺CD301b⁺ cells per entire LN section (F) or the percentage of total CD11c⁺CD301b⁺ within the SCS (G) (identified by podoplanin and CD169 immunohistochemical staining) 24 hr after immunization.

(H) CD301b⁺ DCs within the SCS space of *Ccr8*^{-/-} mice, but not WT mice, 24 hr after Th2 immunization. Panels show single stains of CD301b (red), CD11c (blue), and podoplanin (green) and an overlay of those stains and CD169 (white). Scale bars indicate 10 μ m.

Images (E and H) represent two to three mice per condition and four independent experiments. Bar graphs represent the mean value of individual mouse data points (three to six mice per condition per experiment) taken from a representative of three independent experiments (A, B, D, and F–H) or two independent experiments (C). Error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001, Student's t test. See also Figure S3.

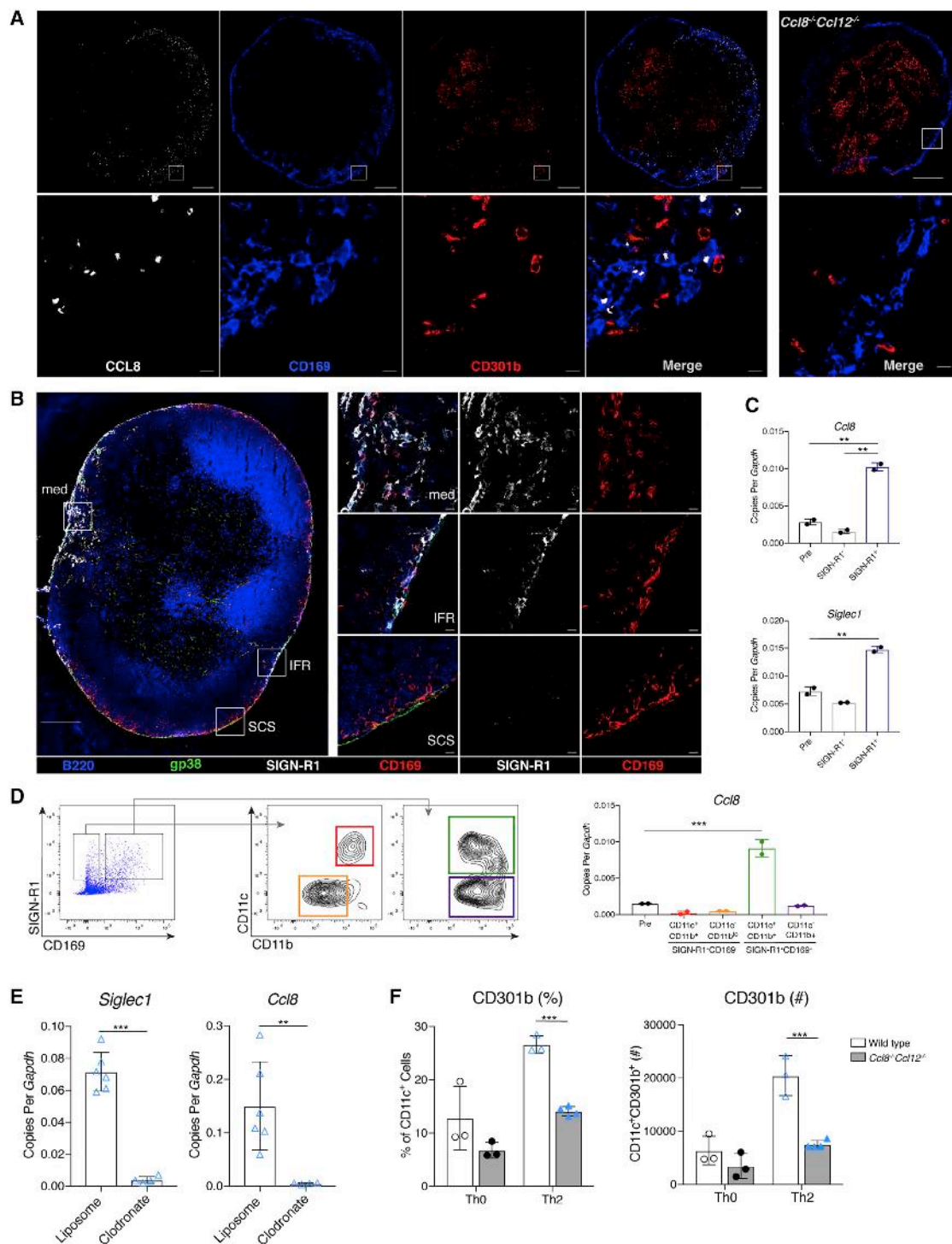


Figure 4. CCL8 Is Produced in the dLN by CD169⁺SIGN-R1⁺ LN Macrophages that Interact with CD301b⁺ DCs

(A) Confocal immunofluorescence of whole popliteal LN (top) and the area of interest (bottom) 24 hr after Th2-skewing immunization in WT or *Ccl8*^{-/-}*Ccl12*^{-/-} mice as indicated. Panels show single stains of CCL8 (white), CD169 (blue), and CD301b (red) and an overlay. Scale bars represent 200 μ m (top) and 10 μ m (bottom). Images represent three mice in each of three independent experiments.

(B) Confocal immunofluorescence of whole popliteal LN. Insets show CD169⁺SIGN-R1⁺ macrophage populations in medullary regions (med) and IFRs, as well as CD169⁺SIGN-R1⁻ SCS macrophages in the SCS. Staining shows CD169 (red), SIGN-R1 (white), gp38 (green), and B220 (blue). Scale bars represent 200 μ m (whole LN) and 10 μ m (insets).

(C) qPCR analysis of total cells (Pre) or MACS positively selected SIGN-R1⁺ or SIGN-R1⁻ cells from popliteal LNs.

(legend continued on next page)

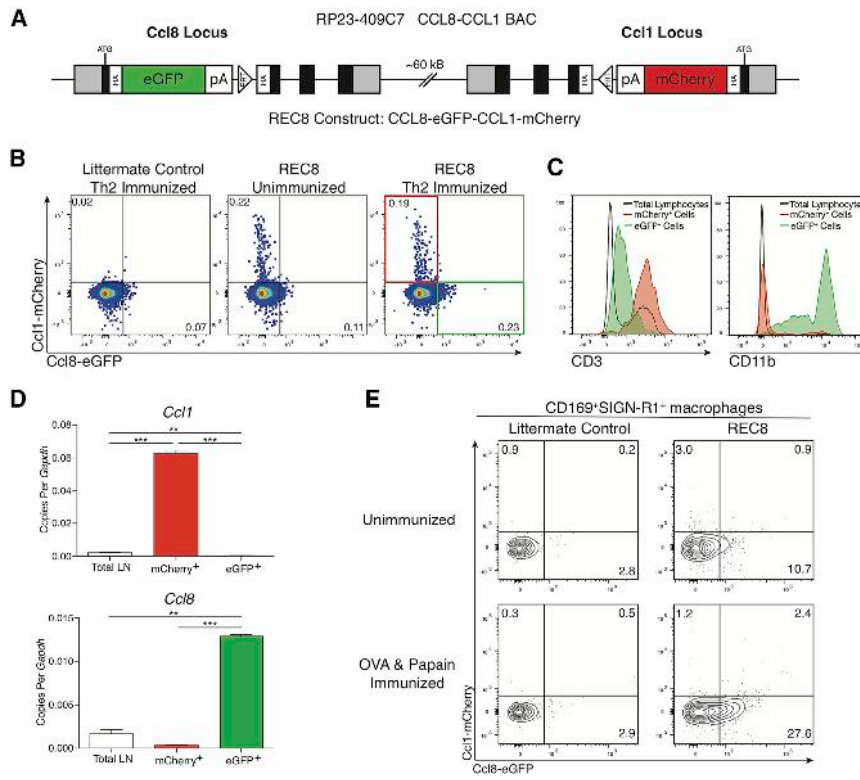


Figure 5. CCR8 Ligands Are Differentially Expressed in the dLN

(A) Schematic of REC8 transgene construct indicates the insertion of eGFP into the *Ccl8* locus and mCherry into the *Ccl1* locus of the RP23-409C7 BAC. Gray box, non-coding exons; black box, endogenous coding exons; green box, eGFP open reading frame (ORF); red box, mCherry ORF; clear box, hemagglutinin tags or SV40 poly(A); clear triangle, flippase recognition target (FRT) site. (B) Expression of mCherry and eGFP in total live dLN cells in unimmunized REC8 mice or in Th2-immunized REC8 mice or littermate control WT mice. Numbers are the percentages of total cells in the indicated gates.

(C) Expression of CD3 and CD11b in total live dLN cells (black), mCherry+ cells (red), or eGFP+ cells (green) gated as in (B) and isolated from REC8 mice 24 hr after Th2-skewing immunization. (D) qPCR analysis of *Ccl1* or *Ccl8* expression in total LN (clear) or fluorescence-activated-cell-sorted mCherry+ cells (red bar) and eGFP+ cells (green bar) gated as in (B) from dLNs harvested from REC8 mice 24 hr after Th2-skewing immunization.

(E) Expression of mCherry and eGFP on live CD11b+CD169+SIGN-R1+ cells harvested from the dLN of unimmunized WT and REC8 mice and 24 hr after Th2-skewing immunization. Bar graphs represent the mean value of replicates (two data points per bar).

Data represent two (D) or three (A–C and E) independent experiments. Error bars represent SEM. ***p* < 0.01, ****p* < 0.001, Student's *t* test. See also Figure S5.

Indeed, the migration synergy induced by murine CCL8 and CCL21 was dependent on CCR8 expression in CD301b+ DCs given that it was absent in *Ccr8*^{−/−} CD301b+ DCs (Figure 6E). To determine whether a CCL8 gradient is necessary for its synergistic effect, we performed a checkerboard chemotaxis assay in which CD301b+ DCs harvested from the dLN of Th2-immunized mice were exposed to combinations of CCL8 and CCL21 in either gradients or fixed concentrations. Whereas CCL21-mediated migration required a CCL21 gradient, the synergistic effect of CCL8 on CCL21-induced migration of CD301b+ DCs was independent of the presence of a CCL8 gradient (Figure 6F). These data suggest that the production of CCL8 is an innate control mechanism to specifically promote the migration of Th2-skewing CD301b+ DCs by altering their responsiveness to CCL21 gradients.

Chemokine receptors are G-protein-coupled receptors (GPCRs) that classically signal through G α i to promote cell migration. We hypothesized that CCL8 augments CCL21-medi-

ated migration in CD301b+ DCs by enhancing GPCR-mediated signaling. To study this, we measured Ca²⁺ flux in CD301b+ DCs isolated from the dLNs of Th2-immunized mice. We were able to detect Ca²⁺ flux in CD301b+ DCs exposed to either CCL8 or CCL21 at 10 and 100 ng/mL concentrations (Figure 6G). Although exposure to either CCL8 or CCL21 at 1 ng/mL did not induce Ca²⁺ flux, the combination of CCL8 and CCL21 at 1 ng/mL did induce Ca²⁺ flux (Figure 6H). This indicates that perhaps CCL8 increases the sensitivity of CD301b+ DCs to low amounts of CCL21 given that we found no augmentation in Ca²⁺ flux at higher chemokine concentrations (Figure S6G). We hypothesized that, in addition to inducing Ca²⁺ signaling, CCL8 might activate other signaling pathways that contribute to cell migration. It was recently reported that prostaglandin E₂ augments CCL21-induced human DC migration by increasing signaling via Src kinase and the tyrosine phosphatase SHP2 (Hauser et al., 2016). Using a human CCR8-transfected cell line (Islam et al., 2011), we found that murine CCL8 stimulation

(D) Gating scheme for identifying macrophage populations from total viable lymphocytes obtained 24 hr after Th2 immunization from dLN. qPCR analysis of the indicated populations shows *Ccl8* expression. Colored histograms correspond to colored gates.

(E) qPCR from total LNs of control liposome- or clodronate-treated mice 24 hr after Th2-mediated immunization. Data points are from four to six individual mice in one representative experiment out of three independent trials.

(F) Flow cytometry of viable CD11c+CD301b+ cells shows a decreased percentage and number of CD301b+ DCs in *Ccl8*^{−/−}*Ccl12*^{−/−} mice after Th2-skewing immunization. Data points are from dLNs of three or four individual mice from one of three independent trials.

Cells in (C) and (D) were obtained by enhanced enzymatic digestion, and those in (F) were obtained by routine enzymatic digestion. qPCR data are presented as copies of the indicated transcript over *Gapdh*. Bar graphs represent the mean value of the indicated data points, and error bars represent SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, Student's *t* test. See also Figures S4 and S5.

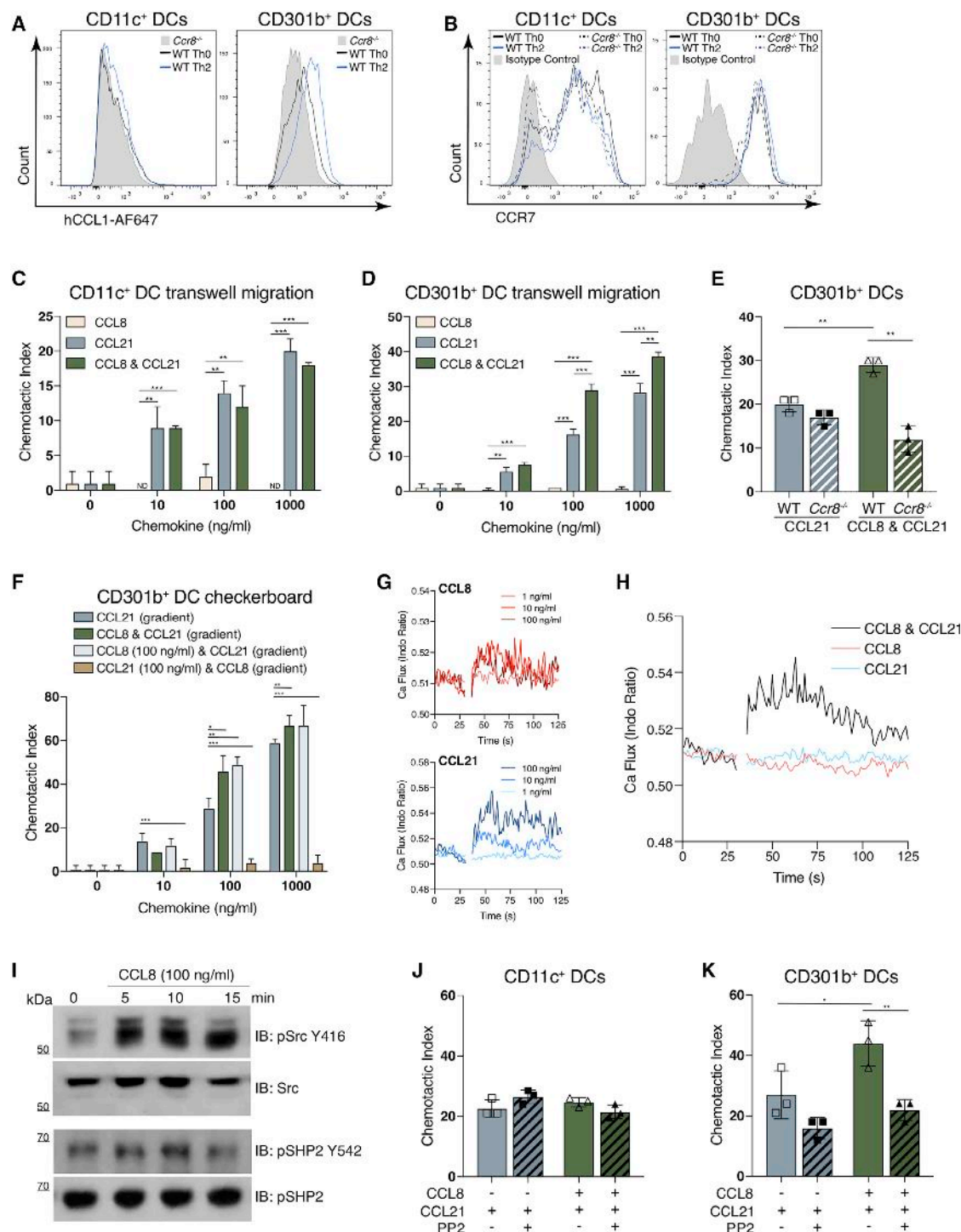


Figure 6. CCL8 Synergizes with CCL21 to Specifically Promote CD301b⁺ DC Migration

(A) Flow-cytometric expression of hCCL1-AF647 binding to CD11c⁺CD11b⁺CD301b⁺ (CD11c⁺ DCs) or CD11c⁺CD11b⁺CD301b⁺ (CD301b⁺ DCs) cells obtained from the dLNs of WT mice after the Th0-skewing (black histogram) or Th2-skewing (blue histogram) immunization or from *Ccr8*^{-/-} mice after Th0 immunization (gray shaded histogram).

(B) Flow-cytometric cell-surface expression of CCR7 on CD11c⁺ DCs or CD301b⁺ DCs from the dLN after Th0 immunization in WT (black solid) or *Ccr8*^{-/-} (black dotted) mice or after Th2 immunization in WT (blue solid) or *Ccr8*^{-/-} (blue dotted) mice. The isotype control from WT Th0-immunized mice is shown (gray shaded histogram).

(C and D) Transwell chemotaxis of CD11c⁺ DCs (C) or CD301b⁺ DCs (D) to the indicated concentrations of CCL8, CCL21, or CCL8 and CCL21.

(E) Transwell chemotaxis of CD301b⁺ DCs isolated from WT or *Ccr8*^{-/-} mice to 100 ng/mL of the indicated chemokines.

(legend continued on next page)

induced the phosphorylation of Src and SHP2 within 5 and 10 min, respectively (Figure 6I). Therefore, we hypothesized that CCL8 could similarly promote signaling through the Src-SHP2 pathway to enhance CCL21-mediated migration in CD301b⁺ DCs. Chemotaxis of CD11c⁺ DCs to CCL21 was unaffected by the presence of the Src inhibitor PP2 (Figure 6J). However, in CD301b⁺ DCs, Src inhibition abolished the synergistic effect of CCL8 on CCL21-mediated migration (Figure 6K). Thus, CCL8 directly promotes Src-SHP2 signaling and augments CCL21-mediated migration in CD301b⁺ DCs in a Src-dependent manner.

CCR8-Mediated Entry of CD301b⁺ DCs into the dLN Is Required for Th2 Differentiation

Our data suggest that the production of CCL8 by CD169⁺SIGN-R1⁺ macrophages promotes the migration of CCR7^{lo}CD301b⁺ DCs by inducing a Src-dependent signaling pathway. This led us to hypothesize that the production of CCL8 by CD169⁺SIGN-R1⁺ macrophages is a mechanism by which the innate immune system controls the polarization of the adaptive immune response. Therefore, we investigated whether CCR8 deficiency leads to alterations in Th development by examining the immune response in *Ccr8*^{-/-} mice that had previously received WT naive OVA-specific CD4⁺ OTII cells. In response to Th2 immunization (OVA and papain), Th2 differentiation displayed a significant defect according to interleukin-4 (IL-4) production during *in vitro* restimulation, but we found no defect in interferon- γ (IFN- γ) production upon restimulation after Th1-skewing immunization (Figure 7A). To assess whether this defect in Th2 differentiation was limited to papain immunization, we next examined CD4⁺ T cell differentiation after immunization with the alternative Th2-skewing agent, OVA emulsified in aluminum hydroxide (OVA-alum), or infection with *Nippostrongylus brasiliensis* (*N. brasiliensis*). Regardless of the Th2-skewing immunization, Th2 differentiation showed a significant defect on the basis of IL-4 and IL-13 production from transferred OVA-specific CD4⁺ OTII cells in *Ccr8*^{-/-} mice (Figures 7B, 7C, and S7). These data indicate that Th2 differentiation in response to cutaneous immune stimuli requires CCR8.

To determine whether this phenotype seen in *Ccr8*^{-/-} mice is the result of CCR8 deficiency specifically on the CD301b⁺ DC subset, we produced mixed bone marrow (BM) chimeras by utilizing WT, CD301b-DTR (diphtheria toxin receptor), and *Ccr8*^{-/-} mice. As illustrated in Figure 7D, we produced three groups of mice: WT, *Ccr8*^{-/-}, and CD301b^{Ccr8}^{-/-}. WT mice were mixed chimeras of WT and CD301b-DTR cells, allowing us to control for any effect caused by depletion of CD301b⁺ DCs derived

from CD301b-DTR precursors, whereas *Ccr8*^{-/-} mice were standard BM chimeras into WT mice (Figure 7E). CD301b^{Ccr8}^{-/-} mice were mixed chimeras of CD301b-DTR and *Ccr8*^{-/-} BM cells, allowing us to examine mice in which the overall immune compartment was mixed but in which the CD301b⁺ DC compartment was derived from *Ccr8*^{-/-} mice after diphtheria toxin (DT)-mediated depletion (Figure 7E). As expected, *Ccr8*^{-/-} BM chimeras showed a defect in Th2 differentiation, as measured by IL-4 and IL-13 production upon *in vitro* restimulation of previously transferred OVA-specific CD4⁺ T cells (Figure 7F). CD301b^{Ccr8}^{-/-} mice, in which all CD301b⁺ DCs were CCR8-deficient cells but the rest of the immune cells were a mixture of CCR8-sufficient and CCR8-deficient cells, also showed a defect in Th2 differentiation (Figure 7F). Restricting complete CCR8 deficiency to the CD301b⁺ DC population led to a significant loss of IL-4 production that was between those seen in the WT and *Ccr8*^{-/-} groups and a loss of IL-13 production that was indistinguishable from that in *Ccr8*^{-/-} chimeras. Because both Th2 and Tfh cells produce IL-4 but only Th2 cells produce IL-13, we believe that these data indicate that CCR8 expression on CD301b⁺ DCs is required for their migration into the dLN after allergen exposure and is necessary for subsequent Th2 differentiation. However, the intermediate level of IL-4 production seen in CD301b^{Ccr8}^{-/-} chimeras could indicate that another, non-CD301b⁺ DC subset involved in Tfh development requires CCR8 for entry into the dLN and subsequent Tfh differentiation (Kumamoto et al., 2016).

DISCUSSION

A fundamental paradigm in immunity is the role of DCs in bridging the innate and adaptive immune responses. DCs are positioned throughout the periphery, where they are poised for early detection of pathogens. Pathogens induce DC maturation through activation of their PRRs, which leads to CCR7 upregulation and migration into the dLN. Within the dLN, DCs can interact with naive T cells to activate the adaptive immune response by providing antigen presentation, co-stimulation, and skewing signals. However, this paradigm does not address the evidence that specific DC subsets play distinct roles in T cell activation. Dermal IRF4⁺CD301b⁺PDL2⁺ DCs and lung KLF4⁺ DCs migrate to dLNs after allergen exposure and have been shown to play a necessary role in Th2 differentiation, whereas IRF4⁺KLF4⁻ DCs play a specific role in the differentiation of Th17 cells (Gao et al., 2013; Kumamoto et al., 2013; Tussiwand et al., 2015; Williams et al., 2013). Given that the migration of DC subsets is stimulus specific and that DC subsets induce different adaptive immune

(F) Checkerboard Transwell migration assay of CD301b⁺ DCs in response to CCL21 gradient alone (blue), CCL8 and CCL21 combined gradient (green), fixed CCL8 concentration and CCL21 gradient (light blue), and fixed CCL21 concentration and CCL8 gradient (brown).

(G) Analysis of Ca²⁺ flux in CD301b⁺ DCs. The gap indicates the addition of CCL8 or CCL21 at the indicated concentration.

(H) Ca²⁺ flux in CD301b⁺ DCs. The gap indicates the addition of 1 ng/mL of CCL8 and CCL21 (black), CCL8 (pink), and CCL21 (blue).

(I) Western blot of Src and SHP2 phosphorylation in serum-starved 4DE4 cells stably transfected with human CCR8.

(J and K) Transwell chemotaxis of CD11c⁺ DCs (J) or CD301b⁺ DCs (K) in response to 100 ng/mL of the indicated chemokines with or without 10 μ M PP2 (Src inhibitor).

In all panels, cells were harvested from dLNs 24 hr after the indicated immunization. Graphs in (A), (B), (G), and (H) represent four independent experiments with three mice per group. We obtained cells by pooling dLNs from 30–50 mice (C–F, J, and K) or 12 mice (G and H) 24 hr after Th2 immunization and using fluorescence-activated cell sorting to obtain CD11c⁺ DCs or CD301b⁺ DCs. Bar graphs represent the mean value of replicates (two to three data points per bar) and are one representative trial of four independent experiments. Error bars represent SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, Student's *t* test. See also Figure S6.

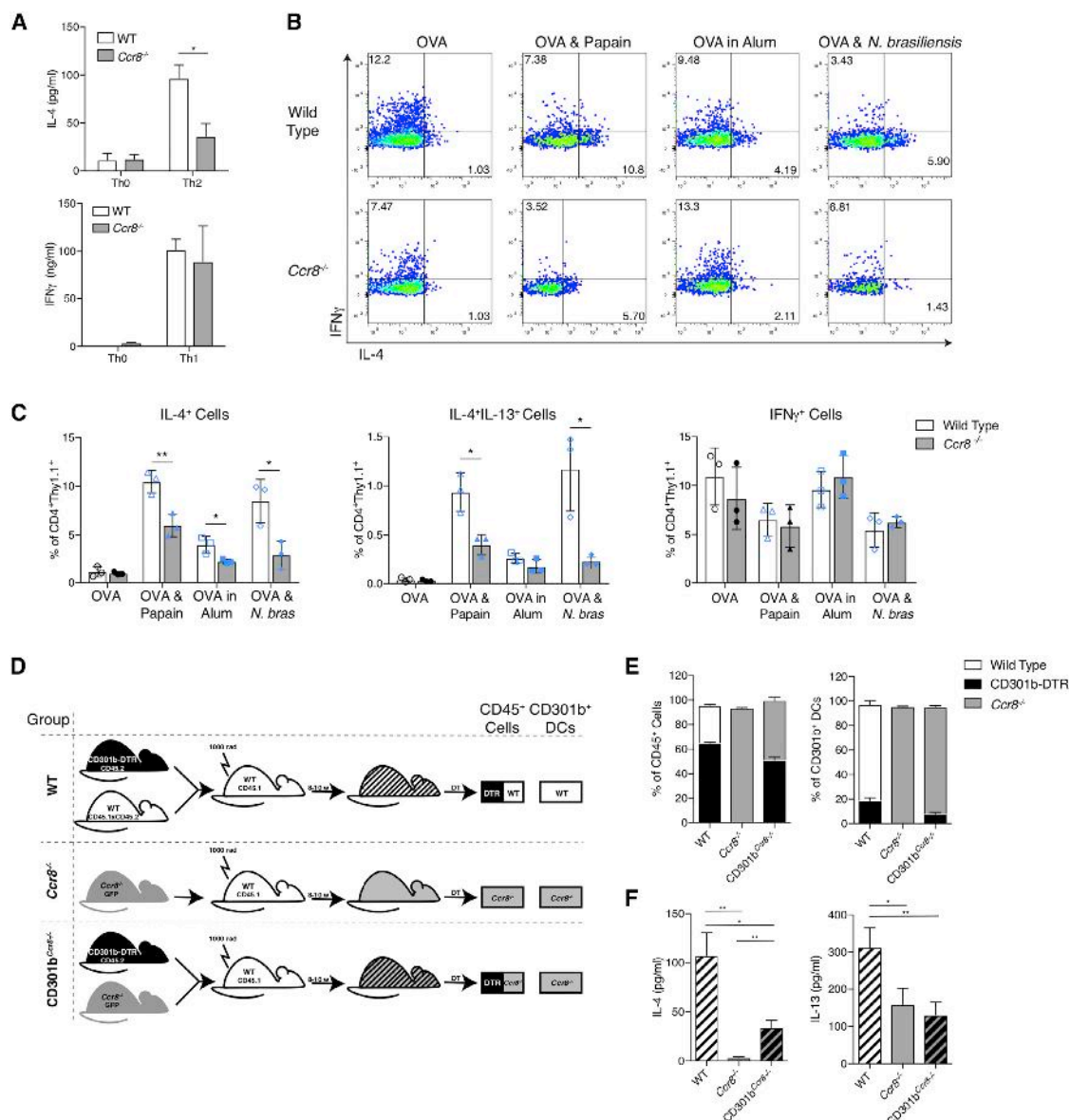


Figure 7. CCR8 on CD301b⁺ DCs Is Required for Allergen-Induced Th2 Differentiation

(A) IL-4 and IFN-γ concentrations in supernatants after *in vitro* restimulation of total dLN cells from WT or *Ccr8*^{-/-} mice after Th0- or Th2-skewing immunizations (IL-4) or Th0- or Th1-skewing immunizations (IFN-γ). Mice received 2.5 × 10⁵ CD4⁺ OTII cells before immunization, dLNs were harvested 5 days after immunization for *in vitro* restimulation with OVA, and supernatants were obtained 4 days later.

(B) Intracellular IL-4 and IFN-γ staining of CD4⁺Thy1.1⁺ viable cells 4 days after OVA, OVA plus papain, or OVA-alum immunization or 5 days after OVA and *N. brasiliensis* infection. Mice received 2.5 × 10⁵ to 5 × 10⁵ naive CD4⁺Thy1.1⁺ OTII cells before immunization. Representative plots are shown with the percentage of cells in the indicated quadrants.

(C) Quantification of IL-4⁺, IL-4⁺IL-13⁺, or IFN-γ⁺ staining of CD4⁺Thy1.1⁺ cells after immunization or infection as in (B).

(D) Generation of BM chimeras.

(E) Chimerism by flow-cytometric analysis of total viable CD45⁺ cells and CD11c⁺CD301b⁺ DCs from popliteal LNs 3 days after last DT injection.

(F) IL-4 and IL-13 concentrations in supernatants from *in vitro* restimulation of total LN cells from the indicated BM chimeras. Chimeric mice received 2.5 × 10⁵ CD4⁺ OTII cells and DT before Th2-skewing immunization. dLNs were harvested 5 days after immunization and cultured with OVA for 4 days before supernatant harvest.

Data represent three (A–C) or five (D and E) independent experiments each with three to five mice per condition. Bar graphs represent the mean value, and error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001, Student's t test. See also Figure S7.

outputs, we were interested in defining the mechanisms that promote this specificity in DC migration. To do this, we utilized subcutaneous papain immunization, which promotes Th2 differ-

entiation via the migration of CD301b⁺ dermal DCs into the dLN. Despite robust migration to the dLN after Th2 immunization, CD301b⁺ DCs did not display more CCR7 than after Th1

immunization, leading us to hypothesize that additional mechanisms might promote CD301b⁺ DC migration *in vivo*.

Chemokines are key regulators controlling the migration and positioning of immune cells. CCR7 and its ligands CCL19 and CCL21 control the migration of mature DCs from the periphery to dLNs (Worbs et al., 2017). Within the dLN, chemokines promote the positioning of DCs, T cells, and accessory cells within specific niches where they allow for optimal T cell activation and effector differentiation (Griffith et al., 2014; Lian and Luster, 2015). There is evidence that CCR8 and its ligand CCL1 could play a role in the migration of monocyte-derived DCs from the skin and of conventional DCs from the lung to the dLN (Jakubczik et al., 2006; Qu et al., 2004). However, we were unable to detect any induction of *Ccl1* transcript or reporter expression in the dLN in response to Th2 immunization. Instead, Th2 immunization was specifically associated with upregulation of the other CCR8 ligand, *Ccl8*, in the dLN, and this temporally correlated with DC migration. CCR8 has been associated with allergic inflammation and is present on Th2 cells, immature DCs, and monocytes. CCR8 has also been shown to play a potential role in the skewing of the adaptive immune response, although the results have been mixed. *Ccr8*^{-/-} mice show attenuated effector type 2 immune responses to *Schistosoma mansoni* soluble egg antigen, OVA, and cockroach antigen-induced allergic airway inflammation, as well as allergic skin inflammation in a model of atopic dermatitis (Chensue et al., 2001; Islam et al., 2011). However, these defects were thought to be the result of defective CCR8 expression by Th2 cells. Arguing in favor of a role for CCR8 expression on DCs, in a model of Th1- and Th17-mediated contact hypersensitivity, *Ccr8*^{-/-} mice were shown to have increased numbers of CD11c⁺ cells in the dLN in association with increased Th1 and Th17 differentiation (Yabe et al., 2015). At the same time, others have not been able to find a role for CCR8 in Th2-mediated inflammation (Chung et al., 2003; Goya et al., 2003). The cause of this discrepancy is unknown, but our data suggest that it could be due to the dependence of different models on CD301b⁺ dermal DCs. We found that disparate Th2-skewing stimuli, including papain, alum, and *N. brasiliensis* infection, all require CCR8 for CD301b⁺ DC migration and Th2 differentiation. Spontaneous migration of CD301b⁺ DCs appears to be unaffected by CCR8 deficiency, raising the question of whether spontaneous migration is functionally different. Additionally, it remains to be determined whether CCR8-mediated migration is a global mechanism used to control the migration of Th2-skewing DCs from non-cutaneous tissues.

We found that, instead of acting to directly promote chemotaxis, CCL8 augments CCL21-induced migration by two potential mechanisms. First, dual exposure of CD301b⁺ DCs to CCL8 and CCL21 leads to Ca²⁺ flux at chemokine concentrations that are individually incapable of inducing a Ca²⁺ flux, suggesting that CCR8 signaling might increase CD301b⁺ DC sensitivity to CCR7-ligand-induced migration. Alternatively, CCL8 directly induces Src-SHP2-mediated signaling in a CCR8-transfected cell line, and the augmentation of CCL21-mediated CD301b⁺ DC chemotaxis by CCL8 requires Src signaling. Prostaglandin-E₂-induced oligomerization of CCR7 has been shown to promote biased signaling through the Src-SHP2 pathway, which is required for optimal migration of human monocyte-derived DCs in response

to CCL21 signaling (Hauser et al., 2016). By promoting Src-SHP2 signaling in CCR8⁺CD301b⁺ DCs, CCL8 could facilitate the ability of CCR7 ligands to induce the migration of CD301b⁺ DCs across the lymphatic endothelium and into the dLN after Th2 immunization.

Why is another signal necessary for CD301b⁺ DCs to cross the border of the LN sinuses into the LN parenchyma? One possibility is based on the observation that chemokine receptors internalize after signaling, thus muting their responsiveness to ligands after signaling. This internalization has been postulated to underlie the role of the chemokine scavenger receptor ACKR4 in permitting conventional DCs to re-express CCR7, allowing them to cross the SCS (Ulvmar et al., 2014). Because of their relatively low CCR7 expression, CD301b⁺ DCs might not be able to recover from such ligand mediated internalization and require an additional chemokine signal. Alternatively, induction of the Src-SHP2 signaling pathway could be a crucial step for traversing the lymphatic endothelium at IFRs. In the absence of CCR8, CCR7 oligomerization can induce this signaling pathway, but oligomerization might require more surface CCR7 than those expressed by CD301b⁺ DCs. Although the precise molecular mechanism remains to be uncovered, we have shown a specific and necessary role for CCR8 in promoting CCR7-mediated migration in subset-specific DC migration.

Beyond showing a role for CCL8-CCR8 in CD301b⁺ DC migration, our data also reveal a role for CD169⁺SIGN-R1⁺ LN macrophages in controlling innate and adaptive immune interactions. SCS macrophages have been shown to efficiently capture lymph-borne virus particles and present them to B lymphocytes, thereby preventing lymphatic spread of virus and promoting antigen-specific immunity (Junt et al., 2007). But the role of other LN macrophage populations during the primary immune response is largely unknown. Through their positive effects on CD301b⁺ DC migration, CD169⁺SIGN-R1⁺ macrophages could play an important role in promoting a Th2-biased response. However, the precise mechanism by which they respond to protease allergen exposure remains unclear. Papain freely flows to the dLN and so might be able to directly activate LN macrophages. Alternatively, papain immunization has been shown to promote epithelial production of reactive oxygen species, thymic stromal lymphopoietin, and IL-33, all of which have been shown to play roles in the induction of the allergic immune response (Halim et al., 2014; Tang et al., 2010). Whether one of these epithelial derived factors activates CD169⁺SIGN-R1⁺ macrophages remains to be elucidated.

We propose a stepwise model for the allergen-induced migration of CD301b⁺ DCs. In the first step, DCs use CCR7-mediated signals to migrate into the draining lymphatics and enter the SCS space. In the second step, CCR8⁺ CD301b⁺ DCs respond to CCL8 produced by CD169⁺SIGN-R1⁺ macrophages, which allows them to transmigrate across the SCS floor and into the LN parenchyma. Such a process was previously identified in a melanoma metastasis model in which tumor cells entered the draining lymphatics and were able to cross the SCS of the dLN only if they expressed CCR8 on their surface (Das et al., 2013). This mirrors the accumulation of CD301b⁺ DCs seen in the LN sinuses of *Ccr8*^{-/-} mice. We propose that this stepwise migration process evolved to specifically promote CD301b⁺ DC migration and adaptive immune bias, but in melanoma

metastasis, this control point is hijacked by cancer cells. Thus, we believe that our model could provide insights that might be generalizable to cell migration and cancer metastasis beyond DC migration.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- [KEY RESOURCES TABLE](#)
- [CONTACT FOR REAGENT AND RESOURCE SHARING](#)
- [EXPERIMENTAL MODEL AND SUBJECT DETAILS](#)
 - Mice
- [METHOD DETAILS](#)
 - Generation of REC8 Mice
 - Immunizations and Cell Transfers
 - Tissue Preparation
 - Generation of Mixed Bone Marrow Chimeras
 - Flow Cytometry
 - Enzyme-Linked Immunosorbent Assay (ELISA)
 - *In Vitro* Restimulation Assays
 - *In Vitro* Chemotaxis Assays
 - RNA Purification and qPCR Analysis
 - Confocal Immunofluorescence
 - Kaede Photoconversion
 - Clodronate-Mediated Cell Depletion
 - Immunoblotting
 - Ca²⁺ Flux Assay
- [QUANTIFICATION AND STATISTICAL ANALYSIS](#)

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at <https://doi.org/10.1016/j.immuni.2018.07.012>.

ACKNOWLEDGMENTS

Work was supported by NIH grants R37AI040618 (to A.D.L.) and K08AI121421 (to C.L.S.). C.L.S. was supported by NIH grant T32HL116275 and the Asthma and Allergy Foundation of America. Flow cytometry was performed in the Department of Pathology Flow and Image Cytometry Research Core at Massachusetts General Hospital with support from the NIH Shared Instrumentation Program (1S10OD012027-01A1, 1S10OD016372-01, 1S10RR020936-01, and 1S10RR023440-01A1). We thank Drs. Sergio Lira, Israel Charo, and Osami Kanagawa for providing breeding pairs of *Ccr8*^{−/−}, *Ccl8*^{−/−}*Ccl12*^{−/−}, and *Kaede* transgenic mice, respectively. We thank Drs. Robert Anthony and Joseph F. Urban for providing *Nippostrongylus brasiliensis*.

AUTHOR CONTRIBUTIONS

Conceptualization, C.L.S. and A.D.L.; Methodology, C.L.S., R.B.C., and A.D.L.; Investigation, C.L.S., R.B.C., M.C.J., and A.D.L.; Writing, C.L.S., R.B.C., and A.D.L.; Funding Acquisition, C.L.S. and A.D.L.; Supervision, A.D.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: August 19, 2017

Revised: June 11, 2018

Accepted: July 23, 2018

Published: August 28, 2018

REFERENCES

- Asano, K., Takahashi, N., Ushiki, M., Monya, M., Aihara, F., Kuboki, E., Moriyama, S., Iida, M., Kitamura, H., Qiu, C.H., et al. (2015). Intestinal CD169(+) macrophages initiate mucosal inflammation by secreting CCL8 that recruits inflammatory monocytes. *Nat. Commun.* 6, 7802.
- Braun, A., Worbs, T., Moschovakis, G.L., Halle, S., Hoffmann, K., Bölter, J., Münk, A., and Förster, R. (2011). Afferent lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. *Nat. Immunol.* 12, 879–887.
- Bromley, S.K., Yan, S., Tomura, M., Kanagawa, O., and Luster, A.D. (2013). Recirculating memory T cells are a unique subset of CD4+ T cells with a distinct phenotype and migratory pattern. *J. Immunol.* 190, 970–976.
- Chensue, S.W., Lukacs, N.W., Yang, T.Y., Shang, X., Frait, K.A., Kunkel, S.L., Kung, T., Wiekowski, M.T., Hedrick, J.A., Cook, D.N., et al. (2001). Aberrant *in vivo* T helper type 2 cell response and impaired eosinophil recruitment in CC chemokine receptor 8 knockout mice. *J. Exp. Med.* 193, 573–584.
- Chung, C.D., Kuo, F., Kumer, J., Motani, A.S., Lawrence, C.E., Henderson, W.R., Jr., and Venkataraman, C. (2003). CCR8 is not essential for the development of inflammation in a mouse model of allergic airway disease. *J. Immunol.* 170, 581–587.
- Das, S., Sarrou, E., Podgrabska, S., Cassella, M., Mungamuri, S.K., Feirt, N., Gordon, R., Nagi, C.S., Wang, Y., Entenberg, D., et al. (2013). Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses. *J. Exp. Med.* 210, 1509–1528.
- Dieu, M.C., Vanbervliet, B., Vicari, A., Bridon, J.M., Oldham, E., Ait-Yahia, S., Brière, F., Zlotnik, A., Lebecque, S., and Caux, C. (1998). Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med.* 188, 373–386.
- Fletcher, A.L., Malhotra, D., Acton, S.E., Lukacs-Kornek, V., Bellemare-Pelletier, A., Curry, M., Armant, M., and Turley, S.J. (2011). Reproducible isolation of lymph node stromal cells reveals site-dependent differences in fibroblastic reticular cells. *Front. Immunol.* 2, 35.
- Gao, Y., Nish, S.A., Jiang, R., Hou, L., Licona-Limón, P., Weinstein, J.S., Zhao, H., and Medzhitov, R. (2013). Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. *Immunity* 39, 722–732.
- Girard, J.P., Moussion, C., and Förster, R. (2012). HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. *Nat. Rev. Immunol.* 12, 762–773.
- Goya, I., Villares, R., Zaballos, A., Gutiérrez, J., Kremer, L., Gonzalo, J.A., Varona, R., Carramolino, L., Serrano, A., Pallarés, P., et al. (2003). Absence of CCR8 does not impair the response to ovalbumin-induced allergic airway disease. *J. Immunol.* 170, 2138–2146.
- Gray, E.E., and Cyster, J.G. (2012). Lymph node macrophages. *J. Innate Immun.* 4, 424–436.
- Gray, E.E., Friend, S., Suzuki, K., Phan, T.G., and Cyster, J.G. (2012). Subcapsular sinus macrophage fragmentation and CD169+ bleb acquisition by closely associated IL-17-committed innate-like lymphocytes. *PLoS ONE* 7, e38258.
- Griffith, J.W., Sokol, C.L., and Luster, A.D. (2014). Chemokines and chemokine receptors: Positioning cells for host defense and immunity. *Annu. Rev. Immunol.* 32, 659–702.
- Halim, T.Y., Steer, C.A., Mathä, L., Gold, M.J., Martinez-Gonzalez, I., McNagny, K.M., McKenzie, A.N., and Takei, F. (2014). Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 40, 425–435.
- Hauser, M.A., Schaeuble, K., Kindinger, I., Impellizzeri, D., Krueger, W.A., Hauck, C.R., Boyman, O., and Legler, D.F. (2016). Inflammation-induced CCR7 oligomers form scaffolds to integrate distinct signaling pathways for efficient cell migration. *Immunity* 44, 59–72.
- Islam, S.A., Chang, D.S., Colvin, R.A., Byrne, M.H., McCully, M.L., Moser, B., Lira, S.A., Charo, I.F., and Luster, A.D. (2011). Mouse CCL8, a CCR8 agonist, promotes atopic dermatitis by recruiting IL-5+ T(H)2 cells. *Nat. Immunol.* 12, 167–177.

- Islam, S.A., Ling, M.F., Leung, J., Shreffler, W.G., and Luster, A.D. (2013). Identification of human CCR8 as a CCL18 receptor. *J. Exp. Med.* **210**, 1889–1898.
- Iwasaki, A., and Medzhitov, R. (2015). Control of adaptive immunity by the innate immune system. *Nat. Immunol.* **16**, 343–353.
- Jakubczik, C., Tacke, F., Llodra, J., van Rooijen, N., and Randolph, G.J. (2006). Modulation of dendritic cell trafficking to and from the airways. *J. Immunol.* **176**, 3578–3584.
- Junt, T., Moseman, E.A., Iannacone, M., Massberg, S., Lang, P.A., Boes, M., Fink, K., Henrickson, S.E., Shayakhmetov, D.M., Di Paolo, N.C., et al. (2007). Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature* **450**, 110–114.
- King, I.L., Kroenke, M.A., and Segal, B.M. (2010). GM-CSF-dependent, CD103+ dermal dendritic cells play a critical role in Th effector cell differentiation after subcutaneous immunization. *J. Exp. Med.* **207**, 953–961.
- Kumamoto, Y., Linehan, M., Weinstein, J.S., Laidlaw, B.J., Craft, J.E., and Iwasaki, A. (2013). CD301b⁺ dermal dendritic cells drive T helper 2 cell-mediated immunity. *Immunity* **39**, 733–743.
- Kumamoto, Y., Hirai, T., Wong, P.W., Kaplan, D.H., and Iwasaki, A. (2016). CD301b⁺ dendritic cells suppress T follicular helper cells and antibody responses to protein antigens. *eLife* **5**, <https://doi.org/10.7554/eLife.17979>.
- León, B., Ballesteros-Tato, A., Browning, J.L., Dunn, R., Randall, T.D., and Lund, F.E. (2012). Regulation of T(H)2 development by CXCR5+ dendritic cells and lymphotoxin-expressing B cells. *Nat. Immunol.* **13**, 681–690.
- Lewis, K.L., Caton, M.L., Bogunovic, M., Greter, M., Grajkowska, L.T., Ng, D., Klinakis, A., Charo, I.F., Jung, S., Gommerman, J.L., et al. (2011). Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity* **35**, 780–791.
- Lian, J., and Luster, A.D. (2015). Chemokine-guided cell positioning in the lymph node orchestrates the generation of adaptive immune responses. *Curr. Opin. Cell Biol.* **36**, 1–6.
- Miller, M.D., and Krangel, M.S. (1992). The human cytokine I-309 is a monocyte chemoattractant. *Proc. Natl. Acad. Sci. USA* **89**, 2950–2954.
- Murakami, R., Denda-Nagai, K., Hashimoto, S., Nagai, S., Hattori, M., and Irimura, T. (2013). A unique dermal dendritic cell subset that skews the immune response toward Th2. *PLoS ONE* **8**, e73270.
- Paoletti, S., Petkovic, V., Sebastiani, S., Danelon, M.G., Uguccioni, M., and Gerber, B.O. (2005). A rich chemokine environment strongly enhances leukocyte migration and activities. *Blood* **105**, 3405–3412.
- Qi, H., Kastenmüller, W., and Germain, R.N. (2014). Spatiotemporal basis of innate and adaptive immunity in secondary lymphoid tissue. *Annu. Rev. Cell Dev. Biol.* **30**, 141–167.
- Qu, C., Edwards, E.W., Tacke, F., Angeli, V., Llodrá, J., Sanchez-Schmitz, G., Garin, A., Haque, N.S., Peters, W., van Rooijen, N., et al. (2004). Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. *J. Exp. Med.* **200**, 1231–1241.
- Sallusto, F., Schaerli, P., Loetscher, P., Scharniel, C., Lenig, D., Mackay, C.R., Qin, S., and Lanzavecchia, A. (1998). Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur. J. Immunol.* **28**, 2760–2769.
- Schlitzer, A., McGovern, N., Teo, P., Zelante, T., Atarashi, K., Low, D., Ho, A.W., See, P., Shin, A., Wasan, P.S., et al. (2013). IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* **38**, 970–983.
- Sokol, C.L., Barton, G.M., Farr, A.G., and Medzhitov, R. (2008). A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat. Immunol.* **9**, 310–318.
- Tal, O., Lim, H.Y., Gurevich, I., Milo, I., Shipony, Z., Ng, L.G., Angeli, V., and Shakhar, G. (2011). DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *J. Exp. Med.* **208**, 2141–2153.
- Tang, H., Cao, W., Kasturi, S.P., Ravindran, R., Nakaya, H.I., Kundu, K., Murthy, N., Kepler, T.B., Malissen, B., and Pulendran, B. (2010). The T helper type 2 response to cysteine proteases requires dendritic cell-basophil cooperation via ROS-mediated signaling. *Nat. Immunol.* **11**, 608–617.
- Tomura, M., Yoshida, N., Tanaka, J., Karasawa, S., Miwa, Y., Miyawaki, A., and Kanagawa, O. (2008). Monitoring cellular movement in vivo with photoconvertible fluorescence protein “Kaede” transgenic mice. *Proc. Natl. Acad. Sci. USA* **105**, 10871–10876.
- Tsou, C.L., Peters, W., Si, Y., Slaymaker, S., Aslanian, A.M., Weisberg, S.P., Mack, M., and Charo, I.F. (2007). Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J. Clin. Invest.* **117**, 902–909.
- Tussiwand, R., Everts, B., Grajales-Reyes, G.E., Kretzer, N.M., Iwata, A., Bagaitkar, J., Wu, X., Wong, R., Anderson, D.A., Murphy, T.L., et al. (2015). Klf4 expression in conventional dendritic cells is required for T helper 2 cell responses. *Immunity* **42**, 916–928.
- Ulvmar, M.H., Werth, K., Braun, A., Kelay, P., Hub, E., Eller, K., Chan, L., Lucas, B., Novitzky-Basso, I., Nakamura, K., et al. (2014). The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. *Nat. Immunol.* **15**, 623–630.
- Weber, M., Hauschild, R., Schwarz, J., Moussion, C., de Vries, I., Legler, D.F., Luther, S.A., Bollenbach, T., and Sixt, M. (2013). Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science* **339**, 328–332.
- Williams, J.W., Tjota, M.Y., Clay, B.S., Vander Lugt, B., Bandukwala, H.S., Hrusch, C.L., Decker, D.C., Blaine, K.M., Fixsen, B.R., Singh, H., et al. (2013). Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. *Nat. Commun.* **4**, 2990.
- Woodruff, M.C., Heesters, B.A., Herndon, C.N., Groom, J.R., Thomas, P.G., Luster, A.D., Turley, S.J., and Carroll, M.C. (2014). Trans-nodal migration of resident dendritic cells into medullary interfollicular regions initiates immunity to influenza vaccine. *J. Exp. Med.* **211**, 1611–1621.
- Worbs, T., Hammerschmidt, S.I., and Förster, R. (2017). Dendritic cell migration in health and disease. *Nat. Rev. Immunol.* **17**, 30–48.
- Yabe, R., Shimizu, K., Shimizu, S., Azechi, S., Choi, B.I., Sudo, K., Kubo, S., Nakae, S., Ishigame, H., Kakuta, S., and Iwakura, Y. (2015). CCR8 regulates contact hypersensitivity by restricting cutaneous dendritic cell migration to the draining lymph nodes. *Int. Immunol.* **27**, 169–181.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	Source	Identifier
Antibodies		
Alexa Fluor 594 Streptavidin	BioLegend	Cat #: 405240
Alexa Fluor 647 Streptavidin	BioLegend	Cat #: 405237
Anti-Mouse CD8 α , Clone: 53-6.7	BioLegend	Cat #: 100725; RRID: AB_493425
Anti-Mouse CD11b, Clone: M1/70	BioLegend	Cat #: 101251; RRID: AB_2562904
Anti-Mouse CD11c, Clone: HL3	BD Biosciences	Cat #: 553801; RRID: AB_395060
Anti-Mouse CD11c, Clone: N418	BioLegend	Cat #: 117346; RRID: AB_2563323
Anti-Mouse CD45.1, Clone: A20	BioLegend	Cat #: 110726; RRID: AB_893345
Anti-Mouse CD45.2, Clone: 104	BioLegend	Cat #: 109807; RRID: AB_313444
Anti-Mouse CD169, Clone: 3D6.112	BioLegend	Cat #: 142419; RRID: AB_2566436
Anti-Mouse CD197 (CCR7), Clone: 4B12	BioLegend	Cat #: 120116; RRID: AB_2291144
Anti-Mouse CD273 (PDL2), Clone: TY25	BD Biosciences	Cat #: 560086; RRID: AB_1645223
Anti-mouse CD301b (MGL2), Clone: URA-1	BioLegend	Cat #: 146806; RRID: AB_2563388
Anti-Mouse F4/80, Clone: BM8	BioLegend	Cat #: 123131; RRID: AB_10901171
Anti-Mouse Lyve-1, Clone: ALY7	eBioscience	Cat #: 53-0443-80; RRID: AB_1633417
Anti-Mouse Podoplanin, Clone: 8.1.1	BioLegend	Cat #: 127406; RRID: AB_2161930
Anti-rabbit IgG, HRP	Cell Signaling	Cat #: 7074S; RRID: AB_2099233
Anti-SHP2	Cell Signaling	Cat #: 3397T
Anti-phospho SHP2 (Tyr 542)	Cell Signaling	Cat #: 3751S; RRID: AB_330825
Anti-Siglec1, biotinylated	R&D Systems	Cat #: BAF5610; RRID: AB_10641427
Anti-SIGN-R1, biotinylated	R&D Systems	Cat #: BAF1836; RRID: AB_2074454
Anti-Src	Cell Signaling	Cat #: 2108S; RRID: AB_10695298
Anti-phospho Src (Tyr 416)	Cell Signaling	Cat #: 2101S; RRID: AB_331697
Biotinylated Anti-mouse MCP-2/CCL8 Antibody	R&D Systems	Cat #: BAF790; RRID: AB_2071685
Human CCL1-AlexaFluor647	Almac	Cat #: CAF-7
Fixable Viability Dye eFluor 780	Invitrogen	Cat #: 65-0865-14
Mouse CCL1/I-309/TCA-3 Biotinylated Antibody	R&D Systems	Cat #: BAF845; RRID: AB_2070619
TruStain fcX anti-mouse CD16/32, Clone: 93	BioLegend	Cat #: 101320; RRID: AB_1574975
Chemicals, Peptides, and Recombinant Proteins		
Macrophage depletion kit of Clodrosome and Encapsome	Encapsula Nano Sciences	Cat #: 8901
Saponin from quillaja bark	Sigma-Aldrich	Cat #: S7900-25G
Recombinant Mouse CCL1/TCA-3	R&D Systems	Cat #: 845-TC
Recombinant Mouse CCL21/6CKine	R&D Systems	Cat #: 457-6C-025
Recombinant Murine MCP-3 (CCL7)	PeproTech	Cat #: 250-08
Recombinant Murine MCP-2 (CCL8)	PeproTech	Cat #: 250-14
Recombinant Mouse CCL2/JE/MCP-1	R&D Systems	Cat #: 479-JE-010
Recombinant Mouse CCL11/Eotaxin	R&D Systems	Cat #: 420-ME
TRIzol Reagent	Invitrogen	Ref #: 15596018
Collagenase P	Sigma-Aldrich	Cat #: 11213857001
Dispase II	Sigma-Aldrich	Cat #: D4693-1G
DNase I, grade II	Sigma-Aldrich	Cat #: 10104159001
Liberase DH	Sigma-Aldrich	Cat #: 5401054001
Streptavidin/Biotin Blocking Kit	Vector Lab	Cat #: SP-2002
Papain	Calbiochem	Cat #: 5125
Ovalbumin	Sigma-Aldrich	Cat #: A5503

(Continued on next page)

Continued

REAGENT or RESOURCE	Source	Identifier
Lipopolysaccharide	InvivoGen	Cat #: tlrl-eklps
Polyinosinic-polycytidylic acid	InvivoGen	Cat #: tlrl-picw
PBS	Corning	Ref #: 21-031-CV
PP2	Sigma-Aldrich	Cat #: 529573-1MG
Indo-1, AM	Invitrogen	Cat #: I1223
RBC Lysis buffer	Sigma-Aldrich	Cat #: R7757
0.5 M EDTA, pH 8.0	Gibco	Cat #: 15575-038
OCT Compound	Tissue-Tek	Cat #: 4583
RPMI 1640, 1×	Corning	Ref #: 15-040-CV
Fetal Calf Serum (FCS)	Atlanta Biologicals	Cat #: S11550
MultiScribe Reverse Transcriptase	Thermo Fisher Scientific	Ref #: 4311235
GeneAMP dNTP mix with dTTP	Thermo Fisher Scientific	Ref #: N8080260
MgCl ₂ Solution 25 mM	Thermo Fisher Scientific	Ref #: 4486224
10× PCR Buffer II	Thermo Fisher Scientific	Ref #: 4486220
Oligio d(T) ₁₆	Thermo Fisher Scientific	Ref #: 100023441
Random Hexamers 50 μM	Thermo Fisher Scientific	Ref #: 100026484
RNase Inhibitor	Thermo Fisher Scientific	Ref #: 100021540
FastStart Essential DNA Green Master	Roche	Cat #: 25595200
Critical Commercial Assays		
Mouse IL-4 ELISA MAX Standard	BioLegend	Cat #: 431103
Mouse IFN-γ ELISA MAX Standard	BioLegend	Cat #: 430803
Mouse IL-13 ELISA Ready-SET-Go!	eBioscience	Ref #: 88-7137-77
RNeasy Plus Micro Kit (50)	QIAGEN	Cat #: 74034
RNeasy Plus Mini Kit (50)	QIAGEN	Cat #: 74134
Naive CD4 ⁺ T Cell Isolation Kit, mouse	Miltenyi Biotec	Cat #: 130-104-453
CD11c Microbeads UltraPure mouse	Miltenyi Biotec	Cat #: 130-108-338
Experimental Models: Organisms/Strains		
Mouse: C57Bl/6	Charles River Laboratory	Strain #: 027
Mouse: C57Bl/6	The Jackson Laboratory	Strain #: 000664
Mouse: B6(FVB)-Mgl2 ^{tm1.1(HBEGF/EGFP)Aiwsk/J} (CD301b-DTR)	The Jackson Laboratory	Strain #: 023822
Mouse: <i>Ccr8</i> ^{-/-}	Chensue et al., 2001	N/A
Mouse: <i>Ccl8</i> ^{-/-} <i>Ccl12</i> ^{-/-}	Tsou et al., 2007	N/A
Mouse: Kaede ⁺	Tomura et al., 2008	N/A
Mouse: <i>Ccr7</i> ^{-/-}	The Jackson Laboratory	Strain #: 006621
Mouse: B6.Cg-Tg (TcrαTcrβ)425Cbn/J (OT2m)	The Jackson Laboratory	Strain #: 004194
Mouse: B6.SJL-Ptprc Pepc/BoyJ (CD45.1)	The Jackson Laboratory	Strain #: 002014
Mouse: REC8	Cyagen Biosciences, this manuscript	N/A
Oligonucleotides		
mCcl1 FWD (5'-GGCTGCCGTGTGGATACAG-3')	This manuscript	N/A
mCcl1 REV (5'-AGGTGATTTTGAACCCACGTTT-3')	This manuscript	N/A
mCcl2 FWD (5'-TGGCTCAGCCAGATGCAGT-3')	This manuscript	N/A
mCcl2 REV (5'-TTGGGATCATCTTGCTGGTG-3')	This manuscript	N/A
mCcl7 FWD (5'- TGGGAAGCTGTTATCTTCAAGACA-3')	This manuscript	N/A
mCcl7 REV (5'- CTCGACCCACTTCTGATGGG-3')	This manuscript	N/A
mCcl8 FWD (5'- GCGAGTGCTTCTTGCCTG-3')	This manuscript	N/A
mCcl8 REV (5'-TCTGGCCCAGTCAGCTTCTC-3')	This manuscript	N/A
mCcl12 FWD (5'-GCTGGACCAGATGCGGTG-3')	This manuscript	N/A
mCcl12 REV (5'- CCGGACGTGAATCTTCTGCT-')	This manuscript	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	Source	Identifier
REC8 genotyping primer P1 FWD (5'- GAACACAGCAG CAGAAAGAACAC-3')	This manuscript	N/A
REC8 genotyping primer P1 REV (5'- CATGGCGTGTAATATGAGAGACGC-3')	This manuscript	N/A
REC8 genotyping primer P2 FWD (5'-GTAATAAATGGA TGCCCTGCGTAAG -3')	This manuscript	N/A
REC8 genotyping primer P2 REV (5'-ACCTGTCAAGAA TGGTGCCAG-3')	This manuscript	N/A
REC8 genotyping primer P3 FWD (5'-ATCAGTCAGGT ACATAACCTCGAG-3')	This manuscript	N/A
REC8 genotyping primer P3 REV (5'-AGTCCATAACC TTCCTCCACG-3')	This manuscript	N/A
REC8 genotyping primer P4 FWD (5'-GATTGGTATCT GCTTCTGCACTTG-3')	This manuscript	N/A
REC8 genotyping primer P4 REV (5'-GTAGATGAAC TCGCCGTCCTG-3')	This manuscript	N/A
REC8 genotyping primer P5 FWD (5'-CCTTCTCACC ACAGCCACTTC-3')	This manuscript	N/A
REC8 genotyping primer P5 REV (5'-TGAGTTTGGAC AAACCACAAC-3')	This manuscript	N/A
REC8 genotyping primer P6 FWD (5'-TGCTTCATGT GGTGCGGGTA-3')	This manuscript	N/A
REC8 genotyping primer P6 REV (5'-GCTTTCATT CCCATTAGGCACA-3')	This manuscript	N/A
Software and Algorithms		
Prism v.7.0c	GraphPad	https://www.graphpad.com/demos
Zen Blue	Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software/zen.html
Imaris	Bitplane	http://www.bitplane.com/download
Softmax Pro	Molecular Devices	https://www.moleculardevices.com/systems/microplate-readers/softmax-pro-7-software
BD FACS Diva 8	BD Biosciences	http://www.bdbiosciences.com/us/instruments/clinical/software/flow-cytometry-acquisition/bd-facsdiva-software/m/333333/overview
FlowJo (v.10)	Tree Star	https://www.flowjo.com/solutions/flowjo/downloads

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Andrew Luster (aluster@mgm.harvard.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Mice**

Animals were bred and maintained under specific pathogen free (SPF) conditions at the Massachusetts General Hospital Center for Comparative Medicine. *Ccr8*^{-/-} mice were bred under SPF plus *Helicobacter* and *Pasteurella Pneumotropica* free conditions and maintained in SPF conditions. All procedures were approved and carried out according to the standards and guidelines set forth by the Institutional Animal Care and Use Committee of Massachusetts General Hospital. All mouse strains were fully backcrossed (>9 generations) to C57BL/6. Mice were used in experiments at 5–14 weeks of age and age matched littermates or purchased C57BL/6 mice were used as WT controls. Male mice were used for all DC migration assays unless otherwise noted. Male and female mice were used interchangeably in other assays, but sex was matched for all conditions within experiments.

METHOD DETAILS

Generation of REC8 Mice

REC8 mice were generated on the C57BL/6 background by Cyagen Biosciences using a PiggyBac on BAC technique by BAC modification in *E. coli*. RP23-409C7 was selected in pBACe3.6 because it contained both *Ccl1* and *Ccl8* target genes. First, a fragment containing 5' and 3' inverted terminal repeat sequences (ITRs) was inserted into pBACe3.6 to allow for eventual integration into TTA short repeat element sites. eGFP-SV40 polyA was placed upstream of an FRT flanked kanamycin resistance gene and this construct was introduced immediately following the start codon of *Ccl8* via recombineering of the modified BAC clone RP23-409C7. After selection of positive colonies, the kanamycin resistance cassette was then removed via Flp-mediated recombination. mCherry-SV40 polyA was placed upstream of rox flanked ampicillin resistance gene and this construct was introduced immediately following the start codon of the *Ccl1* gene via recombineering of the *Ccl8*-eGFP modified BAC clone. Ampicillin resistant clones were selected and then the ampicillin resistance cassette was deleted via Dre-mediated recombination. Recombinant clones were confirmed and monitored by PCR (primers P1–P6) and end-sequencing. The verified circular PiggyBac on BAC clone and helper plasmid was injected into fertilized C57BL/6 eggs and implanted into surrogate mothers. Pups were genotyped by PCR (primers P1–P8) to identify transgene positive pups. Positive pups were screened negative for integration of the helper plasmid (forward primer 5'-CTGGACGAGCAGAACGTGATCG-3' and reverse primer 5'-CGAAGAAGCGTAGATCTCGTCCTC-3'). This yielded 11 founders. Of those, one did not produce offspring and two did not have transgene positive offspring. Of the 8 remaining founders, two had no detectable reporter expression, four had only detectable mCherry expression, and two had detectable expression of both reporters. One line had brighter reporter expression and so was used primarily for data generation, although experiments were confirmed with the alternative line.

Immunizations and Cell Transfers

Mice were immunized subcutaneously in the rear footpads and/or base of tail with 50 μ g of both papain (Calbiochem) and OVA or 50 μ g OVA in 20 μ g Imject Alum (ThermoFisher) for Th2-skewing immunizations, 10 μ g of both LPS (InvivoGen) and poly I:C (InvivoGen) and 50 μ g of OVA for Th1-skewing immunizations, and 50 μ g of OVA for Th0 immunizations. All immunizations except OVA-alum were diluted in sterile PBS and subcutaneously injected at a final volume of 25 μ L. *N. brasiliensis* infection was performed by injecting mice subcutaneously in the base of tail with 500 L3 stage larvae with 50 μ g OVA. Naive CD4⁺ T cells were negatively selected for using Naive CD4⁺ T cell isolation kit (Miltenyi) followed by magnetic cell separation (Miltenyi). Purified naive CD4⁺ T cells were transferred (2.5×10^5 to 5.0×10^5 cells in 100 μ L sterile PBS) into recipient mice via retro-orbital injection. SIGN-R1⁺ cells underwent magnetic cell separation using SIGN-R1-biotin and anti-biotin magnetic beads (Miltenyi).

Tissue Preparation

For CD4⁺ T cell harvest for *in vitro* restimulation assays, whole LNs were placed in 70 μ m sterile filters and mechanically disrupted. Otherwise, for standard LN digestion, whole LNs were harvested, minced, and placed into RPMI media containing Liberase DH (0.26 U/mL) and DNase I (0.1 mg/mL) and incubated at 37°C for 20 min. Following incubation, LNs were mechanically disrupted by passing through a pipette tip before being incubated for another 20 min at 37°C. For extended LN digest to ensure release of LN sinus stroma and entrapped cells, whole LNs were harvested into RPMI media and subjected to digestion in DNase I (100 μ g/mL), Collagenase P (200 μ g/mL), Dispase II (800 μ g/mL), and 1% fetal calf serum (FCS) in RPMI. LNs were placed in the pre-warmed enzyme mixture and incubated at 37°C. At 8 min intervals, any supernatant was removed and added to stop buffer (RPMI/2mM EDTA/1% FCS), and the removed supernatant was replaced with fresh enzyme media. This was repeated at 8 min intervals until no tissue fragments remained. For both protocols, upon completion of digestion, cell suspensions were filtered with a 70 μ m sterile filter.

Generation of Mixed Bone Marrow Chimeras

To generate bone marrow chimeric mice, CD45.1 recipient mice were irradiated with 1,000 rads prior to injection with isolated bone marrow. Bone marrow was harvested from 5- to 8-week-old mice by mechanical disruption using a mortar and pestle followed by incubation with RBC lysis buffer. Lysis was stopped by adding excess PBS, centrifugation, and resuspension of pellet in sterile PBS. Within 6 hr of irradiation, recipient mice received a total of 1×10^7 cells in a 1:1 mix of CD301b-DTR (CD45.2) and CD45.1 $\times$ CD45.2 cells, CD301b-DTR (CD45.2) and Kaede⁺Ccr8^{-/-} cells or all Kaede⁺Ccr8^{-/-} cells. Bone marrow cells were injected via retro-orbital injection in 100 μ L. After 8 to 10 weeks, mice received purified CD4⁺ T cells (2.5×10^5) via retro-orbital injection as well as 0.5 μ g of Diphtheria toxin intraperitoneally. 16–24 hr following T cell transfer, mice underwent footpad immunization and received a second dose of Diphtheria toxin at 0.5 μ g. 48 hr following immunizations, mice were given a final injection of Diphtheria toxin at 0.5 μ g. 5 days after immunization, popliteal LNs were collected. Reconstitution and depletion of CD301b⁺ DCs were assessed in a subset of mice one day after immunization and in all other mice at time of final harvest.

Flow Cytometry

Enzymatically digested cell suspensions were incubated for 15 min at 4°C in PBS with 0.5% FCS with the following antibodies: anti-CD16/32, anti-CD8 α , anti-CD11b, anti-CD11c, anti-CD45.1, anti-CD45.2, anti-CD103, anti-CD169, anti-CD301b, anti-Thy1.1 (all from Biolegend), anti-PDL2 (BD Biosciences), or anti-SIGN-R1 (R&D Systems). Viability was determined using Fixable Viability

Dye eFluor 780 (Invitrogen). Experiments examining CCR7 were done by first incubating cells with anti-CCR7 (BD Biosciences) for 30 min at 37°C prior to additional surface staining as above. Intracellular cytokine staining was done by first incubating cells in PMA/Ionomycin (Sigma) in the presence of GolgiPlug for 4 hr prior to surface staining followed by intracellular staining using BD Cytofix/Cytoperm kit (BD Biosciences). hCCL1-AF647 (Almac) binding was accomplished by first incubating 2×10^6 cells in 50 μ L of 0.25 μ g/mL hCCL1-AF647 in PBS/0.5% FCS/20mM HEPES for 1 hr at 37°C. Cells then underwent additional antibody staining as above. All samples were run on BD Fortessa X-20 or BD LSRII and analyzed using FACS Diva 8 (BD Biosciences) and FlowJo (Version 10) (TreeStar).

Enzyme-Linked Immunosorbent Assay (ELISA)

Mouse IL-4 and IFN- γ were detected using a mouse IL-4 and IFN- γ ELISA MAX standard set (both from Biolegend). Mouse IL-13 was detected using a Mouse IL-13 ELISA Ready-SET-Go! kit (eBioscience). All procedures were carried out according to the manufacturer's protocols. Briefly, samples were incubated overnight at 4°C on antibody-coated plates. Detection antibodies were allowed to incubate for 1 hr at room temperature, followed by addition of streptavidin-horseradish peroxidase for 30 min. Finally, TMB substrate solution was added and the reaction was stopped with 2M sulfuric acid. Samples were assayed on Softmax Pro Software in triplicates and concentrations were determined from a standard curve.

In Vitro Restimulation Assays

To achieve restimulation of antigen-specific T cells, single cell suspensions of 5×10^5 isolated leukocytes of whole LNs were added to 96-well round bottom microwell plates with 100 μ g/mL OVA in 200 μ L of media. Plates were incubated at 37°C in 5% CO₂ between four and five days at which time cytokine production was analyzed.

In Vitro Chemotaxis Assays

Chemotaxis of sorted CD11c⁺CD11b⁺CD301b⁺ (CD11c⁺) DCs and CD11c⁺CD11b⁺CD301b⁺ DCs was performed using 5- μ m pore size Transwell membranes (Neuro Probe). Briefly, 29 μ L of diluted chemokines in media, 0.5% (wt/vol) BSA in RPMI, or media alone were added to the lower chamber. To the upper chamber, 25,000 cells were added in triplicate in 50 μ L of media with or without chemokine. Plates were incubated for 2 hr at 37°C in 5% CO₂ after which point the upper membrane was washed with PBS. A brief spin was performed to collect any migrated adherent cells, the upper chamber was removed, and migrated cells were counted. When applicable, cells were pretreated for 30 min with 10 μ M of the Src inhibitor PP2 and chemotaxis was performed in the presence of this or DMSO as a control.

RNA Purification and qPCR Analysis

Total RNA was collected from whole LN or skin using a Trizol (Invitrogen) reagent protocol in accordance with the manufacturer. Total RNA from sorted cells was collected using the QIAGEN RNeasy Micro or Mini Kit, based on the manufacturer's protocol. For both methods, RNA was diluted in RNase-free water and stored at -80°C until prepared for cDNA using reverse transcription. 50 μ L of cDNA per reaction was generated using random hexamers, Oligio(dT), magnesium chloride, dNTPs (10mM), Reverse Transcriptase, and RNase Inhibitor (all from Fisher). A Roche LightCycler 96 Real-Time PCR System was utilized to quantitate gene expression using SYBR Green Master Mix (Roche). The reaction cycles were carried out in the following manner: 95°C for 600 s, then 95°C, 60°C, and 72°C for 10 s each for 45 total cycles, followed by 95°C for 10 s, 65°C for 60 s, and finally 97°C for 1 s. Fluorescence was measured during each amplification step and Roche LightCycler 96 Software 1.1 was utilized to determine quantification cycle (Cq) values for each sample. In Excel, copy values were determined based off of the Cq values which were then divided by values from the housekeeping genes β 2-microglobulin or Gapdh to generate ratios.

Confocal Immunofluorescence

Lymph nodes were fixed at room temperature for 2-3 hr in periodate-lysine-paraformaldehyde fixative. Organs then were placed sequentially in 10%, 20% and 30% sucrose (wt/vol) in PBS for cryoprotection, then embedded in optimum cutting temperature (OCT) compound (Tissue-Tek) and frozen in 2-methylbutane over liquid nitrogen. A Cryostat (Microm HM 505 E, GMI) was used to generate 6-9 μ m sections, which were kept at -20°C. On the day of staining, sections were thawed, rehydrated in PBS for 20 min at room temperature, blocked in PBS containing 0.1% (vol/vol) Tween-20 and 5% (vol/vol) Normal Goat Serum (Jackson ImmunoResearch), and were stained with biotinylated anti-Siglec1 or anti-SIGN-R1 (both from R&D Systems) or fluorochrome-conjugated anti-mouse CD11c (BD Biosciences), anti-mouse LYVE-1 (eBioscience), anti-mouse CD301b, anti-mouse podoplanin, anti-mouse CD169, or anti-mouse F4/80 (all from BioLegend). Streptavidin-Alexa Fluor 647 or Alexa Fluor 594 (both from BioLegend) were used as secondary antibodies. Secondary antibodies were allowed to incubate for 1 h at room temperature. Sections were also stained with biotinylated anti-mouse MCP-2/CCL8 or CCL1 (both from R&D Systems) which were blocked using a streptavidin/biotin blocking kit (Vector Laboratories) to block endogenous biotin. All dilutions were made in PBS containing 0.1% (wt/vol) Saponin and 5% rabbit serum (vol/vol), with the exception of primary antibody which was diluted in PBS containing 0.1% (w/v) Saponin only. Primary antibody was allowed to incubate overnight at 4°C. A biotinylated anti-goat IgG secondary antibody (Vector Laboratories) incubated at room temperature for 30 min followed by addition of fluorescein streptavidin (1:200) for 15 min at 4°C. All slides were mounted in Prolong Gold to prevent fading of staining. A Zeiss LSM confocal microscope was used to acquire images that were generated by collecting consecutive scans with 1 laser line active per scan to prevent excitation crosstalk.

Kaede Photoconversion

All Kaede transgenic mice were anesthetized to shave a $\sim 2 \times 2$ cm patch of skin above the base of tail. A chemical depilatory agent (Veet) was applied to the shaved patch for 1 min, followed by multiple washings with PBS. To perform photoconversion, the exposed skin was subjected to violet light (420 nm) using a Bluewave LED visible light curing unit (Dymax) that expressed a 420 nm bandpass filter (Andover Corp). Skin was exposed to this wavelength with the light source at a maximum power, approximately 7.5 cm away from the skin for 5 min. Following photoconversion, mice were immunized subcutaneously in the shaved region as described above. 24 hr following immunization, dLNs were isolated and analyzed for skin DC migration using flow cytometry.

Clodronate-Mediated Cell Depletion

Mice were injected subcutaneously in the rear footpads with 50 μ L of either Clodrosome Liposomal Clodronate or Encapsome Control Liposome (Encapsula Nano Sciences). Footpad immunizations were performed four days following liposome administration, as described above, and draining lymph nodes were isolated 24 hr following immunization.

Immunoblotting

4DE4 cells stably transfected with human CCR8 as previously described were serum starved for 4 hr and then stimulated with CCL8 (Islam et al., 2011). Cells were lysed and lysates were immunoblotted for presence of phosphorylated Src (Tyr416) and SHP2 (Tyr542) (Cell Signaling).

Ca²⁺ Flux Assay

To access intracellular Ca²⁺ flux in primary dendritic cells, MACS isolated CD11c positive cells from the dLN of Th2-immunized mice were washed with PBS (with Ca/Mg) supplemented with 1% FCS. Indo-1, AM (Invitrogen) was prepared immediately before use by dissolving each vial according to the manufacturer's recommendations. 5 μ L of freshly diluted Indo-1 was added to 500 μ L of PBS 1% FCS (with Ca/Mg) per every two million cells. Cells were allowed to incubate for 45 min at 37°C in the dark and then washed with cold PBS (with Ca/Mg) and re-suspended in 500 μ L cold PBS (with Ca/Mg). Ca²⁺ flux was then assessed by flow cytometry measuring Indo-1 ratio over a time course of 90 s following chemokine stimulation.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses, as specified in figure legends, were performed with Prism 7 (GraphPad software). Data were analyzed with the non-paired Student's t test. A p value < 0.05 was considered significant (*p < 0.05, **p < 0.01, ***p < 0.001).

Immunity, Volume 49

Supplemental Information

**The Chemokine Receptor CCR8 Promotes the Migration
of Dendritic Cells into the Lymph Node Parenchyma
to Initiate the Allergic Immune Response**

Caroline L. Sokol, Ryan B. Camire, Michael C. Jones, and Andrew D. Luster

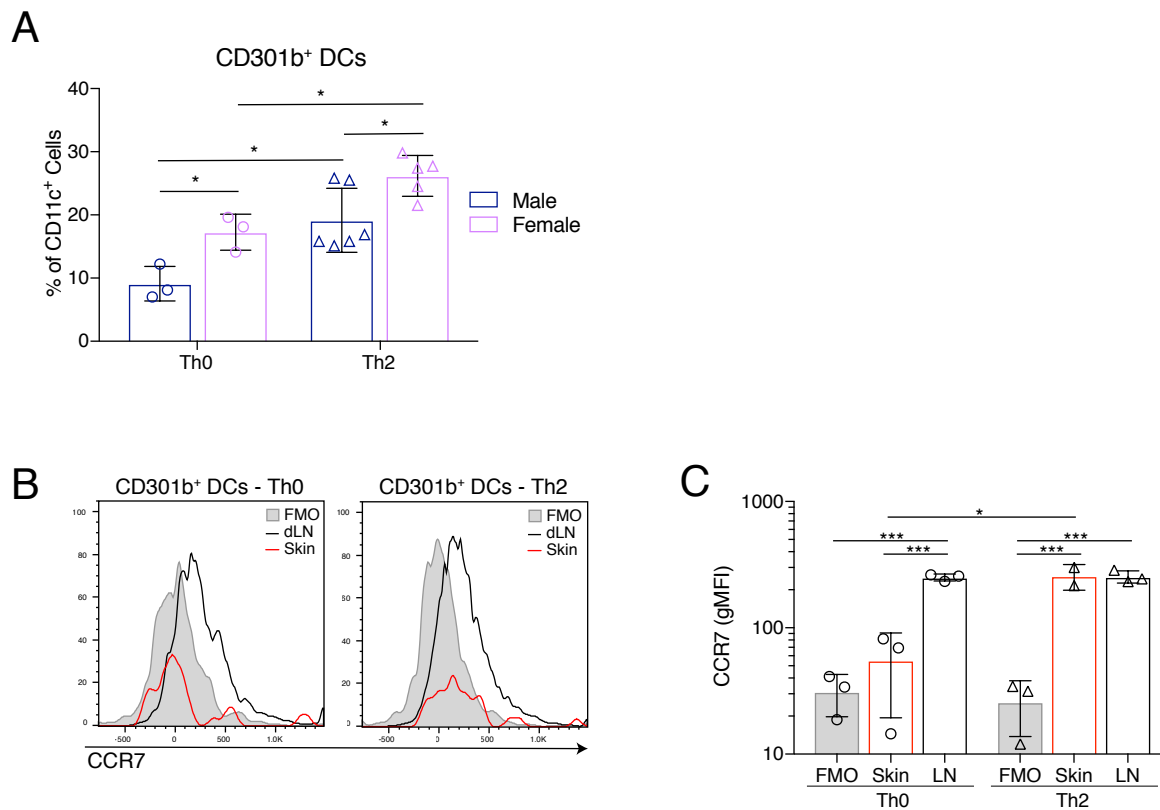


Figure S1 | Related to Figure 1

(A) CD301b⁺ DCs as a percentage of total viable CD11c⁺ cells from the popliteal LN 24 hrs after Th0 or Th2-skewing immunization in male or female mice. (B & C) CCR7 expression compared to fluorescence minus one (FMO) on viable CD45⁺CD11c⁺CD301b⁺ cells from the immunization site (skin) or popliteal LN (dLN) 24 hrs after the indicated immunization. Bar graphs represent mean value of indicated data points, error bars represent SEM. Data shown is representative of 3 independent experiments with 2-6 individual mice per condition per experiment. * p<0.05, ** p<0.01, *** p<0.001, Student's T test.

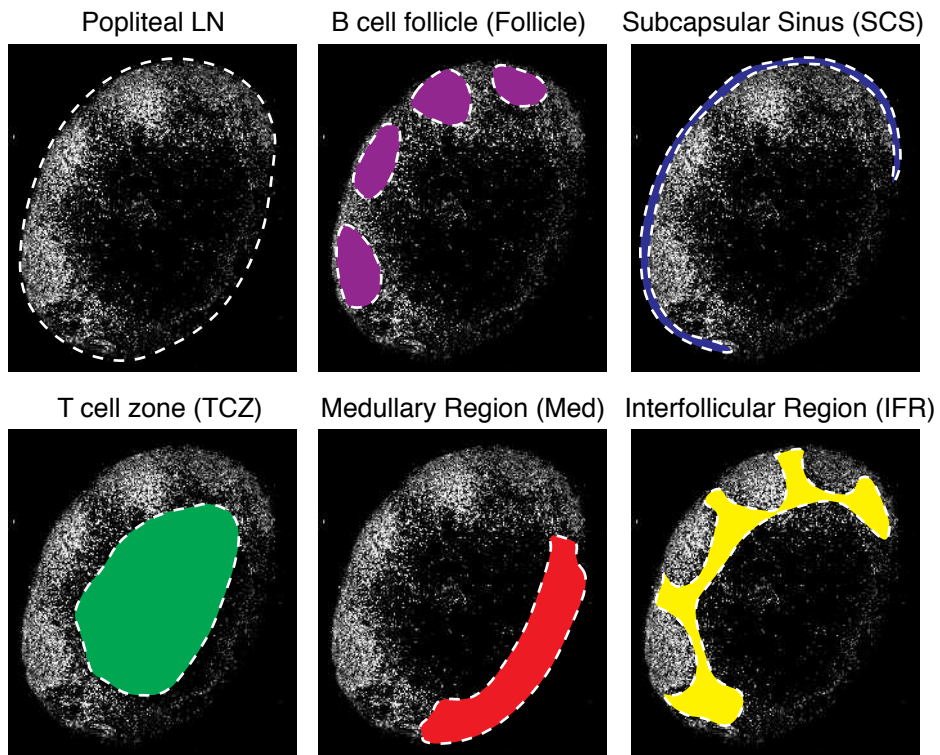


Figure S2 | Related to Figure 2.

Representative section of draining popliteal LN is shown (top left). Color regions indicated LN areas analyzed as determined by B220 staining (white) and LN architecture. Scale bars = 200 μm .

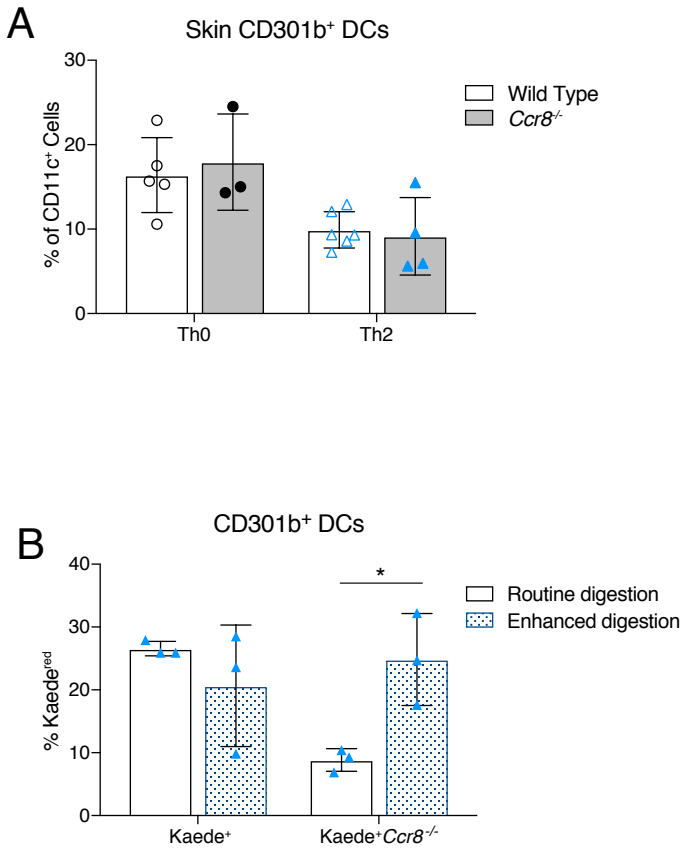


Figure S3 | Related to Figure 3.

(A) CD301b⁺ DCs as a percentage of total viable CD45⁺CD11c⁺ cells from the cutaneous immunization site (skin, footpad) 24 hrs after the indicated immunization of Wild Type or *Ccr8*^{-/-} mice. (B) Percentage of Kaede^{red} cells out of total CD11c⁺CD301b⁺ cells from the dLN 24 hours after photoconversion and Th2-skewing immunization of Kaede⁺ or Kaede⁺*Ccr8*^{-/-} mice. dLNs were harvested and divided for either routine enzymatic digestion (clear black bar) or enhanced enzymatic digestion (dotted blue bar). Bar graphs represent mean value of indicated data points, error bars represent SEM. Data shown is representative of 2 independent experiments with 2-3 individual mice per condition per experiment. * p<0.05, ** p<0.01, *** p<0.001, Student's T test.

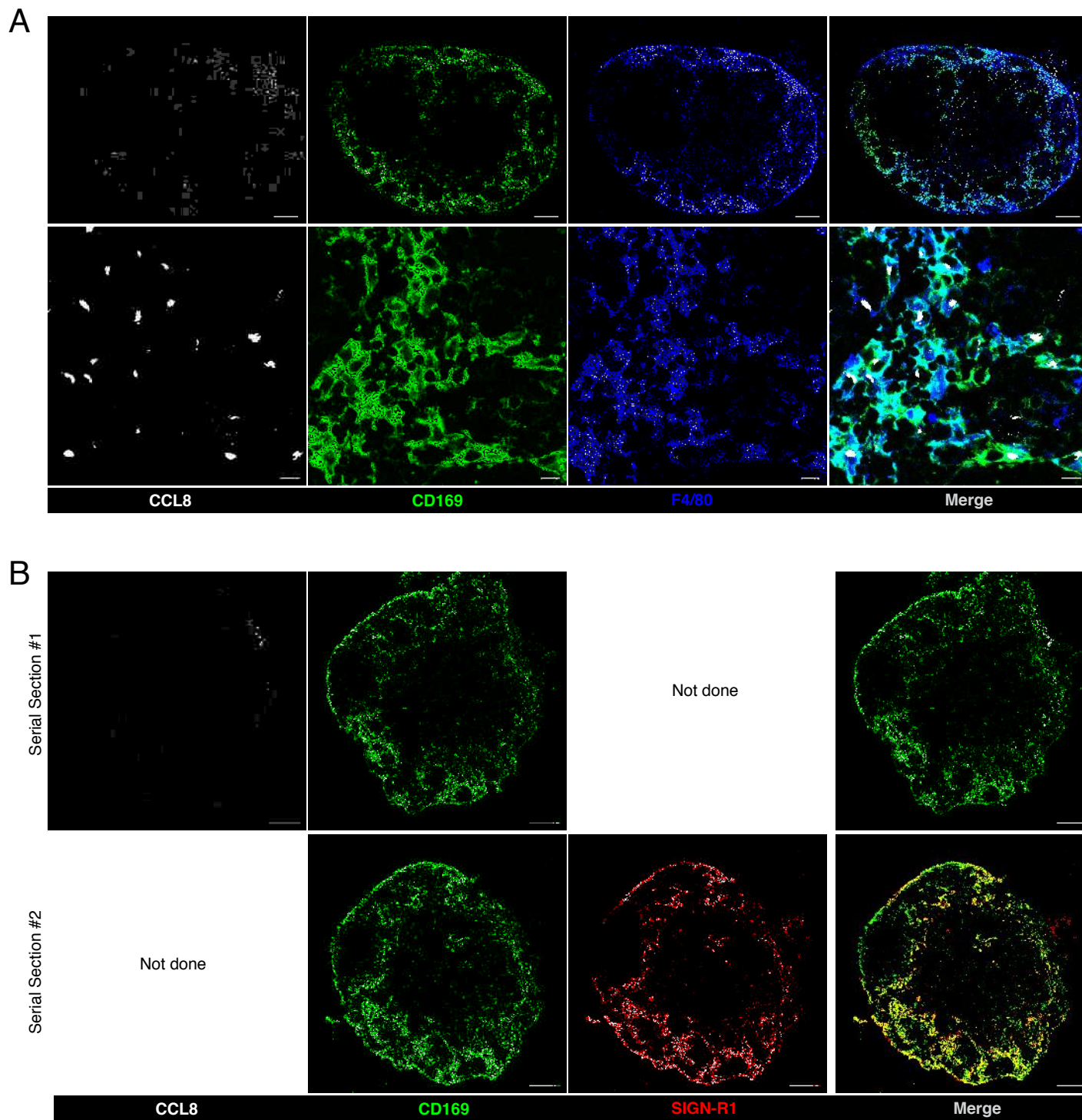


Figure S4 | Related to Figure 4.

(A) CCL8⁺ cells co-express CD169 and F4/80 on their surfaces. Top panels show CCL8 (white), CD169 (green) and F4/80 (blue) staining of entire LN, scale bar = 200 μ m. Bottom panels show zoom in of LN area of interest, scale bar = 10 μ m. (B) CD169⁺CCL8⁺ cells are colocalized with CD169⁺SIGNR1⁺ cells in serial sections. Top panels show CD169 (green) and CCL8 (white) in dLN 24 hrs after Th2-skewing immunization. Bottom panels are of serial section showing CD169 (green) and SIGN-R1 (red) staining in the same dLN. SIGN-R1 and CCL8 could not be done in the same sections due to antibody cross-reactivity and so are labeled “Not done” where appropriate. Scale bars = 200 μ m. Images shown are representative of two independent experiments with 3 mice in each experiment and images are one of six sections per mouse.

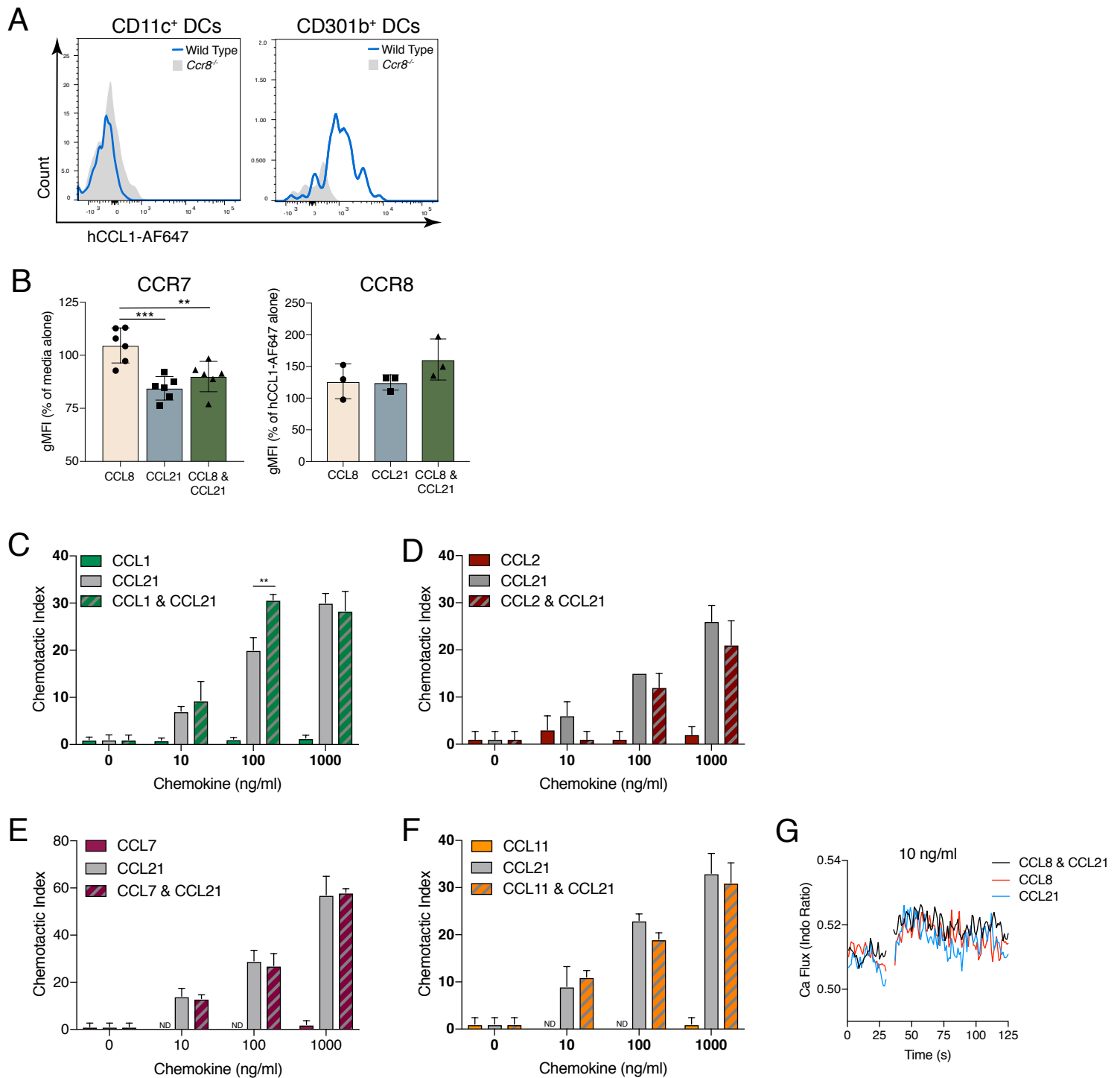


Figure S6 | Related to Figure 6.

(A) CCR8 expression as a measure of hCCL1-AF647 binding after overnight exposure of wild type (blue histogram) or *Ccr8*^{-/-} (shaded gray histogram) CD11c⁺ DCs or CD301b⁺ DCs to hCCL1-AF647. (B) CCR7 expression and hCCL1-AF647 binding of ex vivo CD301b⁺ DCs after 2 hours stimulation with the specified chemokine over that of the media control (gray shaded histogram). gMFI data shown is normalized to that of the media control. Transwell chemotaxis of CD301b⁺ DCs to (C) CCL1 (green bar), (D) CCL2 (red bar), (E) CCL7 (magenta bar), (F) CCL11 (orange bar), (C-F) CCL21 (gray bar), or combined respective chemokines (hatched bars). (G) Ca²⁺ flux expressed as the Indo-1 AM ratio from CD301b⁺ DCs stimulated ex vivo as indicated. In all cases cells were harvested from dLNs of mice 24 hrs after Th2-immunization. Representative graphs are shown from one of three (A, B, C) or one of two (D-G) independent experiments. Cells in (C-D) were pooled from 50 mice and cells in (G) were pooled from 12 mice. Bar graphs represent mean value of 2-3 data points/replicates per bar, error bars represent SEM. ** p<0.01, Student's T test.

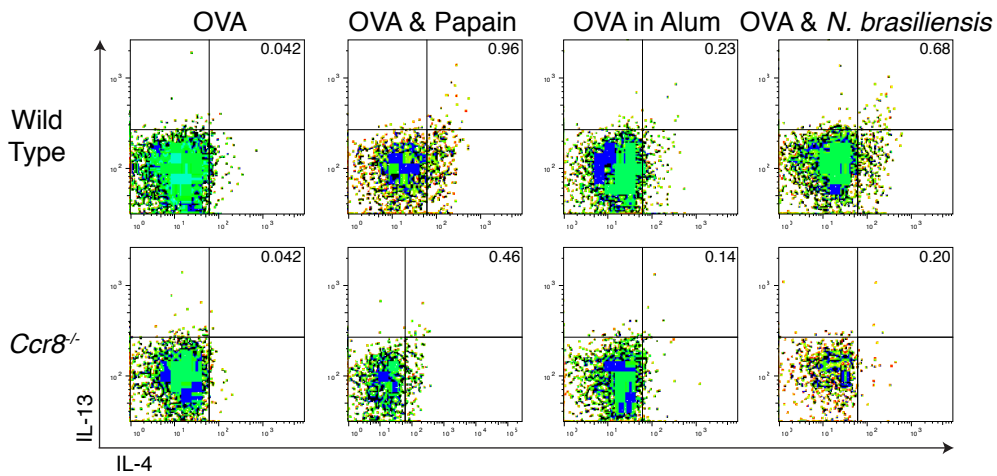


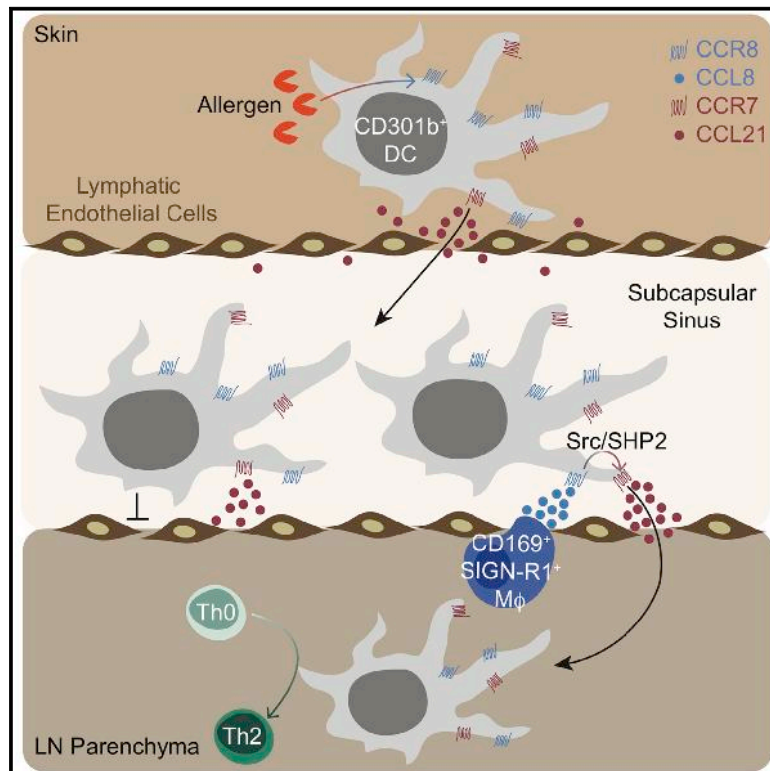
Figure S7 | Related to Figure 7.

Intracellular IL-4 and IL-13 staining of CD4⁺Thy1.1⁺ viable cells 4 days after OVA, OVA & Papain, or OVA in Alum immunization or 5 days after OVA & *N. brasiliensis* infection. Mice received 2.5-5x10⁵ naïve CD4⁺Thy1.1⁺ OTII cells prior to immunization. Representative plots are shown with percentage of cells in quadrants indicated. Data shown is representative of 3 independent experiments.

Immunity

The Chemokine Receptor CCR8 Promotes the Migration of Dendritic Cells into the Lymph Node Parenchyma to Initiate the Allergic Immune Response

Graphical Abstract



Authors

Caroline L. Sokol, Ryan B. Camire, Michael C. Jones, Andrew D. Luster

Correspondence

aluster@mgh.harvard.edu

In Brief

Migration of dendritic cells (DCs) into the lymph node (LN) initiates adaptive immunity and is thought to be solely dependent on CCR7. Sokol et al. show that in a cutaneous type 2 immune response, CCR8 synergizes with CCR7 and is necessary to promote the entry of allergen-activated DCs into the LN parenchyma, which is required for Th2 differentiation.

Highlights

- Allergen exposure promotes CD301b⁺ DC LN migration without upregulating CCR7
- CD169⁺SIGN-R1⁺ macrophages produce CCL8 in the LN in response to allergen exposure
- CCL8 and CCL21 synergize in a Src-dependent manner to augment CD301b⁺ DC migration
- CD301b⁺ DCs require CCR8 expression to enter the LN and promote Th2 differentiation



The Chemokine Receptor CCR8 Promotes the Migration of Dendritic Cells into the Lymph Node Parenchyma to Initiate the Allergic Immune Response

Caroline L. Sokol,¹ Ryan B. Camire,¹ Michael C. Jones,^{1,2} and Andrew D. Luster^{1,3,*}

¹Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy, and Immunology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA

²Present address: Department of Pathology, University of Massachusetts Medical School, Worcester, MA 01605, USA

³Lead Contact

*Correspondence: aluster@mgh.harvard.edu

<https://doi.org/10.1016/j.immuni.2018.07.012>

SUMMARY

The migration of mature dendritic cells (DCs) into the draining lymph node (dLN) is thought to depend solely on the chemokine receptor CCR7. CD301b⁺ DCs migrate into the dLN after cutaneous allergen exposure and are required for T helper 2 (Th2) differentiation. We found that CD301b⁺ DCs poorly upregulated CCR7 expression after allergen exposure and required a second chemokine signal, mediated by CCR8 on CD301b⁺ DCs and its ligand CCL8, to exit the subcapsular sinus (SCS) and enter the lymph node (LN) parenchyma. After allergen exposure, CD169⁺SIGN-R1⁺ macrophages in interfollicular regions produced CCL8, which synergized with CCL21 in a Src-kinase-dependent manner to promote CD301b⁺ DC migration. In CCR8-deficient mice, CD301b⁺ DCs remained in the SCS and were unable to enter the LN parenchyma, resulting in defective Th2 differentiation. We have defined a CCR8-dependent stepwise mechanism of DC-subset-specific migration through which LN CD169⁺SIGN-R1⁺ macrophages control the polarization of the adaptive immune response.

INTRODUCTION

Dendritic cells (DCs) play a central role in linking innate sensing with the activation and skewing of the adaptive immune response. The activation of pattern-recognition receptors (PRRs) in DCs leads to a coordinated response known as DC maturation. DC maturation is classically characterized by a decrease in DC endocytosis, migration to the draining lymph nodes (dLNs), an increase in the expression of major histocompatibility type II (MHC II) and co-stimulatory molecules, and the production of skewing cytokines involved in CD4⁺ T helper (Th) cell differentiation and activation. By reducing endocytosis and upregulating MHC II presentation, DC maturation creates a snapshot of the antigens encountered by DCs at the time of PRR activation. Once in the dLN, DCs display antigens as well

as co-stimulatory molecules and skewing cytokines that are influenced by the inflammatory stimuli previously encountered. Thus, DCs can independently provide the precise co-stimulation and skewing cytokines required for promoting the ideal adaptive immune response.

Although all conventional DCs share a robust capacity for antigen uptake and presentation, DC subsets show functional heterogeneity. Multiple subsets of DCs have been described to have different skewing effects on Th differentiation (Iwasaki and Medzhitov, 2015). For example, CD11b[−]CD103⁺ DCs have been shown to preferentially induce Th1 differentiation while CD11b⁺CD103⁺ DCs preferentially induce Th17 differentiation (King et al., 2010; Lewis et al., 2011; Schlitzer et al., 2013). In contrast, the CD11b⁺CD301b⁺PDL2⁺ DC population has been shown to be required for Th2 differentiation after cutaneous exposure to Th2-skewing stimuli (Gao et al., 2013; Kumamoto et al., 2013; Murakami et al., 2013). Given that Th differentiation is dependent on the DC subset, the migration of DC subsets must be differentially regulated in order to effectively skew the adaptive immune response. But how is this subset-specific migration controlled? One mechanism would be for DC subsets to express distinct PRRs, thereby allowing only certain subsets to be activated by any specific stimulus. However, although some subset-specific PRR expression has been described, most PRRs are widely expressed across DC subsets (Iwasaki and Medzhitov, 2015). Therefore, we hypothesized that subset-specific DC migration could be controlled by a distinct mechanism.

The processes governing DC positioning and migration are largely dependent on chemokines. Immature DCs express a wide variety of chemokine receptors, including CCR1, CCR2, CCR5, CCR6, CCR8, CXCR1, CXCR2, and CXCR4, on their surface (Dieu et al., 1998; Griffith et al., 2014; Sallusto et al., 1998). The reason for this broad expression remains unclear, but it could be involved in the positioning of DC precursors in peripheral tissues as well as the migration of immature DCs to sites of inflammation. Once DCs are activated and undergo maturation, they upregulate CCR7 surface expression. Expression of CCR7 permits the maturing DCs to sense the CCR7 ligand CCL21, which is produced by the lymphatic endothelium (Worbs et al., 2017). This promotes their emigration from the peripheral tissues into the draining lymphatics, where they travel via lymphatic flow into the LN sinuses, including the subcapsular sinus (SCS) (Tal et al., 2011; Weber et al., 2013). Once in the LN sinuses, DCs



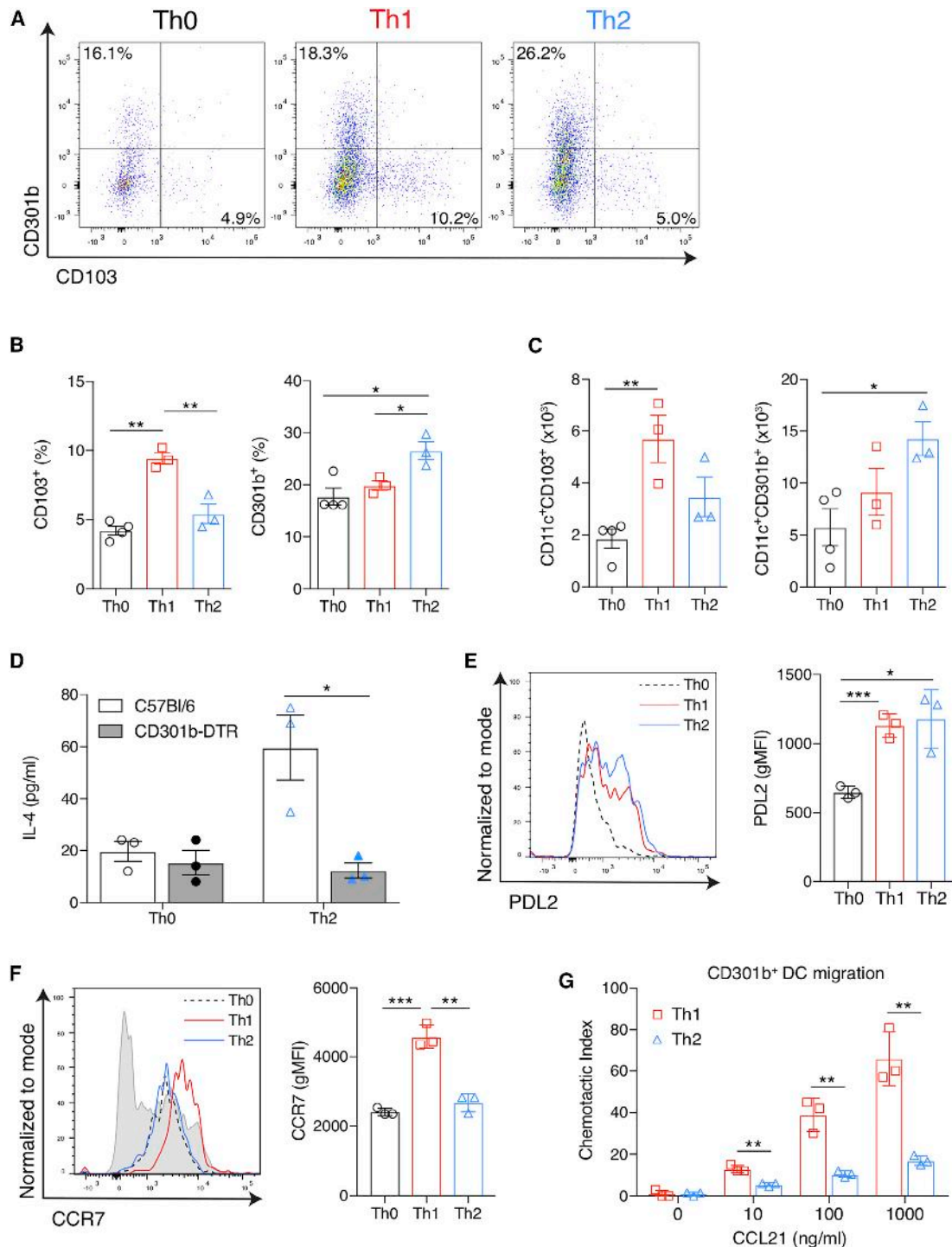


Figure 1. CD301b⁺ DCs Specifically Migrate in Response to Th2-Skewing Immunization

(A) Flow-cytometric analysis of viable CD11c⁺ cells from the popliteal LNs of mice 24 hr after footpad immunization with Th0-skewing (OVA), Th1-skewing (OVA, LPS, and poly I:C), or Th2-skewing (OVA and papain) immunizations. Numbers indicate percentages of CD11c⁺ cells in the indicated quadrant.

(B and C) Quantification of the percentage of CD103⁺ or CD301b⁺ cells out of the total viable CD11c⁺ cells (B) and the total CD11c⁺CD103⁺ or CD11c⁺CD301b⁺ cells from the dLN (C) as gated in (A) 24 hr after the indicated immunization.

(D) ELISA of IL-4 in the supernatant of LN cultures from WT (white bars) or CD301b-DTR (gray bars) mice after *in vitro* restimulation with OVA. All mice received DT and 2.5×10^5 CD4⁺ OTII cells before the indicated immunization.

(E and F) Surface expression of PDL2 (E) and CCR7 (F) from viable CD11c⁺CD301b⁺ cells taken from the dLN 24 hr after the indicated immunization and displayed in comparison with the total CD11c⁺ cells from a Th0-immunized mouse (gray shaded histogram).

(legend continued on next page)

again follow CCL21 gradients that in the SCS are established by the expression of the atypical chemokine scavenger receptor ACKR4 on the SCS ceiling (Ulvmar et al., 2014). After crossing the floor of the LN sinuses in the interfollicular regions (IFRs), DCs continue to follow gradients of the CCR7 ligands CCL19 and CCL21 to enter the T cell area where they can interact with naive T cells (Braun et al., 2011). However, CCR7 might not be sufficient for proper DC positioning within the LN. In a *Heligmosomoides polygyrus* infection model, CXCR5 expression on DCs was shown to be necessary for their localization in IFRs of the dLN and induction of follicular T helper (T_{fh}) and Th2 responses (León et al., 2012). Thus, other chemokines could play a role in DC positioning and adaptive immune outcomes.

We hypothesized that alternative, non-CCR7 chemokine networks could be involved in subset-specific DC migration and entry into the dLN. We found that the Th2-skewing stimulus papain induced robust migration of CD301b⁺ DCs into the dLN despite low surface expression of CCR7. Coinciding with CD301b⁺ DC entry, allergen immunization led to an upregulation in CCL8 expression from CD169⁺SIGN-R1⁺F4/80⁺ macrophages present within the IFRs of the dLN. CCL8 enhanced CCL21-mediated migration of CD301b⁺ DCs in a Src-dependent manner, and both CCR7 and CCR8 were required for the entry of CD301b⁺ DCs into the dLN after papain administration. CD301b⁺ DCs lacking CCR8 expression trafficked to the dLN but were retained in the SCS space, leading to a loss of Th2 differentiation in response to multiple Th2-skewing stimuli. These data reveal a chemokine-mediated mechanism by which LN macrophages control DC-subset migration and the outcome of the adaptive immune response.

RESULTS

The Migration of DC Subsets Is Context Dependent

To understand how the innate immune system might control the migration of DC subsets, we first analyzed the migration of cutaneous DC subsets after immunization with ovalbumin (OVA) alone (Th0 immunization), OVA plus lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (poly I:C) (Th1 immunization), or OVA plus papain (Th2 immunization). As others have shown, subcutaneous Th2 immunization promoted the specific migration of CD301b⁺ DCs into the dLN (Figures 1A–1C) (Kumamoto et al., 2013). Conversely, Th1 immunization specifically promoted the migration of CD103⁺ DCs into the dLN (Figures 1A–1C). Because female mice had a higher frequency of CD301b⁺ DCs in the dLN after both Th0 and Th2 immunization (Figure S1A), we used male mice for all DC migration experiments to control baseline DC migration. As previously shown, CD301b⁺ DCs were absolutely required for Th2 differentiation after papain immunization (Figure 1D) (Kumamoto et al., 2013). Thus, allergen immunization specifically promoted the migration of CD301b⁺ DCs, which are required for Th2 differentiation.

We next examined the phenotype of the CD301b⁺ DCs in the dLN 24 hr after Th0, Th1, or Th2 immunization. As expected, Th2

immunization led to upregulated expression of PDL2, an activation marker, on CD301b⁺ DCs (Figure 1E) (Gao et al., 2013; Kumamoto et al., 2013). This was not significantly different from that seen after a Th1 immunization, indicating that PDL2 upregulation is a common feature of CD301b⁺ DC activation by both allergens and PRRs (Figure 1E). Despite evidence of CD301b⁺ DC maturation and migration after Th2 immunization, CD301b⁺ DCs from the dLN of Th2-immunized mice did not upregulate CCR7 expression, whereas DCs from Th1-immunized mice did (Figure 1F). CCR7 expression on intracutaneous CD301b⁺ DCs after Th2 immunization was slightly higher than after Th0 immunization, but these levels did not approach those seen on CD301b⁺ DCs after Th1 immunization (Figures S1B and S1C). This lower level of CCR7 expression had functional consequences: CD301b⁺ DCs from the dLN of Th2-immunized mice exhibited less chemotaxis toward CCL21 at all concentrations tested than CD301b⁺ DCs isolated from the dLN of Th1-immunized mice (Figure 1G).

Th2-Skewing Immunization Promotes a Specific Chemokine Signature in the dLN

Given that Th2 immunization promoted robust CD301b⁺ DC migration without concurrently inducing optimal CCR7 expression, we hypothesized that additional allergen-induced chemokines could be involved in promoting CD301b⁺ DC migration from the periphery into the dLN. We focused on two potential sites of chemokine-mediated control: the point of DC entry into lymphatics (i.e., the skin) and the point of LN entry from the lymphatics. We focused our examination at 24 hr after immunization, which corresponded with DC entry into the dLN (Sokol et al., 2008). In the skin, both Th1 and Th2 immunizations promoted upregulation of the CCR2 chemokine ligands *Ccl2*, *Ccl7*, and *Ccl12* (Figure 2A). Th2 immunization induced the CCR8 ligand *Ccl8*, but this occurred past the window of DC emigration, making it unlikely to play a role in CD301b⁺ DC migration (Figure 2A). Therefore, we turned our attention to the dLN. Within 24 hr of footpad immunization, Th1 immunization led to upregulation of the CCR2 ligands, whereas Th2 immunization specifically promoted upregulation of *Ccl8* (Figure 2B). *Ccl8* upregulation was specific to Th2 immunization; Th1 immunization reduced the expression of both CCR8 ligands *Ccl1* and *Ccl8* (Figure 2B).

After leaving the afferent lymphatics, DCs enter the SCS space and the LN sinuses, which are lined by lymphatic endothelium and LN sinus macrophages, which must be traversed for DCs to enter the LN parenchyma (Girard et al., 2012; Qi et al., 2014). Using *Ccl8*^{−/−}*Ccl12*^{−/−} mice as a negative control, we examined the localization of CCL8 within dLN sections. In Th0-immunized wild-type (WT) mice, we detected CCL8 in two locations (Figure S2): adjacent to gp38⁺ lymphatic endothelium in the medullary region (Figure 2Ci) and within SCS regions, specifically where the SCS met the medullary region (Figure 2Cii). Concordant with the qPCR data, the number of discrete CCL8-expressing cells significantly increased after Th2 immunization in WT mice but not in *Ccl8*^{−/−}*Ccl12*^{−/−} mice (Figures 2C and 2D). In

(G) Transwell chemotaxis to CCL21 of CD11c⁺CD301b⁺ cells sorted from the dLN 24 hr after Th2-skewing immunization. The chemotactic index reflects the number of cells migrating through the Transwell at the indicated concentration over that of media alone. In all experiments, bars indicate mean values of the individual data points presented. Error bars indicate SEM. Data are representative experiments from three independent experiments with three to four mice (A–F) or three replicates (G) each. *p < 0.05, **p < 0.01, ***p < 0.001, Student's t test. See also Figure S1.

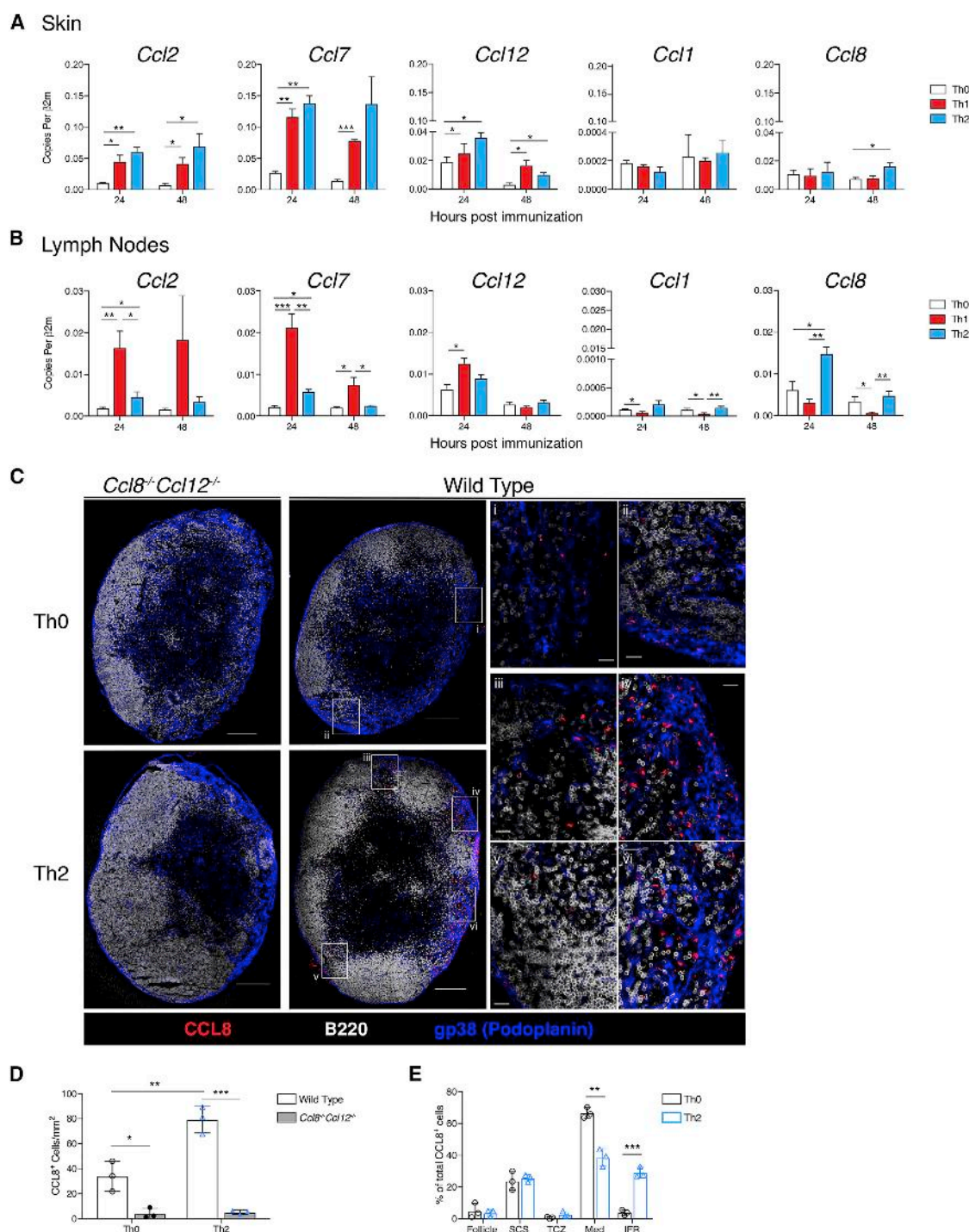


Figure 2. Allergen Immunization Promotes a Specific Chemokine Signature in the dLN

(A and B) qPCR analysis of the footpad skin immunization site (A) and popliteal LN (B) 24 and 48 hr after immunization with Th0-, Th1-, or Th2-skewing immunization. Data are expressed as cDNA copies of the indicated gene per copies of $\beta 2$ m. Each bar represents the mean of 3–6 mice from a representative of four independent experiments. Error bars indicate SEM

(C) CCL8 staining in whole LNs. Popliteal LNs of WT or *Ccl8*^{−/−}*Ccl12*^{−/−} mice were harvested 24 hr after immunization. Confocal immunofluorescence of whole LNs shows CCL8 (red), B220 (white), and gp38 (blue). Insets show the medullary region (i) and the medullary-follicular border (ii) in a Th0-immunized WT mouse. In a Th2-immunized WT mouse, insets show interfollicular regions (IFRs) (iii, iv, and v) and the medullary region (vi). Scale bars indicate 200 μ m (main panels) and 20 μ m (insets).

(legend continued on next page)

addition to this absolute increase in CCL8⁺ cells, we found that Th2 immunization specifically promoted CCL8 expression within the IFRs of the dLN (Figures 2Ciii, 2Civ, 2Cv, and 2E). CCL8⁺ cells were still readily appreciated within the medullary region after Th2 immunization (Figure 2Cvi). However, given observations by others that IFRs are the site of DC entry, we hypothesized that CCL8 produced in the IFRs could play a role in the allergen-induced movement of CD301b⁺ DCs across LN sinuses and into the LN parenchyma (Braun et al., 2011; Woodruff et al., 2014).

CD301b⁺ DC Entry into the dLN Requires CCR8 Signals

CCL8 and its functional human analog CCL18 bind specifically to the CCR8 receptor (Islam et al., 2011, 2013). We hypothesized that CD301b⁺ DCs express CCR8 and that this chemokine system promotes CD301b⁺ DC trafficking into the dLN in response to Th2-skewing stimuli. *Ccr8*^{-/-} mice showed decreased migration of mature, PDL2⁺CD301b⁺ DCs into the dLN at baseline (i.e., Th0 immunization) and after Th2 immunization (Figure 3A). This was not due to defective exit from or a decreased amount of CD301b⁺ DCs within the skin given that there was no significant difference in the frequency of CD301b⁺ DCs in the skin of *Ccr8*^{-/-} mice (Figure S3A). Perhaps as a result of the loss of CD301b⁺ DC migration, CD103⁺ DCs made a larger percentage of the total DC population in the dLN of *Ccr8*^{-/-} mice but were not present in significantly greater numbers (Figure 3B). In order to directly assess the migration of CD301b⁺ DCs from the skin to the dLN, we utilized Kaede transgenic mice, which express a photoconvertible protein (Kaede) that can be converted from green to red fluorescence (Kaede^{red}) (Bromley et al., 2013; Tomura et al., 2008). As expected, we found that migration of CD301b⁺ DCs from the skin to the dLN (i.e., Kaede^{red} cells) was completely dependent on CCR7 (Figure 3C). However, although some baseline migration of CD301b⁺ DCs was detected in *Ccr8*^{-/-} Kaede⁺ mice, CCR8 was required for the allergen-induced DC emigration from the skin to the dLN (Figure 3D).

Our data suggest that CD301b⁺ DCs specifically require CCR8 for entry into the dLN after Th2 immunization. However, by immunofluorescence of the dLN after Th2 immunization, we found no significant difference in the total number of CD301b⁺ DCs between WT and *Ccr8*^{-/-} mice (Figures 3E and 3F). Instead, whereas CD301b⁺ DCs generally localized to the T-B interface of the dLN in WT mice after Th2 immunization, CD301b⁺ DCs in Th2-immunized *Ccr8*^{-/-} mice appeared to be excluded from entering the dLN and were retained at the periphery of the LN (Figure 3E). Closer inspection after Th2 immunization revealed an accumulation of CD301b⁺ DCs in the SCS space of *Ccr8*^{-/-} mice (Figures 3G and 3H). Because standard LN digestion protocols do not adequately release SCS stroma, these SCS CD301b⁺ DCs were unintentionally excluded in flow-cytometric experiments, leading to a decrease in CD301b⁺ DC numbers by flow cytometry but not immunofluorescence (Fletcher et al.,

2011; Gray et al., 2012). We were able to confirm this by using enhanced digestion methods designed to release LN stromal cells (Fletcher et al., 2011). Although standard digestion methods showed a decreased percentage of Kaede^{red} skin emigrant CD301b⁺ DCs in *Ccr8*^{-/-} mice (Figure 3D), no such defect was seen after enhanced digestion, indicating that enhanced digestion released CD301b⁺ DCs from the SCS (Figure S3B). Together, these data indicate that whereas CCR7 is essential for CD301b⁺ DC entry into the draining lymphatics, CCR8 plays an important role in promoting their migration across the floor of the LN sinus and into the dLN after Th2 immunization.

CD169⁺SIGN-R1⁺ Macrophages Produce *Ccl8*, which Is Required for CD301b⁺ DC Entry

Our initial CCL8 staining showed it to be closely associated with the lymphatic endothelium of the dLN, but we were unable to detect CCL8 or *Ccl8* RNA in Lyve-1⁺ or gp38⁺ lymphatic endothelium (data not shown). We next hypothesized that LN sinus macrophages found in association with lymphatic endothelium were the source of CCL8. LN sinus macrophages include CD169⁺SIGN-R1⁻F4/80⁻ SCS macrophages and F4/80⁺ medullary macrophages, which have been divided into CD169⁻F4/80⁺ medullary cord macrophages and CD169⁺SIGN-R1⁺F4/80⁺ medullary sinus macrophages (Gray and Cyster, 2012). In accordance with their name, medullary cord macrophages are present in the LN medullary region, and SCS macrophages make up the floor of the SCS overlying B cell follicles. However, CD169⁺SIGN-R1⁺ medullary sinus macrophages can be found along the lymphatic endothelium of the medullary region as well as IFRs (Gray and Cyster, 2012). CD169⁺ macrophages in the lamina propria have been shown to produce CCL8 in murine colitis models (Asano et al., 2015). Similarly, we detected CCL8 within CD169⁺F4/80⁺ macrophages after Th2-skewing immunization and visualized these cells in apposition with CD301b⁺ DCs (Figures 4A and S4A). Given their localization in serial sections, CCL8⁺CD169⁺ cells also expressed SIGN-R1, consistent with the F4/80⁺CD169⁺SIGN-R1⁺ macrophage population (Figure S4B). CD169⁺SIGN-R1⁻ SCS macrophages were specifically localized in the SCS floor adjacent to B cell follicles (Figure 4B, SCS), whereas CD169⁺SIGN-R1⁺ macrophages were present in the medullary region (Figure 4B, med) as well as in IFRs (Figure 4B, IFR). To confirm that CD169⁺SIGN-R1⁺ macrophages were the source of *Ccl8*, we utilized enhanced enzymatic digestion techniques to free LN-resident macrophages for further analysis (Fletcher et al., 2011). Using SIGN-R1⁺ selection by magnetic beads, we found that *Ccl8* and *Siglec1* (CD169) were both enriched in the SIGN-R1⁺ population (Figure 4C). We next performed flow-cytometric sorting of SIGN-R1⁺ cells, which identified the CD169⁺SIGN-R1⁺CD11c⁺CD11b⁺ macrophages as the source of *Ccl8* (Figure 4D). We confirmed this enrichment of *Ccl8* expression by qPCR by using an alternative sorting scheme to identify the CCL8-producing macrophages on the basis of CD11c⁺CD103⁺CD11b^{high}CD169⁺F4/80⁺

(D) Total CCL8⁺ cells per mm² of LN section from WT and *Ccl8*^{-/-} *Ccl12*^{-/-} mice 24 hr after Th0 or Th2 immunization.

(E) Percentage of CCL8⁺ cells in each indicated region out of the total CCL8⁺ cells per LN section in WT Th0- or Th2-immunized mice. Abbreviations are as follows: Follicle, B cell follicle; SCS, subcapsular sinus; TCZ, T cell zone; Med, medullary region; and IFR, interfollicular region.

Data represent three mice from each of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, Student's t test. See also Figure S2.

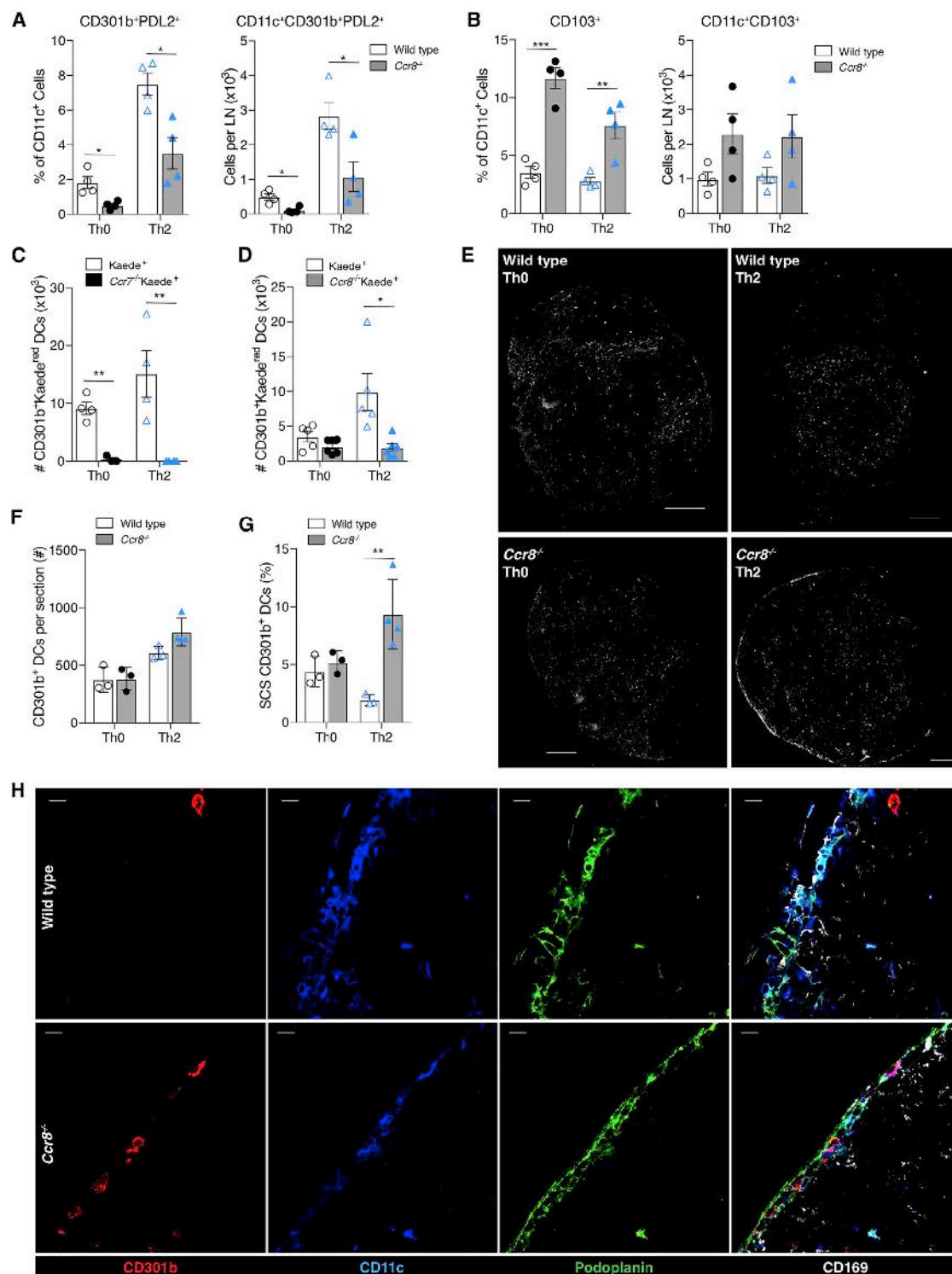


Figure 3. CCR8 Is Required for CD301b⁺ DC Entry into the dLN

(A and B) Quantification of CD301b⁺PDL2⁺ DCs (A) and CD103⁺ DCs (B) in the dLN of Th0- or Th2-immunized WT (clear bars) or *Ccr8*^{-/-} (gray bars) mice as determined by flow cytometry. Percentages reflect CD301b⁺PDL2⁺ or CD103⁺ cells out of the total CD11c⁺ cells, and numbers reflect total cells per popliteal LN. (C and D) Flow cytometry of inguinal LNs was performed 24 hr after immunization of photoconverted flank skin of Kaede⁺ transgenic mice. Total CD11c⁺CD301b⁺Kaede^{red} (i.e., photoconverted) cells per inguinal LN are shown in comparison with (C) *Ccr7*^{-/-}Kaede⁺ and (D) *Ccr8*^{-/-}Kaede⁺ groups. (E) CD301b staining (white) of whole popliteal LNs of WT or *Ccr8*^{-/-} mice 24 hr after Th0 or Th2 immunization. Scale bars indicate 200 μ m.

(legend continued on next page)

expression (Figures S5A and S5B). Finally, to confirm our cell-sorting data, we depleted CD169⁺SIGN-R1⁺F4/80⁺ macrophages by using clodronate-mediated depletion (Figure S5A). Clodronate treatment completely abolished *Siglec1* and *Ccl8* expression within the dLN (Figure 4E). Together, these data indicate that CD169⁺SIGN-R1⁺ macrophages, located in the IFRs and LN medullary area, are the source of CCL8 in the dLN.

CCR8 has two ligands in mice: CCL1 and CCL8 (Griffith et al., 2014). CCL1 has been shown to be produced by the lymphatic endothelium on the floor of the SCS and to promote entry of monocyte-derived DCs into the dLN (Qu et al., 2004). We found that Th2-skewing immunization did not promote *Ccl1* production in the dLN (Figure 2B). However, to ensure that the defect in CD301b⁺ DC migration was due to CCL8 and not CCL1, we examined CD301b⁺ DC migration in *Ccl8*^{-/-} mice. Because of the genes' proximity, *Ccl8*^{-/-} mice are only currently available as *Ccl8*^{-/-}*Ccl12*^{-/-} dual-knockout mice (Tsou et al., 2007). Consistent with our data from *Ccr8*^{-/-} mice with standard enzymatic digestion, we found that *Ccl8*^{-/-}*Ccl12*^{-/-} mice exhibited defective CD301b⁺ DC migration by flow cytometry in both total frequency and number after Th2-skewing immunization (Figure 4F). Therefore, we conclude that CCL8 production from CD169⁺SIGN-R1⁺ macrophages is required for CD301b⁺ DC entry into the dLN after Th2-skewing immunization.

Analysis of *Ccl1*-*Ccl8* Dual-Reporter Transgenic Mice Confirms *Ccl8* Production by CD169⁺SIGN-R1⁺ Macrophages

In order to verify our CCL8 expression data, we generated a transgenic mouse, called REC8 (reporting the expression of CCR8 ligands), that reports the expression of both CCL1 and CCL8. To allow for detection of *Ccl1* and *Ccl8* expression, we inserted mCherry at the start codon of *Ccl1* and eGFP at the start codon of *Ccl8* in a bacterial artificial chromosome (BAC) that contained *Ccl1* and *Ccl8* (Figure 5A). Consistent with RNA data from dLNs (Figure 2B), *Ccl1*-mCherry expression was unaffected, whereas *Ccl8*-eGFP expression was induced by Th2 immunization (Figure 5B). CCL1 has been described to be produced by CD3⁺ T cells as well as the lymphatic endothelium (Miller and Krangel, 1992; Qu et al., 2004). We were unable to detect *Ccl1*-mCherry expression in lymphatic endothelial cells, blood endothelial cells, or fibroblastic reticular cells of the dLN (Figure S5C). Instead, we found that *Ccl1*-mCherry⁺ cells were uniformly CD3⁺, whereas *Ccl8*-eGFP⁺ cells were CD11b⁺ (Figure 5C). Accurate *Ccl1* and *Ccl8* reporting by REC8 mice was confirmed by enrichment of *Ccl1* and *Ccl8* RNA within the mCherry⁺ and eGFP⁺ populations, respectively (Figure 5D). The expression of CD11b on *Ccl8*-eGFP⁺ cells suggested that *Ccl8*-eGFP marked CD169⁺SIGN-R1⁺ macrophages. Confirming this, CD169⁺SIGN-R1⁺ macrophages exhibited *Ccl8*-eGFP expression, which was induced by Th2 immunization, but not *Ccl1*-mCherry expression

(Figure 5E). These data indicate that Th2 immunization specifically induces *Ccl8* expression by CD169⁺SIGN-R1⁺ macrophages.

CCR8 Promotes CCR7-Mediated Migration of CD301b⁺ DCs

Our data suggest that whereas both CCR7 and CCR8 are necessary for CD301b⁺ DC migration, CCR8 plays a specific role in promoting CD301b⁺ DC migration across the SCS and into the LN parenchyma. *Ccr8* mRNA has been detected in dermal DC populations, but the detection of CCR8 has been hampered by the lack of suitable reagents (Jakubzick et al., 2006; Qu et al., 2004; Sallusto et al., 1998; Yabe et al., 2015). Flow-cytometric staining using commercially available anti-mouse CCR8 antibodies consistently showed staining of DCs from *Ccr8*^{-/-} mice (data not shown). Therefore, to determine the expression of CCR8 on CD301b⁺ DCs, we utilized a fluorescently labeled ligand of CCR8, human CCL1 (hCCL1). hCCL1 binds to the murine CCR8 receptor, inducing calcium (Ca²⁺) flux and chemotaxis in transfected cell lines (Islam et al., 2011). Using this reagent to fluorescently "label" CCR8, we found that, compared with DCs from *Ccr8*^{-/-} mice, CD11c⁺CD11b⁺CD301b⁻ DCs (CD11c⁺ DCs) did not express detectable CCR8 but that CD11c⁺CD11b⁺CD301b⁺ DCs (i.e., CD301b⁺ DCs) from the dLNs of Th0-immunized mice expressed detectable CCR8 that increased after Th2 immunization (Figures 6A and S6A). We found no significant difference in CCR7 expression between WT and *Ccr8*^{-/-} DCs after Th0 or Th2 immunization (Figure 6B). As expected, *ex vivo* CCL21 stimulation of CD301b⁺ DCs promoted CCR7 internalization, but this was unaffected by co-stimulation with CCL8 (Figure S6B). Likewise, CCL21 stimulation had no effect on CCR8 expression (Figure S6B). These data suggest that CCR8 is expressed by CD301b⁺ DCs and that CCR8 expression does not affect CCR7 expression.

We next performed *in vitro* chemotaxis assays to determine what role CCR8 and its ligand, CCL8, play in CD301b⁺ DC migration. CCL8 had no effect on migration of the CCR8⁻CD11c⁺ DCs (Figure 6C). Despite expression of CCR8, CD301b⁺ DCs showed no migration in response to CCL8 alone (Figure 6D). However, the combination of CCL8 and the CCR7 ligand CCL21 was synergistic in CD301b⁺ DCs, leading to an almost doubling of migration at the physiologic 100 ng/mL concentration of these chemokines (Figure 6D). This synergy was also seen with the other CCR8 ligand, CCL1 (Figure S6C). *In vitro* assays with μ M chemokine concentrations have shown that human CCL1, CCL2, CCL7, CCL8, and CCL11 synergize with CCL21 by forming heteromeric chemokine complexes that promote the migration of mature human DCs (Paoletti et al., 2005). Therefore, we examined whether this synergistic effect on CCR7-mediated CD301b⁺ DC migration is similarly indiscriminate but found no effect of murine CCL2, CCL7, or CCL11 on CD301b⁺ DC migration in the presence or absence of CCL21 (Figures S6D–S6F).

(F and G) Quantification of the total number CD11c⁺CD301b⁺ cells per entire LN section (F) or the percentage of total CD11c⁺CD301b⁺ within the SCS (G) (identified by podoplanin and CD169 immunohistochemical staining) 24 hr after immunization.

(H) CD301b⁺ DCs within the SCS space of *Ccr8*^{-/-} mice, but not WT mice, 24 hr after Th2 immunization. Panels show single stains of CD301b (red), CD11c (blue), and podoplanin (green) and an overlay of those stains and CD169 (white). Scale bars indicate 10 μ m.

Images (E and H) represent two to three mice per condition and four independent experiments. Bar graphs represent the mean value of individual mouse data points (three to six mice per condition per experiment) taken from a representative of three independent experiments (A, B, D, and F–H) or two independent experiments (C). Error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001, Student's t test. See also Figure S3.

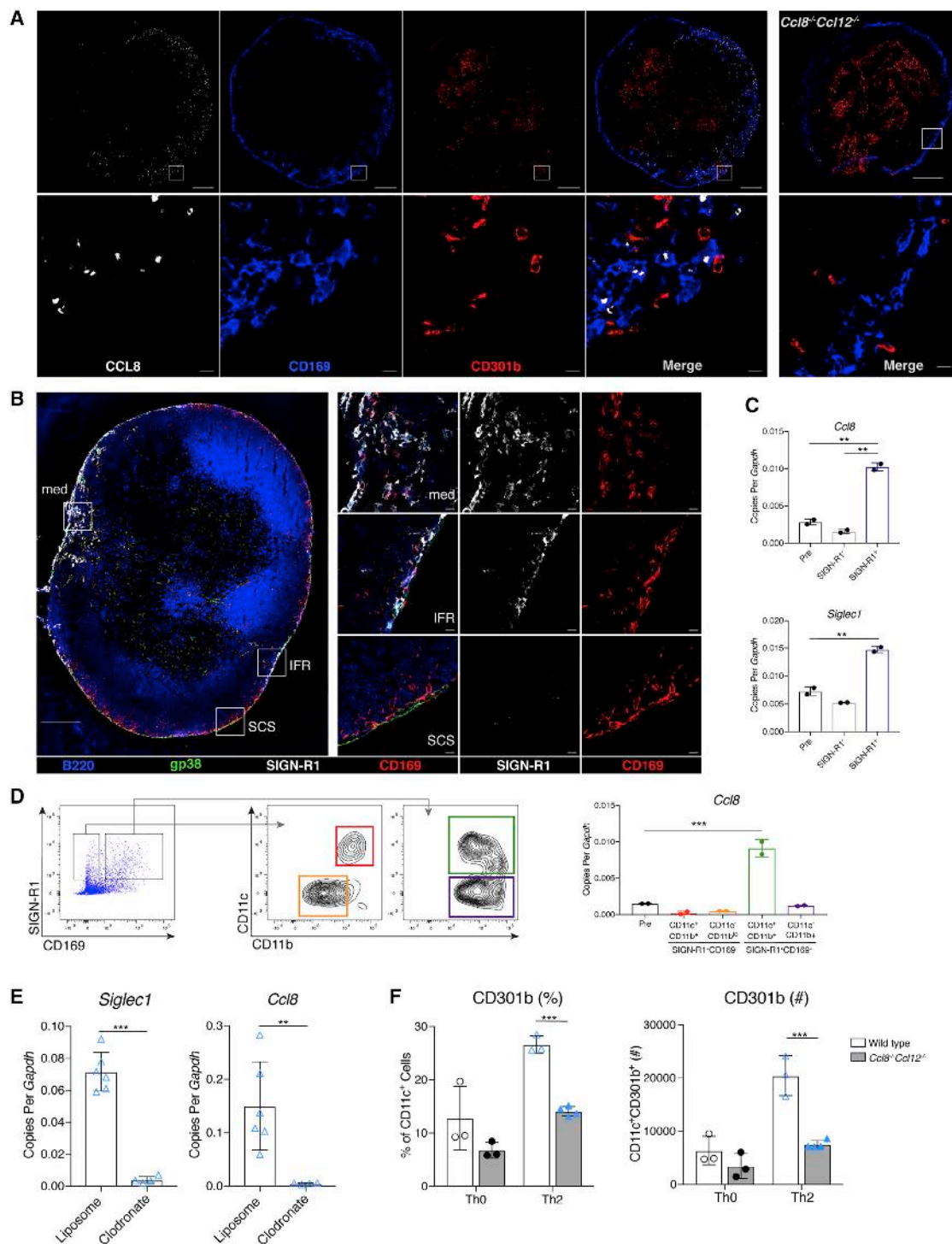


Figure 4. CCL8 Is Produced in the dLN by CD169⁺SIGN-R1⁺ LN Macrophages that Interact with CD301b⁺ DCs

(A) Confocal immunofluorescence of whole popliteal LN (top) and the area of interest (bottom) 24 hr after Th2-skewing immunization in WT or *Ccl8*^{-/-}*Ccl12*^{-/-} mice as indicated. Panels show single stains of CCL8 (white), CD169 (blue), and CD301b (red) and an overlay. Scale bars represent 200 μ m (top) and 10 μ m (bottom). Images represent three mice in each of three independent experiments.

(B) Confocal immunofluorescence of whole popliteal LN. Insets show CD169⁺SIGN-R1⁺ macrophage populations in medullary regions (med) and IFRs, as well as CD169⁺SIGN-R1⁻ SCS macrophages in the SCS. Staining shows CD169 (red), SIGN-R1 (white), gp38 (green), and B220 (blue). Scale bars represent 200 μ m (whole LN) and 10 μ m (insets).

(C) qPCR analysis of total cells (Pre) or MACS positively selected SIGN-R1⁺ or SIGN-R1⁻ cells from popliteal LNs.

(legend continued on next page)

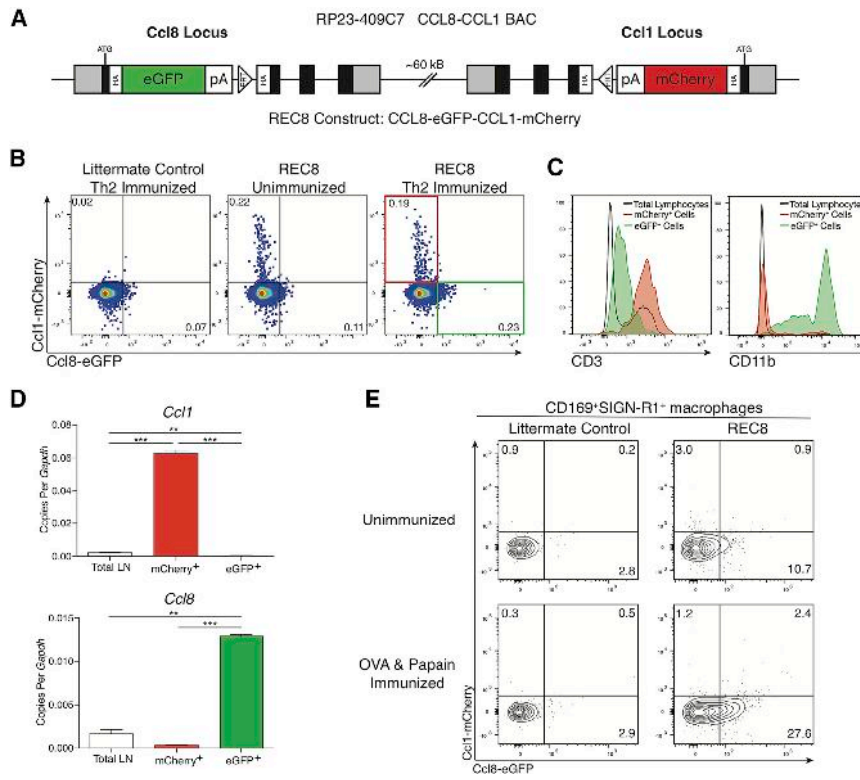


Figure 5. CCR8 Ligands Are Differentially Expressed in the dLN

(A) Schematic of REC8 transgene construct indicates the insertion of eGFP into the *Ccl8* locus and mCherry into the *Ccl1* locus of the RP23-409C7 BAC. Gray box, non-coding exons; black box, endogenous coding exons; green box, eGFP open reading frame (ORF); red box, mCherry ORF; clear box, hemagglutinin tags or SV40 poly(A); clear triangle, flippase recognition target (FRT) site. (B) Expression of mCherry and eGFP in total live dLN cells in unimmunized REC8 mice or in Th2-immunized REC8 mice or littermate control WT mice. Numbers are the percentages of total cells in the indicated gates.

(C) Expression of CD3 and CD11b in total live dLN cells (black), mCherry⁺ cells (red), or eGFP⁺ cells (green) gated as in (B) and isolated from REC8 mice 24 hr after Th2-skewing immunization. (D) qPCR analysis of *Ccl1* or *Ccl8* expression in total LN (clear) or fluorescence-activated-cell-sorted mCherry⁺ cells (red bar) and eGFP⁺ cells (green bar) gated as in (B) from dLNs harvested from REC8 mice 24 hr after Th2-skewing immunization.

(E) Expression of mCherry and eGFP on live CD11b⁺CD169⁺SIGN-R1⁺ cells harvested from the dLN of unimmunized WT and REC8 mice and 24 hr after Th2-skewing immunization. Bar graphs represent the mean value of replicates (two data points per bar).

Data represent two (D) or three (A–C and E) independent experiments. Error bars represent SEM. **p < 0.01, ***p < 0.001, Student's t test. See also Figure S5.

Indeed, the migration synergy induced by murine CCL8 and CCL21 was dependent on CCR8 expression in CD301b⁺ DCs given that it was absent in *Ccr8*^{−/−} CD301b⁺ DCs (Figure 6E). To determine whether a CCL8 gradient is necessary for its synergistic effect, we performed a checkerboard chemotaxis assay in which CD301b⁺ DCs harvested from the dLN of Th2-immunized mice were exposed to combinations of CCL8 and CCL21 in either gradients or fixed concentrations. Whereas CCL21-mediated migration required a CCL21 gradient, the synergistic effect of CCL8 on CCL21-induced migration of CD301b⁺ DCs was independent of the presence of a CCL8 gradient (Figure 6F). These data suggest that the production of CCL8 is an innate control mechanism to specifically promote the migration of Th2-skewing CD301b⁺ DCs by altering their responsiveness to CCL21 gradients.

Chemokine receptors are G-protein-coupled receptors (GPCRs) that classically signal through G α i to promote cell migration. We hypothesized that CCL8 augments CCL21-medi-

ated migration in CD301b⁺ DCs by enhancing GPCR-mediated signaling. To study this, we measured Ca²⁺ flux in CD301b⁺ DCs isolated from the dLNs of Th2-immunized mice. We were able to detect Ca²⁺ flux in CD301b⁺ DCs exposed to either CCL8 or CCL21 at 10 and 100 ng/mL concentrations (Figure 6G). Although exposure to either CCL8 or CCL21 at 1 ng/mL did not induce Ca²⁺ flux, the combination of CCL8 and CCL21 at 1 ng/mL did induce Ca²⁺ flux (Figure 6H). This indicates that perhaps CCL8 increases the sensitivity of CD301b⁺ DCs to low amounts of CCL21 given that we found no augmentation in Ca²⁺ flux at higher chemokine concentrations (Figure S6G). We hypothesized that, in addition to inducing Ca²⁺ signaling, CCL8 might activate other signaling pathways that contribute to cell migration. It was recently reported that prostaglandin E₂ augments CCL21-induced human DC migration by increasing signaling via Src kinase and the tyrosine phosphatase SHP2 (Hauser et al., 2016). Using a human CCR8-transfected cell line (Islam et al., 2011), we found that murine CCL8 stimulation

(D) Gating scheme for identifying macrophage populations from total viable lymphocytes obtained 24 hr after Th2 immunization from dLN. qPCR analysis of the indicated populations shows *Ccl8* expression. Colored histograms correspond to colored gates.

(E) qPCR from total LNs of control liposome- or clodronate-treated mice 24 hr after Th2-mediated immunization. Data points are from four to six individual mice in one representative experiment out of three independent trials.

(F) Flow cytometry of viable CD11c⁺CD301b⁺ cells shows a decreased percentage and number of CD301b⁺ DCs in *Ccl8*^{−/−}*Ccl12*^{−/−} mice after Th2-skewing immunization. Data points are from dLNs of three or four individual mice from one of three independent trials.

Cells in (C) and (D) were obtained by enhanced enzymatic digestion, and those in (F) were obtained by routine enzymatic digestion. qPCR data are presented as copies of the indicated transcript over *Gapdh*. Bar graphs represent the mean value of the indicated data points, and error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001, Student's t test. See also Figures S4 and S5.

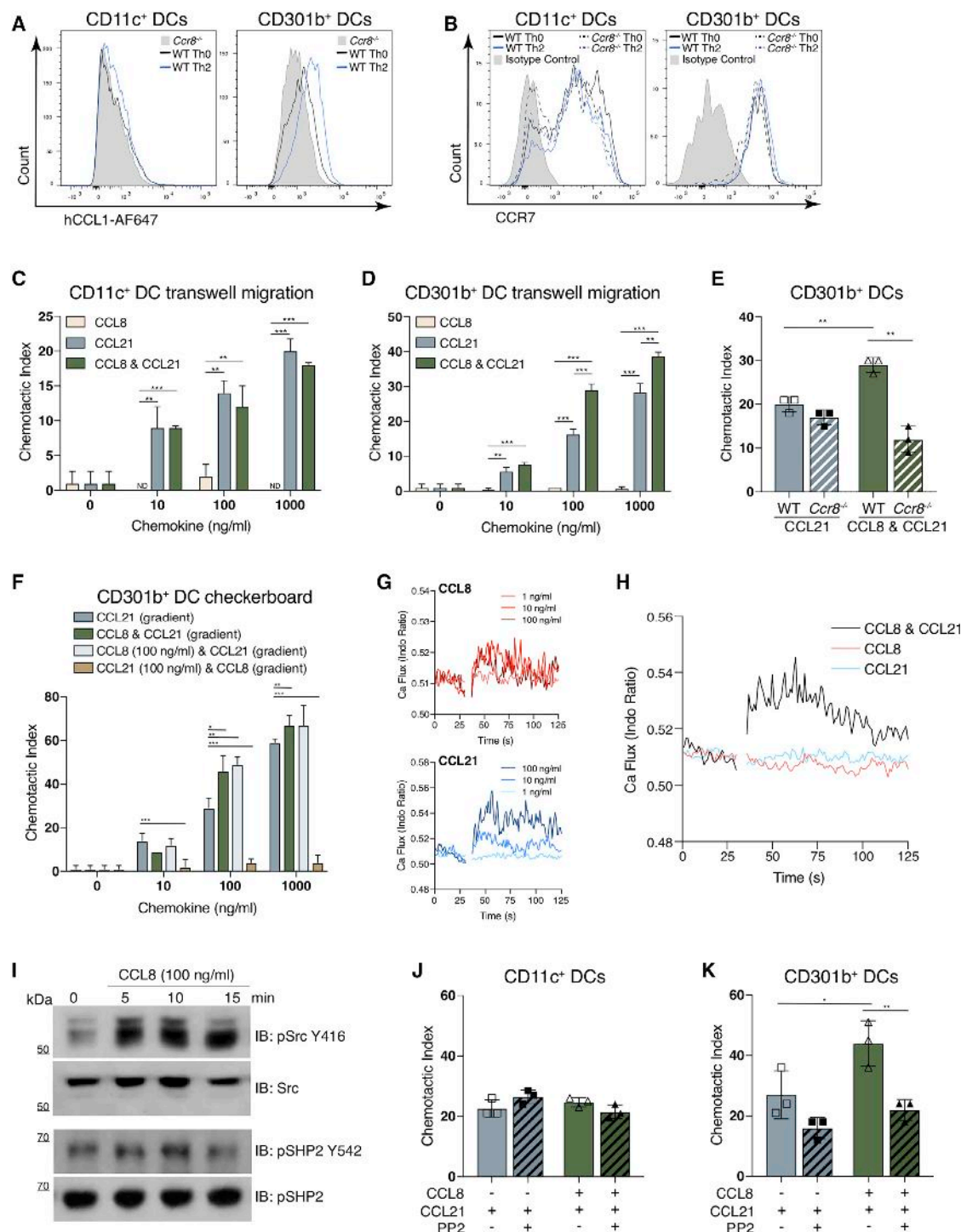


Figure 6. CCL8 Synergizes with CCL21 to Specifically Promote CD301b⁺ DC Migration

(A) Flow-cytometric expression of hCCL1-AF647 binding to CD11c⁺CD11b⁺CD301b⁺ (CD11c⁺ DCs) or CD11c⁺CD11b⁺CD301b⁺ (CD301b⁺ DCs) cells obtained from the dLNs of WT mice after the Th0-skewing (black histogram) or Th2-skewing (blue histogram) immunization or from *Ccr8*^{-/-} mice after Th0 immunization (gray shaded histogram).

(B) Flow-cytometric cell-surface expression of CCR7 on CD11c⁺ DCs or CD301b⁺ DCs from the dLN after Th0 immunization in WT (black solid) or *Ccr8*^{-/-} (black dotted) mice or after Th2 immunization in WT (blue solid) or *Ccr8*^{-/-} (blue dotted) mice. The isotype control from WT Th0-immunized mice is shown (gray shaded histogram).

(C and D) Transwell chemotaxis of CD11c⁺ DCs (C) or CD301b⁺ DCs (D) to the indicated concentrations of CCL8, CCL21, or CCL8 and CCL21.

(E) Transwell chemotaxis of CD301b⁺ DCs isolated from WT or *Ccr8*^{-/-} mice to 100 ng/mL of the indicated chemokines.

(legend continued on next page)

induced the phosphorylation of Src and SHP2 within 5 and 10 min, respectively (Figure 6I). Therefore, we hypothesized that CCL8 could similarly promote signaling through the Src-SHP2 pathway to enhance CCL21-mediated migration in CD301b⁺ DCs. Chemotaxis of CD11c⁺ DCs to CCL21 was unaffected by the presence of the Src inhibitor PP2 (Figure 6J). However, in CD301b⁺ DCs, Src inhibition abolished the synergistic effect of CCL8 on CCL21-mediated migration (Figure 6K). Thus, CCL8 directly promotes Src-SHP2 signaling and augments CCL21-mediated migration in CD301b⁺ DCs in a Src-dependent manner.

CCR8-Mediated Entry of CD301b⁺ DCs into the dLN Is Required for Th2 Differentiation

Our data suggest that the production of CCL8 by CD169⁺SIGN-R1⁺ macrophages promotes the migration of CCR7^{lo}CD301b⁺ DCs by inducing a Src-dependent signaling pathway. This led us to hypothesize that the production of CCL8 by CD169⁺SIGN-R1⁺ macrophages is a mechanism by which the innate immune system controls the polarization of the adaptive immune response. Therefore, we investigated whether CCR8 deficiency leads to alterations in Th development by examining the immune response in *Ccr8*^{-/-} mice that had previously received WT naive OVA-specific CD4⁺ OTII cells. In response to Th2 immunization (OVA and papain), Th2 differentiation displayed a significant defect according to interleukin-4 (IL-4) production during *in vitro* restimulation, but we found no defect in interferon- γ (IFN- γ) production upon restimulation after Th1-skewing immunization (Figure 7A). To assess whether this defect in Th2 differentiation was limited to papain immunization, we next examined CD4⁺ T cell differentiation after immunization with the alternative Th2-skewing agent, OVA emulsified in aluminum hydroxide (OVA-alum), or infection with *Nippostrongylus brasiliensis* (*N. brasiliensis*). Regardless of the Th2-skewing immunization, Th2 differentiation showed a significant defect on the basis of IL-4 and IL-13 production from transferred OVA-specific CD4⁺ OTII cells in *Ccr8*^{-/-} mice (Figures 7B, 7C, and S7). These data indicate that Th2 differentiation in response to cutaneous immune stimuli requires CCR8.

To determine whether this phenotype seen in *Ccr8*^{-/-} mice is the result of CCR8 deficiency specifically on the CD301b⁺ DC subset, we produced mixed bone marrow (BM) chimeras by utilizing WT, CD301b-DTR (diphtheria toxin receptor), and *Ccr8*^{-/-} mice. As illustrated in Figure 7D, we produced three groups of mice: WT, *Ccr8*^{-/-}, and CD301b^{Ccr8}^{-/-}. WT mice were mixed chimeras of WT and CD301b-DTR cells, allowing us to control for any effect caused by depletion of CD301b⁺ DCs derived

from CD301b-DTR precursors, whereas *Ccr8*^{-/-} mice were standard BM chimeras into WT mice (Figure 7E). CD301b^{Ccr8}^{-/-} mice were mixed chimeras of CD301b-DTR and *Ccr8*^{-/-} BM cells, allowing us to examine mice in which the overall immune compartment was mixed but in which the CD301b⁺ DC compartment was derived from *Ccr8*^{-/-} mice after diphtheria toxin (DT)-mediated depletion (Figure 7E). As expected, *Ccr8*^{-/-} BM chimeras showed a defect in Th2 differentiation, as measured by IL-4 and IL-13 production upon *in vitro* restimulation of previously transferred OVA-specific CD4⁺ T cells (Figure 7F). CD301b^{Ccr8}^{-/-} mice, in which all CD301b⁺ DCs were CCR8-deficient cells but the rest of the immune cells were a mixture of CCR8-sufficient and CCR8-deficient cells, also showed a defect in Th2 differentiation (Figure 7F). Restricting complete CCR8 deficiency to the CD301b⁺ DC population led to a significant loss of IL-4 production that was between those seen in the WT and *Ccr8*^{-/-} groups and a loss of IL-13 production that was indistinguishable from that in *Ccr8*^{-/-} chimeras. Because both Th2 and Tfh cells produce IL-4 but only Th2 cells produce IL-13, we believe that these data indicate that CCR8 expression on CD301b⁺ DCs is required for their migration into the dLN after allergen exposure and is necessary for subsequent Th2 differentiation. However, the intermediate level of IL-4 production seen in CD301b^{Ccr8}^{-/-} chimeras could indicate that another, non-CD301b⁺ DC subset involved in Tfh development requires CCR8 for entry into the dLN and subsequent Tfh differentiation (Kumamoto et al., 2016).

DISCUSSION

A fundamental paradigm in immunity is the role of DCs in bridging the innate and adaptive immune responses. DCs are positioned throughout the periphery, where they are poised for early detection of pathogens. Pathogens induce DC maturation through activation of their PRRs, which leads to CCR7 upregulation and migration into the dLN. Within the dLN, DCs can interact with naive T cells to activate the adaptive immune response by providing antigen presentation, co-stimulation, and skewing signals. However, this paradigm does not address the evidence that specific DC subsets play distinct roles in T cell activation. Dermal IRF4⁺CD301b⁺PDL2⁺ DCs and lung KLF4⁺ DCs migrate to dLNs after allergen exposure and have been shown to play a necessary role in Th2 differentiation, whereas IRF4⁺KLF4⁻ DCs play a specific role in the differentiation of Th17 cells (Gao et al., 2013; Kumamoto et al., 2013; Tussiwand et al., 2015; Williams et al., 2013). Given that the migration of DC subsets is stimulus specific and that DC subsets induce different adaptive immune

(F) Checkerboard Transwell migration assay of CD301b⁺ DCs in response to CCL21 gradient alone (blue), CCL8 and CCL21 combined gradient (green), fixed CCL8 concentration and CCL21 gradient (light blue), and fixed CCL21 concentration and CCL8 gradient (brown).

(G) Analysis of Ca²⁺ flux in CD301b⁺ DCs. The gap indicates the addition of CCL8 or CCL21 at the indicated concentration.

(H) Ca²⁺ flux in CD301b⁺ DCs. The gap indicates the addition of 1 ng/mL of CCL8 and CCL21 (black), CCL8 (pink), and CCL21 (blue).

(I) Western blot of Src and SHP2 phosphorylation in serum-starved 4DE4 cells stably transfected with human CCR8.

(J and K) Transwell chemotaxis of CD11c⁺ DCs (J) or CD301b⁺ DCs (K) in response to 100 ng/mL of the indicated chemokines with or without 10 μ M PP2 (Src inhibitor).

In all panels, cells were harvested from dLNs 24 hr after the indicated immunization. Graphs in (A), (B), (G), and (H) represent four independent experiments with three mice per group. We obtained cells by pooling dLNs from 30–50 mice (C–F, J, and K) or 12 mice (G and H) 24 hr after Th2 immunization and using fluorescence-activated cell sorting to obtain CD11c⁺ DCs or CD301b⁺ DCs. Bar graphs represent the mean value of replicates (two to three data points per bar) and are one representative trial of four independent experiments. Error bars represent SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, Student's *t* test. See also Figure S6.

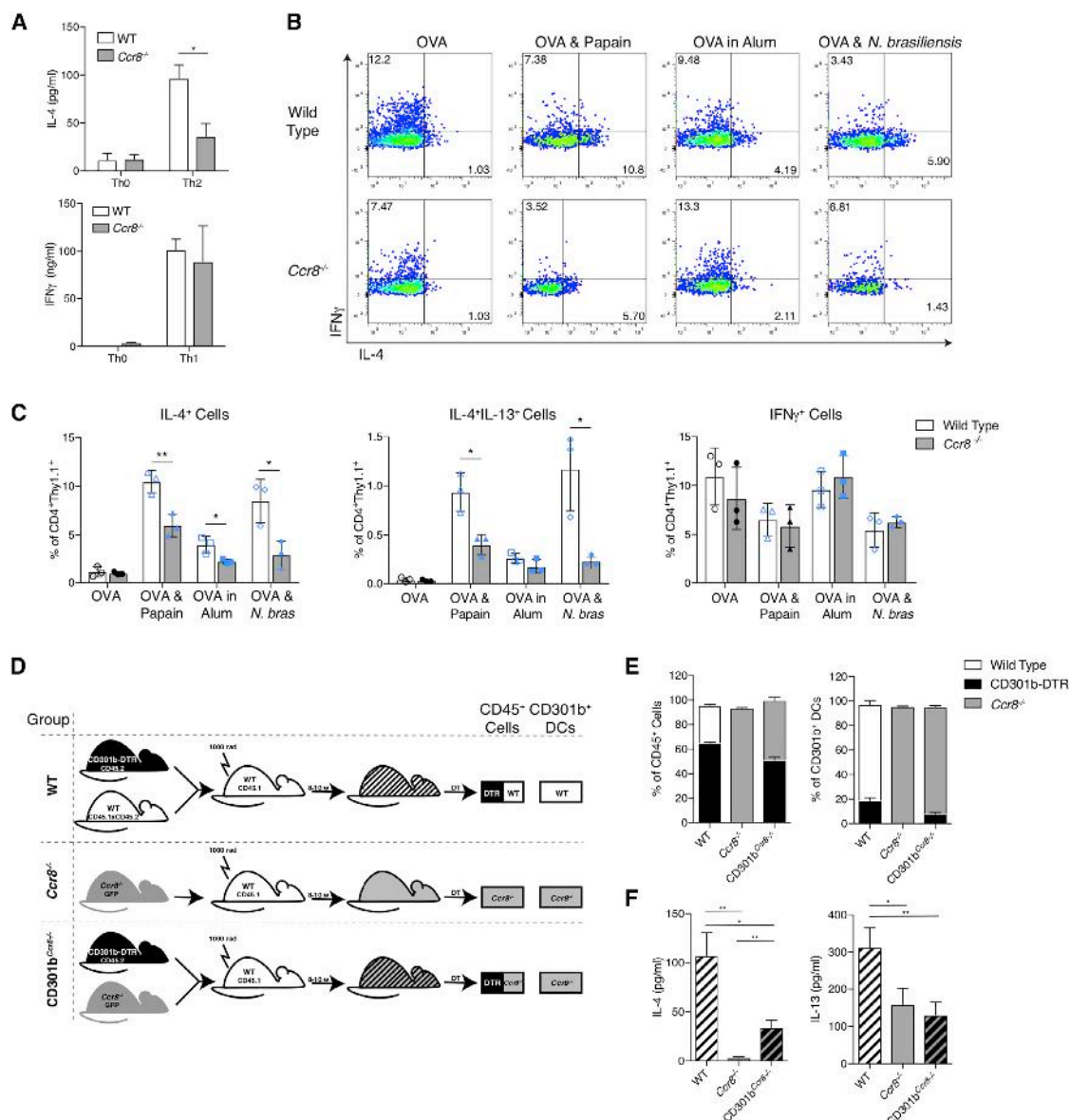


Figure 7. CCR8 on CD301b⁺ DCs Is Required for Allergen-Induced Th2 Differentiation

(A) IL-4 and IFN-γ concentrations in supernatants after *in vitro* restimulation of total dLN cells from WT or *Ccr8*^{-/-} mice after Th0- or Th2-skewing immunizations (IL-4) or Th0- or Th1-skewing immunizations (IFN-γ). Mice received 2.5 × 10⁵ CD4⁺ OTII cells before immunization, dLNs were harvested 5 days after immunization for *in vitro* restimulation with OVA, and supernatants were obtained 4 days later.

(B) Intracellular IL-4 and IFN-γ staining of CD4⁺Thy1.1⁺ viable cells 4 days after OVA, OVA plus papain, or OVA-alum immunization or 5 days after OVA and *N. brasiliensis* infection. Mice received 2.5 × 10⁵ to 5 × 10⁵ naive CD4⁺Thy1.1⁺ OTII cells before immunization. Representative plots are shown with the percentage of cells in the indicated quadrants.

(C) Quantification of IL-4⁺, IL-4⁺IL-13⁺, or IFN-γ⁺ staining of CD4⁺Thy1.1⁺ cells after immunization or infection as in (B).

(D) Generation of BM chimeras.

(E) Chimerism by flow-cytometric analysis of total viable CD45⁺ cells and CD11c⁺CD301b⁺ DCs from popliteal LNs 3 days after last DT injection.

(F) IL-4 and IL-13 concentrations in supernatants from *in vitro* restimulation of total LN cells from the indicated BM chimeras. Chimeric mice received 2.5 × 10⁵ CD4⁺ OTII cells and DT before Th2-skewing immunization. dLNs were harvested 5 days after immunization and cultured with OVA for 4 days before supernatant harvest.

Data represent three (A–C) or five (D and E) independent experiments each with three to five mice per condition. Bar graphs represent the mean value, and error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001, Student's t test. See also Figure S7.

outputs, we were interested in defining the mechanisms that promote this specificity in DC migration. To do this, we utilized subcutaneous papain immunization, which promotes Th2 differ-

entiation via the migration of CD301b⁺ dermal DCs into the dLN. Despite robust migration to the dLN after Th2 immunization, CD301b⁺ DCs did not display more CCR7 than after Th1

immunization, leading us to hypothesize that additional mechanisms might promote CD301b⁺ DC migration *in vivo*.

Chemokines are key regulators controlling the migration and positioning of immune cells. CCR7 and its ligands CCL19 and CCL21 control the migration of mature DCs from the periphery to dLNs (Worbs et al., 2017). Within the dLN, chemokines promote the positioning of DCs, T cells, and accessory cells within specific niches where they allow for optimal T cell activation and effector differentiation (Griffith et al., 2014; Lian and Luster, 2015). There is evidence that CCR8 and its ligand CCL1 could play a role in the migration of monocyte-derived DCs from the skin and of conventional DCs from the lung to the dLN (Jakubczik et al., 2006; Qu et al., 2004). However, we were unable to detect any induction of *Ccl1* transcript or reporter expression in the dLN in response to Th2 immunization. Instead, Th2 immunization was specifically associated with upregulation of the other CCR8 ligand, *Ccl8*, in the dLN, and this temporally correlated with DC migration. CCR8 has been associated with allergic inflammation and is present on Th2 cells, immature DCs, and monocytes. CCR8 has also been shown to play a potential role in the skewing of the adaptive immune response, although the results have been mixed. *Ccr8*^{-/-} mice show attenuated effector type 2 immune responses to *Schistosoma mansoni* soluble egg antigen, OVA, and cockroach antigen-induced allergic airway inflammation, as well as allergic skin inflammation in a model of atopic dermatitis (Chensue et al., 2001; Islam et al., 2011). However, these defects were thought to be the result of defective CCR8 expression by Th2 cells. Arguing in favor of a role for CCR8 expression on DCs, in a model of Th1- and Th17-mediated contact hypersensitivity, *Ccr8*^{-/-} mice were shown to have increased numbers of CD11c⁺ cells in the dLN in association with increased Th1 and Th17 differentiation (Yabe et al., 2015). At the same time, others have not been able to find a role for CCR8 in Th2-mediated inflammation (Chung et al., 2003; Goya et al., 2003). The cause of this discrepancy is unknown, but our data suggest that it could be due to the dependence of different models on CD301b⁺ dermal DCs. We found that disparate Th2-skewing stimuli, including papain, alum, and *N. brasiliensis* infection, all require CCR8 for CD301b⁺ DC migration and Th2 differentiation. Spontaneous migration of CD301b⁺ DCs appears to be unaffected by CCR8 deficiency, raising the question of whether spontaneous migration is functionally different. Additionally, it remains to be determined whether CCR8-mediated migration is a global mechanism used to control the migration of Th2-skewing DCs from non-cutaneous tissues.

We found that, instead of acting to directly promote chemotaxis, CCL8 augments CCL21-induced migration by two potential mechanisms. First, dual exposure of CD301b⁺ DCs to CCL8 and CCL21 leads to Ca²⁺ flux at chemokine concentrations that are individually incapable of inducing a Ca²⁺ flux, suggesting that CCR8 signaling might increase CD301b⁺ DC sensitivity to CCR7-ligand-induced migration. Alternatively, CCL8 directly induces Src-SHP2-mediated signaling in a CCR8-transfected cell line, and the augmentation of CCL21-mediated CD301b⁺ DC chemotaxis by CCL8 requires Src signaling. Prostaglandin-E₂-induced oligomerization of CCR7 has been shown to promote biased signaling through the Src-SHP2 pathway, which is required for optimal migration of human monocyte-derived DCs in response

to CCL21 signaling (Hauser et al., 2016). By promoting Src-SHP2 signaling in CCR8⁺CD301b⁺ DCs, CCL8 could facilitate the ability of CCR7 ligands to induce the migration of CD301b⁺ DCs across the lymphatic endothelium and into the dLN after Th2 immunization.

Why is another signal necessary for CD301b⁺ DCs to cross the border of the LN sinuses into the LN parenchyma? One possibility is based on the observation that chemokine receptors internalize after signaling, thus muting their responsiveness to ligands after signaling. This internalization has been postulated to underlie the role of the chemokine scavenger receptor ACKR4 in permitting conventional DCs to re-express CCR7, allowing them to cross the SCS (Ulvmar et al., 2014). Because of their relatively low CCR7 expression, CD301b⁺ DCs might not be able to recover from such ligand mediated internalization and require an additional chemokine signal. Alternatively, induction of the Src-SHP2 signaling pathway could be a crucial step for traversing the lymphatic endothelium at IFRs. In the absence of CCR8, CCR7 oligomerization can induce this signaling pathway, but oligomerization might require more surface CCR7 than those expressed by CD301b⁺ DCs. Although the precise molecular mechanism remains to be uncovered, we have shown a specific and necessary role for CCR8 in promoting CCR7-mediated migration in subset-specific DC migration.

Beyond showing a role for CCL8-CCR8 in CD301b⁺ DC migration, our data also reveal a role for CD169⁺SIGN-R1⁺ LN macrophages in controlling innate and adaptive immune interactions. SCS macrophages have been shown to efficiently capture lymph-borne virus particles and present them to B lymphocytes, thereby preventing lymphatic spread of virus and promoting antigen-specific immunity (Junt et al., 2007). But the role of other LN macrophage populations during the primary immune response is largely unknown. Through their positive effects on CD301b⁺ DC migration, CD169⁺SIGN-R1⁺ macrophages could play an important role in promoting a Th2-biased response. However, the precise mechanism by which they respond to protease allergen exposure remains unclear. Papain freely flows to the dLN and so might be able to directly activate LN macrophages. Alternatively, papain immunization has been shown to promote epithelial production of reactive oxygen species, thymic stromal lymphopoietin, and IL-33, all of which have been shown to play roles in the induction of the allergic immune response (Halim et al., 2014; Tang et al., 2010). Whether one of these epithelial derived factors activates CD169⁺SIGN-R1⁺ macrophages remains to be elucidated.

We propose a stepwise model for the allergen-induced migration of CD301b⁺ DCs. In the first step, DCs use CCR7-mediated signals to migrate into the draining lymphatics and enter the SCS space. In the second step, CCR8⁺ CD301b⁺ DCs respond to CCL8 produced by CD169⁺SIGN-R1⁺ macrophages, which allows them to transmigrate across the SCS floor and into the LN parenchyma. Such a process was previously identified in a melanoma metastasis model in which tumor cells entered the draining lymphatics and were able to cross the SCS of the dLN only if they expressed CCR8 on their surface (Das et al., 2013). This mirrors the accumulation of CD301b⁺ DCs seen in the LN sinuses of *Ccr8*^{-/-} mice. We propose that this stepwise migration process evolved to specifically promote CD301b⁺ DC migration and adaptive immune bias, but in melanoma

metastasis, this control point is hijacked by cancer cells. Thus, we believe that our model could provide insights that might be generalizable to cell migration and cancer metastasis beyond DC migration.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- [KEY RESOURCES TABLE](#)
- [CONTACT FOR REAGENT AND RESOURCE SHARING](#)
- [EXPERIMENTAL MODEL AND SUBJECT DETAILS](#)
 - Mice
- [METHOD DETAILS](#)
 - Generation of REC8 Mice
 - Immunizations and Cell Transfers
 - Tissue Preparation
 - Generation of Mixed Bone Marrow Chimeras
 - Flow Cytometry
 - Enzyme-Linked Immunosorbent Assay (ELISA)
 - *In Vitro* Restimulation Assays
 - *In Vitro* Chemotaxis Assays
 - RNA Purification and qPCR Analysis
 - Confocal Immunofluorescence
 - Kaede Photoconversion
 - Clodronate-Mediated Cell Depletion
 - Immunoblotting
 - Ca²⁺ Flux Assay
- [QUANTIFICATION AND STATISTICAL ANALYSIS](#)

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at <https://doi.org/10.1016/j.immuni.2018.07.012>.

ACKNOWLEDGMENTS

Work was supported by NIH grants R37AI040618 (to A.D.L.) and K08AI121421 (to C.L.S.). C.L.S. was supported by NIH grant T32HL116275 and the Asthma and Allergy Foundation of America. Flow cytometry was performed in the Department of Pathology Flow and Image Cytometry Research Core at Massachusetts General Hospital with support from the NIH Shared Instrumentation Program (1S10OD012027-01A1, 1S10OD016372-01, 1S10RR020936-01, and 1S10RR023440-01A1). We thank Drs. Sergio Lira, Israel Charo, and Osami Kanagawa for providing breeding pairs of *Ccr8*^{−/−}, *Ccl8*^{−/−}*Ccl12*^{−/−}, and *Kaede* transgenic mice, respectively. We thank Drs. Robert Anthony and Joseph F. Urban for providing *Nippostrongylus brasiliensis*.

AUTHOR CONTRIBUTIONS

Conceptualization, C.L.S. and A.D.L.; Methodology, C.L.S., R.B.C., and A.D.L.; Investigation, C.L.S., R.B.C., M.C.J., and A.D.L.; Writing, C.L.S., R.B.C., and A.D.L.; Funding Acquisition, C.L.S. and A.D.L.; Supervision, A.D.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: August 19, 2017

Revised: June 11, 2018

Accepted: July 23, 2018

Published: August 28, 2018

REFERENCES

- Asano, K., Takahashi, N., Ushiki, M., Monya, M., Aihara, F., Kuboki, E., Moriyama, S., Iida, M., Kitamura, H., Qiu, C.H., et al. (2015). Intestinal CD169(+) macrophages initiate mucosal inflammation by secreting CCL8 that recruits inflammatory monocytes. *Nat. Commun.* 6, 7802.
- Braun, A., Worbs, T., Moschovakis, G.L., Halle, S., Hoffmann, K., Bölter, J., Münk, A., and Förster, R. (2011). Afferent lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. *Nat. Immunol.* 12, 879–887.
- Bromley, S.K., Yan, S., Tomura, M., Kanagawa, O., and Luster, A.D. (2013). Recirculating memory T cells are a unique subset of CD4+ T cells with a distinct phenotype and migratory pattern. *J. Immunol.* 190, 970–976.
- Chensue, S.W., Lukacs, N.W., Yang, T.Y., Shang, X., Frait, K.A., Kunkel, S.L., Kung, T., Wiekowski, M.T., Hedrick, J.A., Cook, D.N., et al. (2001). Aberrant *in vivo* T helper type 2 cell response and impaired eosinophil recruitment in CC chemokine receptor 8 knockout mice. *J. Exp. Med.* 193, 573–584.
- Chung, C.D., Kuo, F., Kumer, J., Motani, A.S., Lawrence, C.E., Henderson, W.R., Jr., and Venkataraman, C. (2003). CCR8 is not essential for the development of inflammation in a mouse model of allergic airway disease. *J. Immunol.* 170, 581–587.
- Das, S., Sarrou, E., Podgrabska, S., Cassella, M., Mungamuri, S.K., Feirt, N., Gordon, R., Nagi, C.S., Wang, Y., Entenberg, D., et al. (2013). Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses. *J. Exp. Med.* 210, 1509–1528.
- Dieu, M.C., Vanbervliet, B., Vicari, A., Bridon, J.M., Oldham, E., Ait-Yahia, S., Brière, F., Zlotnik, A., Lebecque, S., and Caux, C. (1998). Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med.* 188, 373–386.
- Fletcher, A.L., Malhotra, D., Acton, S.E., Lukacs-Kornek, V., Bellemare-Pelletier, A., Curry, M., Armant, M., and Turley, S.J. (2011). Reproducible isolation of lymph node stromal cells reveals site-dependent differences in fibroblastic reticular cells. *Front. Immunol.* 2, 35.
- Gao, Y., Nish, S.A., Jiang, R., Hou, L., Licona-Limón, P., Weinstein, J.S., Zhao, H., and Medzhitov, R. (2013). Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. *Immunity* 39, 722–732.
- Girard, J.P., Moussion, C., and Förster, R. (2012). HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. *Nat. Rev. Immunol.* 12, 762–773.
- Goya, I., Villares, R., Zaballos, A., Gutiérrez, J., Kremer, L., Gonzalo, J.A., Varona, R., Carramolino, L., Serrano, A., Pallarés, P., et al. (2003). Absence of CCR8 does not impair the response to ovalbumin-induced allergic airway disease. *J. Immunol.* 170, 2138–2146.
- Gray, E.E., and Cyster, J.G. (2012). Lymph node macrophages. *J. Innate Immun.* 4, 424–436.
- Gray, E.E., Friend, S., Suzuki, K., Phan, T.G., and Cyster, J.G. (2012). Subcapsular sinus macrophage fragmentation and CD169+ bleb acquisition by closely associated IL-17-committed innate-like lymphocytes. *PLoS ONE* 7, e38258.
- Griffith, J.W., Sokol, C.L., and Luster, A.D. (2014). Chemokines and chemokine receptors: Positioning cells for host defense and immunity. *Annu. Rev. Immunol.* 32, 659–702.
- Halim, T.Y., Steer, C.A., Mathä, L., Gold, M.J., Martinez-Gonzalez, I., McNaghy, K.M., McKenzie, A.N., and Takei, F. (2014). Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 40, 425–435.
- Hauser, M.A., Schaeuble, K., Kindinger, I., Impellizzeri, D., Krueger, W.A., Hauck, C.R., Boyman, O., and Legler, D.F. (2016). Inflammation-induced CCR7 oligomers form scaffolds to integrate distinct signaling pathways for efficient cell migration. *Immunity* 44, 59–72.
- Islam, S.A., Chang, D.S., Colvin, R.A., Byrne, M.H., McCully, M.L., Moser, B., Lira, S.A., Charo, I.F., and Luster, A.D. (2011). Mouse CCL8, a CCR8 agonist, promotes atopic dermatitis by recruiting IL-5+ T(H)2 cells. *Nat. Immunol.* 12, 167–177.

- Islam, S.A., Ling, M.F., Leung, J., Shreffler, W.G., and Luster, A.D. (2013). Identification of human CCR8 as a CCL18 receptor. *J. Exp. Med.* **210**, 1889–1898.
- Iwasaki, A., and Medzhitov, R. (2015). Control of adaptive immunity by the innate immune system. *Nat. Immunol.* **16**, 343–353.
- Jakubczik, C., Tacke, F., Llodra, J., van Rooijen, N., and Randolph, G.J. (2006). Modulation of dendritic cell trafficking to and from the airways. *J. Immunol.* **176**, 3578–3584.
- Junt, T., Moseman, E.A., Iannacone, M., Massberg, S., Lang, P.A., Boes, M., Fink, K., Henrickson, S.E., Shayakhmetov, D.M., Di Paolo, N.C., et al. (2007). Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature* **450**, 110–114.
- King, I.L., Kroenke, M.A., and Segal, B.M. (2010). GM-CSF-dependent, CD103⁺ dermal dendritic cells play a critical role in Th effector cell differentiation after subcutaneous immunization. *J. Exp. Med.* **207**, 953–961.
- Kumamoto, Y., Linehan, M., Weinstein, J.S., Laidlaw, B.J., Craft, J.E., and Iwasaki, A. (2013). CD301b⁺ dermal dendritic cells drive T helper 2 cell-mediated immunity. *Immunity* **39**, 733–743.
- Kumamoto, Y., Hirai, T., Wong, P.W., Kaplan, D.H., and Iwasaki, A. (2016). CD301b⁺ dendritic cells suppress T follicular helper cells and antibody responses to protein antigens. *eLife* **5**, <https://doi.org/10.7554/eLife.17979>.
- León, B., Ballesteros-Tato, A., Browning, J.L., Dunn, R., Randall, T.D., and Lund, F.E. (2012). Regulation of T(H)2 development by CXCR5⁺ dendritic cells and lymphotoxin-expressing B cells. *Nat. Immunol.* **13**, 681–690.
- Lewis, K.L., Caton, M.L., Bogunovic, M., Greter, M., Grajkowska, L.T., Ng, D., Klinakis, A., Charo, I.F., Jung, S., Gommerman, J.L., et al. (2011). Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity* **35**, 780–791.
- Lian, J., and Luster, A.D. (2015). Chemokine-guided cell positioning in the lymph node orchestrates the generation of adaptive immune responses. *Curr. Opin. Cell Biol.* **36**, 1–6.
- Miller, M.D., and Krangel, M.S. (1992). The human cytokine I-309 is a monocyte chemoattractant. *Proc. Natl. Acad. Sci. USA* **89**, 2950–2954.
- Murakami, R., Denda-Nagai, K., Hashimoto, S., Nagai, S., Hattori, M., and Irimura, T. (2013). A unique dermal dendritic cell subset that skews the immune response toward Th2. *PLoS ONE* **8**, e73270.
- Paoletti, S., Petkovic, V., Sebastiani, S., Danelon, M.G., Uguccioni, M., and Gerber, B.O. (2005). A rich chemokine environment strongly enhances leukocyte migration and activities. *Blood* **105**, 3405–3412.
- Qi, H., Kastenmüller, W., and Germain, R.N. (2014). Spatiotemporal basis of innate and adaptive immunity in secondary lymphoid tissue. *Annu. Rev. Cell Dev. Biol.* **30**, 141–167.
- Qu, C., Edwards, E.W., Tacke, F., Angeli, V., Llodrá, J., Sanchez-Schmitz, G., Garin, A., Haque, N.S., Peters, W., van Rooijen, N., et al. (2004). Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. *J. Exp. Med.* **200**, 1231–1241.
- Sallusto, F., Schaerli, P., Loetscher, P., Scharniel, C., Lenig, D., Mackay, C.R., Qin, S., and Lanzavecchia, A. (1998). Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur. J. Immunol.* **28**, 2760–2769.
- Schlitzer, A., McGovern, N., Teo, P., Zelante, T., Atarashi, K., Low, D., Ho, A.W., See, P., Shin, A., Wasan, P.S., et al. (2013). IRF4 transcription factor-dependent CD11b⁺ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* **38**, 970–983.
- Sokol, C.L., Barton, G.M., Farr, A.G., and Medzhitov, R. (2008). A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat. Immunol.* **9**, 310–318.
- Tal, O., Lim, H.Y., Gurevich, I., Milo, I., Shipony, Z., Ng, L.G., Angeli, V., and Shakhar, G. (2011). DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *J. Exp. Med.* **208**, 2141–2153.
- Tang, H., Cao, W., Kasturi, S.P., Ravindran, R., Nakaya, H.I., Kundu, K., Murthy, N., Kepler, T.B., Malissen, B., and Pulendran, B. (2010). The T helper type 2 response to cysteine proteases requires dendritic cell-basophil cooperation via ROS-mediated signaling. *Nat. Immunol.* **11**, 608–617.
- Tomura, M., Yoshida, N., Tanaka, J., Karasawa, S., Miwa, Y., Miyawaki, A., and Kanagawa, O. (2008). Monitoring cellular movement in vivo with photoconvertible fluorescence protein “Kaede” transgenic mice. *Proc. Natl. Acad. Sci. USA* **105**, 10871–10876.
- Tsou, C.L., Peters, W., Si, Y., Slaymaker, S., Aslanian, A.M., Weisberg, S.P., Mack, M., and Charo, I.F. (2007). Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J. Clin. Invest.* **117**, 902–909.
- Tussiwand, R., Everts, B., Grajales-Reyes, G.E., Kretzer, N.M., Iwata, A., Bagaitkar, J., Wu, X., Wong, R., Anderson, D.A., Murphy, T.L., et al. (2015). Klf4 expression in conventional dendritic cells is required for T helper 2 cell responses. *Immunity* **42**, 916–928.
- Ulvmar, M.H., Werth, K., Braun, A., Kelay, P., Hub, E., Eller, K., Chan, L., Lucas, B., Novitzky-Basso, I., Nakamura, K., et al. (2014). The atypical chemokine receptor CXCR1 shapes functional CCL21 gradients in lymph nodes. *Nat. Immunol.* **15**, 623–630.
- Weber, M., Hauschild, R., Schwarz, J., Moussion, C., de Vries, I., Legler, D.F., Luther, S.A., Bollenbach, T., and Sixt, M. (2013). Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science* **339**, 328–332.
- Williams, J.W., Tjota, M.Y., Clay, B.S., Vander Lugt, B., Bandukwala, H.S., Hrusch, C.L., Decker, D.C., Blaine, K.M., Fixsen, B.R., Singh, H., et al. (2013). Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. *Nat. Commun.* **4**, 2990.
- Woodruff, M.C., Heesters, B.A., Herndon, C.N., Groom, J.R., Thomas, P.G., Luster, A.D., Turley, S.J., and Carroll, M.C. (2014). Trans-nodal migration of resident dendritic cells into medullary interfollicular regions initiates immunity to influenza vaccine. *J. Exp. Med.* **211**, 1611–1621.
- Worbs, T., Hammerschmidt, S.I., and Förster, R. (2017). Dendritic cell migration in health and disease. *Nat. Rev. Immunol.* **17**, 30–48.
- Yabe, R., Shimizu, K., Shimizu, S., Azechi, S., Choi, B.I., Sudo, K., Kubo, S., Nakae, S., Ishigame, H., Kakuta, S., and Iwakura, Y. (2015). CCR8 regulates contact hypersensitivity by restricting cutaneous dendritic cell migration to the draining lymph nodes. *Int. Immunol.* **27**, 169–181.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	Source	Identifier
Antibodies		
Alexa Fluor 594 Streptavidin	BioLegend	Cat #: 405240
Alexa Fluor 647 Streptavidin	BioLegend	Cat #: 405237
Anti-Mouse CD8 α , Clone: 53-6.7	BioLegend	Cat #: 100725; RRID: AB_493425
Anti-Mouse CD11b, Clone: M1/70	BioLegend	Cat #: 101251; RRID: AB_2562904
Anti-Mouse CD11c, Clone: HL3	BD Biosciences	Cat #: 553801; RRID: AB_395060
Anti-Mouse CD11c, Clone: N418	BioLegend	Cat #: 117346; RRID: AB_2563323
Anti-Mouse CD45.1, Clone: A20	BioLegend	Cat #: 110726; RRID: AB_893345
Anti-Mouse CD45.2, Clone: 104	BioLegend	Cat #: 109807; RRID: AB_313444
Anti-Mouse CD169, Clone: 3D6.112	BioLegend	Cat #: 142419; RRID: AB_2566436
Anti-Mouse CD197 (CCR7), Clone: 4B12	BioLegend	Cat #: 120116; RRID: AB_2291144
Anti-Mouse CD273 (PDL2), Clone: TY25	BD Biosciences	Cat #: 560086; RRID: AB_1645223
Anti-mouse CD301b (MGL2), Clone: URA-1	BioLegend	Cat #: 146806; RRID: AB_2563388
Anti-Mouse F4/80, Clone: BM8	BioLegend	Cat #: 123131; RRID: AB_10901171
Anti-Mouse Lyve-1, Clone: ALY7	eBioscience	Cat #: 53-0443-80; RRID: AB_1633417
Anti-Mouse Podoplanin, Clone: 8.1.1	BioLegend	Cat #: 127406; RRID: AB_2161930
Anti-rabbit IgG, HRP	Cell Signaling	Cat #: 7074S; RRID: AB_2099233
Anti-SHP2	Cell Signaling	Cat #: 3397T
Anti-phospho SHP2 (Tyr 542)	Cell Signaling	Cat #: 3751S; RRID: AB_330825
Anti-Siglec1, biotinylated	R&D Systems	Cat #: BAF5610; RRID: AB_10641427
Anti-SIGN-R1, biotinylated	R&D Systems	Cat #: BAF1836; RRID: AB_2074454
Anti-Src	Cell Signaling	Cat #: 2108S; RRID: AB_10695298
Anti-phospho Src (Tyr 416)	Cell Signaling	Cat #: 2101S; RRID: AB_331697
Biotinylated Anti-mouse MCP-2/CCL8 Antibody	R&D Systems	Cat #: BAF790; RRID: AB_2071685
Human CCL1-AlexaFluor647	Almac	Cat #: CAF-7
Fixable Viability Dye eFluor 780	Invitrogen	Cat #: 65-0865-14
Mouse CCL1/I-309/TCA-3 Biotinylated Antibody	R&D Systems	Cat #: BAF845; RRID: AB_2070619
TruStain fcX anti-mouse CD16/32, Clone: 93	BioLegend	Cat #: 101320; RRID: AB_1574975
Chemicals, Peptides, and Recombinant Proteins		
Macrophage depletion kit of Clodrosome and Encapsome	Encapsula Nano Sciences	Cat #: 8901
Saponin from quillaja bark	Sigma-Aldrich	Cat #: S7900-25G
Recombinant Mouse CCL1/TCA-3	R&D Systems	Cat #: 845-TC
Recombinant Mouse CCL21/6Ckine	R&D Systems	Cat #: 457-6C-025
Recombinant Murine MCP-3 (CCL7)	PeproTech	Cat #: 250-08
Recombinant Murine MCP-2 (CCL8)	PeproTech	Cat #: 250-14
Recombinant Mouse CCL2/JE/MCP-1	R&D Systems	Cat #: 479-JE-010
Recombinant Mouse CCL11/Eotaxin	R&D Systems	Cat #: 420-ME
TRIzol Reagent	Invitrogen	Ref #: 15596018
Collagenase P	Sigma-Aldrich	Cat #: 11213857001
Dispase II	Sigma-Aldrich	Cat #: D4693-1G
DNase I, grade II	Sigma-Aldrich	Cat #: 10104159001
Liberase DH	Sigma-Aldrich	Cat #: 5401054001
Streptavidin/Biotin Blocking Kit	Vector Lab	Cat #: SP-2002
Papain	Calbiochem	Cat #: 5125
Ovalbumin	Sigma-Aldrich	Cat #: A5503

(Continued on next page)

Continued

REAGENT or RESOURCE	Source	Identifier
Lipopolysaccharide	InvivoGen	Cat #: tlrl-eklps
Polyinosinic-polycytidylic acid	InvivoGen	Cat #: tlrl-picw
PBS	Corning	Ref #: 21-031-CV
PP2	Sigma-Aldrich	Cat #: 529573-1MG
Indo-1, AM	Invitrogen	Cat #: I1223
RBC Lysis buffer	Sigma-Aldrich	Cat #: R7757
0.5 M EDTA, pH 8.0	Gibco	Cat #: 15575-038
OCT Compound	Tissue-Tek	Cat #: 4583
RPMI 1640, 1×	Corning	Ref #: 15-040-CV
Fetal Calf Serum (FCS)	Atlanta Biologicals	Cat #: S11550
MultiScribe Reverse Transcriptase	Thermo Fisher Scientific	Ref #: 4311235
GeneAMP dNTP mix with dTTP	Thermo Fisher Scientific	Ref #: N8080260
MgCl ₂ Solution 25 mM	Thermo Fisher Scientific	Ref #: 4486224
10× PCR Buffer II	Thermo Fisher Scientific	Ref #: 4486220
Oligio d(T) ₁₆	Thermo Fisher Scientific	Ref #: 100023441
Random Hexamers 50 μM	Thermo Fisher Scientific	Ref #: 100026484
RNase Inhibitor	Thermo Fisher Scientific	Ref #: 100021540
FastStart Essential DNA Green Master	Roche	Cat #: 25595200
Critical Commercial Assays		
Mouse IL-4 ELISA MAX Standard	BioLegend	Cat #: 431103
Mouse IFN-γ ELISA MAX Standard	BioLegend	Cat #: 430803
Mouse IL-13 ELISA Ready-SET-Go!	eBioscience	Ref #: 88-7137-77
RNeasy Plus Micro Kit (50)	QIAGEN	Cat #: 74034
RNeasy Plus Mini Kit (50)	QIAGEN	Cat #: 74134
Naive CD4 ⁺ T Cell Isolation Kit, mouse	Miltenyi Biotec	Cat #: 130-104-453
CD11c Microbeads UltraPure mouse	Miltenyi Biotec	Cat #: 130-108-338
Experimental Models: Organisms/Strains		
Mouse: C57Bl/6	Charles River Laboratory	Strain #: 027
Mouse: C57Bl/6	The Jackson Laboratory	Strain #: 000664
Mouse: B6(FVB)-Mgl2 ^{tm1.1(HBEGF/EGFP)Aiwsk/J} (CD301b-DTR)	The Jackson Laboratory	Strain #: 023822
Mouse: <i>Ccr8</i> ^{-/-}	Chensue et al., 2001	N/A
Mouse: <i>Ccl8</i> ^{-/-} <i>Ccl12</i> ^{-/-}	Tsou et al., 2007	N/A
Mouse: Kaede ⁺	Tomura et al., 2008	N/A
Mouse: <i>Ccr7</i> ^{-/-}	The Jackson Laboratory	Strain #: 006621
Mouse: B6.Cg-Tg (TcraTcrb)425Cbn/J (OT2m)	The Jackson Laboratory	Strain #: 004194
Mouse: B6.SJL-Ptprc Pepc/BoyJ (CD45.1)	The Jackson Laboratory	Strain #: 002014
Mouse: REC8	Cyagen Biosciences, this manuscript	N/A
Oligonucleotides		
mCcl1 FWD (5'-GGGTGCCGTGTGGATACAG-3')	This manuscript	N/A
mCcl1 REV (5'-AGGTGATTTTGAACCCACGTTT-3')	This manuscript	N/A
mCcl2 FWD (5'-TGGCTCAGCCAGATGCAGT-3')	This manuscript	N/A
mCcl2 REV (5'-TTGGGATCATCTTGCTGGTG-3')	This manuscript	N/A
mCcl7 FWD (5'- TGGGAAGCTGTTATCTTCAAGACA-3')	This manuscript	N/A
mCcl7 REV (5'- CTCGACCCACTTCTGATGGG-3')	This manuscript	N/A
mCcl8 FWD (5'- GCGAGTGCTTCTTGCCTG-3')	This manuscript	N/A
mCcl8 REV (5'-TCTGGCCCAGTCAGCTTCTC-3')	This manuscript	N/A
mCcl12 FWD (5'-GCTGGACCAGATGCGGTG-3')	This manuscript	N/A
mCcl12 REV (5'- CCGGACGTGAATCTTCTGCT-')	This manuscript	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	Source	Identifier
REC8 genotyping primer P1 FWD (5'- GAACACAGCAG CAGAAAGAACAC-3')	This manuscript	N/A
REC8 genotyping primer P1 REV (5'- CATGGCGTGTAATATGAGAGACGC-3')	This manuscript	N/A
REC8 genotyping primer P2 FWD (5'-GTAATAAATGGA TGCCCTGCGTAAG -3')	This manuscript	N/A
REC8 genotyping primer P2 REV (5'-ACCTGTCAAGAA TGGTGCCAG-3')	This manuscript	N/A
REC8 genotyping primer P3 FWD (5'-ATCAGTCAGGT ACATAACCTCGAG-3')	This manuscript	N/A
REC8 genotyping primer P3 REV (5'-AGTCCATAACC TTCCTCCACG-3')	This manuscript	N/A
REC8 genotyping primer P4 FWD (5'-GATTGGTATCT GCTTCTGCACTTG-3')	This manuscript	N/A
REC8 genotyping primer P4 REV (5'-GTAGATGAAC TCGCCGTCCTG-3')	This manuscript	N/A
REC8 genotyping primer P5 FWD (5'-CCTTCTCACC ACAGCCACTTC-3')	This manuscript	N/A
REC8 genotyping primer P5 REV (5'-TGAGTTTGGAC AAACCACAAC-3')	This manuscript	N/A
REC8 genotyping primer P6 FWD (5'-TGCTTCATGT GGTGCGGGTA-3')	This manuscript	N/A
REC8 genotyping primer P6 REV (5'-GCTTTCATT CCCATTAGGCACA-3')	This manuscript	N/A
Software and Algorithms		
Prism v.7.0c	GraphPad	https://www.graphpad.com/demos
Zen Blue	Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software/zen.html
Imaris	Bitplane	http://www.bitplane.com/download
Softmax Pro	Molecular Devices	https://www.moleculardevices.com/systems/microplate-readers/softmax-pro-7-software
BD FACS Diva 8	BD Biosciences	http://www.bdbiosciences.com/us/instruments/clinical/software/flow-cytometry-acquisition/bd-facsdiva-software/m/333333/overview
FlowJo (v.10)	Tree Star	https://www.flowjo.com/solutions/flowjo/downloads

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Andrew Luster (aluster@mgm.harvard.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Mice**

Animals were bred and maintained under specific pathogen free (SPF) conditions at the Massachusetts General Hospital Center for Comparative Medicine. *Ccr8*^{-/-} mice were bred under SPF plus *Helicobacter* and *Pasteurella Pneumotropica* free conditions and maintained in SPF conditions. All procedures were approved and carried out according to the standards and guidelines set forth by the Institutional Animal Care and Use Committee of Massachusetts General Hospital. All mouse strains were fully backcrossed (>9 generations) to C57BL/6. Mice were used in experiments at 5–14 weeks of age and age matched littermates or purchased C57BL/6 mice were used as WT controls. Male mice were used for all DC migration assays unless otherwise noted. Male and female mice were used interchangeably in other assays, but sex was matched for all conditions within experiments.

METHOD DETAILS

Generation of REC8 Mice

REC8 mice were generated on the C57BL/6 background by Cyagen Biosciences using a PiggyBac on BAC technique by BAC modification in *E. coli*. RP23-409C7 was selected in pBACe3.6 because it contained both *Ccl1* and *Ccl8* target genes. First, a fragment containing 5' and 3' inverted terminal repeat sequences (ITRs) was inserted into pBACe3.6 to allow for eventual integration into TTA short repeat element sites. eGFP-SV40 polyA was placed upstream of an FRT flanked kanamycin resistance gene and this construct was introduced immediately following the start codon of *Ccl8* via recombineering of the modified BAC clone RP23-409C7. After selection of positive colonies, the kanamycin resistance cassette was then removed via Flp-mediated recombination. mCherry-SV40 polyA was placed upstream of rox flanked ampicillin resistance gene and this construct was introduced immediately following the start codon of the *Ccl1* gene via recombineering of the *Ccl8*-eGFP modified BAC clone. Ampicillin resistant clones were selected and then the ampicillin resistance cassette was deleted via Dre-mediated recombination. Recombinant clones were confirmed and monitored by PCR (primers P1–P6) and end-sequencing. The verified circular PiggyBac on BAC clone and helper plasmid was injected into fertilized C57BL/6 eggs and implanted into surrogate mothers. Pups were genotyped by PCR (primers P1–P8) to identify transgene positive pups. Positive pups were screened negative for integration of the helper plasmid (forward primer 5'-CTGGACGAGCAGAACGTGATCG-3' and reverse primer 5'-CGAAGAAGCGTAGATCTCGTCCTC-3'). This yielded 11 founders. Of those, one did not produce offspring and two did not have transgene positive offspring. Of the 8 remaining founders, two had no detectable reporter expression, four had only detectable mCherry expression, and two had detectable expression of both reporters. One line had brighter reporter expression and so was used primarily for data generation, although experiments were confirmed with the alternative line.

Immunizations and Cell Transfers

Mice were immunized subcutaneously in the rear footpads and/or base of tail with 50 μ g of both papain (Calbiochem) and OVA or 50 μ g OVA in 20 μ g Imject Alum (ThermoFisher) for Th2-skewing immunizations, 10 μ g of both LPS (InvivoGen) and poly I:C (InvivoGen) and 50 μ g of OVA for Th1-skewing immunizations, and 50 μ g of OVA for Th0 immunizations. All immunizations except OVA-alum were diluted in sterile PBS and subcutaneously injected at a final volume of 25 μ L. *N. brasiliensis* infection was performed by injecting mice subcutaneously in the base of tail with 500 L3 stage larvae with 50 μ g OVA. Naive CD4⁺ T cells were negatively selected for using Naive CD4⁺ T cell isolation kit (Miltenyi) followed by magnetic cell separation (Miltenyi). Purified naive CD4⁺ T cells were transferred (2.5×10^5 to 5.0×10^5 cells in 100 μ L sterile PBS) into recipient mice via retro-orbital injection. SIGN-R1⁺ cells underwent magnetic cell separation using SIGN-R1-biotin and anti-biotin magnetic beads (Miltenyi).

Tissue Preparation

For CD4⁺ T cell harvest for *in vitro* restimulation assays, whole LNs were placed in 70 μ m sterile filters and mechanically disrupted. Otherwise, for standard LN digestion, whole LNs were harvested, minced, and placed into RPMI media containing Liberase DH (0.26 U/mL) and DNase I (0.1 mg/mL) and incubated at 37°C for 20 min. Following incubation, LNs were mechanically disrupted by passing through a pipette tip before being incubated for another 20 min at 37°C. For extended LN digest to ensure release of LN sinus stroma and entrapped cells, whole LNs were harvested into RPMI media and subjected to digestion in DNase I (100 μ g/mL), Collagenase P (200 μ g/mL), Dispase II (800 μ g/mL), and 1% fetal calf serum (FCS) in RPMI. LNs were placed in the pre-warmed enzyme mixture and incubated at 37°C. At 8 min intervals, any supernatant was removed and added to stop buffer (RPMI/2mM EDTA/1% FCS), and the removed supernatant was replaced with fresh enzyme media. This was repeated at 8 min intervals until no tissue fragments remained. For both protocols, upon completion of digestion, cell suspensions were filtered with a 70 μ m sterile filter.

Generation of Mixed Bone Marrow Chimeras

To generate bone marrow chimeric mice, CD45.1 recipient mice were irradiated with 1,000 rads prior to injection with isolated bone marrow. Bone marrow was harvested from 5- to 8-week-old mice by mechanical disruption using a mortar and pestle followed by incubation with RBC lysis buffer. Lysis was stopped by adding excess PBS, centrifugation, and resuspension of pellet in sterile PBS. Within 6 hr of irradiation, recipient mice received a total of 1×10^7 cells in a 1:1 mix of CD301b-DTR (CD45.2) and CD45.1 $\times$ CD45.2 cells, CD301b-DTR (CD45.2) and Kaede⁺Ccr8^{-/-} cells or all Kaede⁺Ccr8^{-/-} cells. Bone marrow cells were injected via retro-orbital injection in 100 μ L. After 8 to 10 weeks, mice received purified CD4⁺ T cells (2.5×10^5) via retro-orbital injection as well as 0.5 μ g of Diphtheria toxin intraperitoneally. 16–24 hr following T cell transfer, mice underwent footpad immunization and received a second dose of Diphtheria toxin at 0.5 μ g. 48 hr following immunizations, mice were given a final injection of Diphtheria toxin at 0.5 μ g. 5 days after immunization, popliteal LNs were collected. Reconstitution and depletion of CD301b⁺ DCs were assessed in a subset of mice one day after immunization and in all other mice at time of final harvest.

Flow Cytometry

Enzymatically digested cell suspensions were incubated for 15 min at 4°C in PBS with 0.5% FCS with the following antibodies: anti-CD16/32, anti-CD8 α , anti-CD11b, anti-CD11c, anti-CD45.1, anti-CD45.2, anti-CD103, anti-CD169, anti-CD301b, anti-Thy1.1 (all from Biolegend), anti-PDL2 (BD Biosciences), or anti-SIGN-R1 (R&D Systems). Viability was determined using Fixable Viability

Dye eFluor 780 (Invitrogen). Experiments examining CCR7 were done by first incubating cells with anti-CCR7 (BD Biosciences) for 30 min at 37°C prior to additional surface staining as above. Intracellular cytokine staining was done by first incubating cells in PMA/Ionomycin (Sigma) in the presence of GolgiPlug for 4 hr prior to surface staining followed by intracellular staining using BD Cytofix/Cytoperm kit (BD Biosciences). hCCL1-AF647 (Almac) binding was accomplished by first incubating 2×10^6 cells in 50 μ L of 0.25 μ g/mL hCCL1-AF647 in PBS/0.5% FCS/20mM HEPES for 1 hr at 37°C. Cells then underwent additional antibody staining as above. All samples were run on BD Fortessa X-20 or BD LSRII and analyzed using FACS Diva 8 (BD Biosciences) and FlowJo (Version 10) (TreeStar).

Enzyme-Linked Immunosorbent Assay (ELISA)

Mouse IL-4 and IFN- γ were detected using a mouse IL-4 and IFN- γ ELISA MAX standard set (both from Biolegend). Mouse IL-13 was detected using a Mouse IL-13 ELISA Ready-SET-Go! kit (eBioscience). All procedures were carried out according to the manufacturer's protocols. Briefly, samples were incubated overnight at 4°C on antibody-coated plates. Detection antibodies were allowed to incubate for 1 hr at room temperature, followed by addition of streptavidin-horseradish peroxidase for 30 min. Finally, TMB substrate solution was added and the reaction was stopped with 2M sulfuric acid. Samples were assayed on Softmax Pro Software in triplicates and concentrations were determined from a standard curve.

In Vitro Restimulation Assays

To achieve restimulation of antigen-specific T cells, single cell suspensions of 5×10^5 isolated leukocytes of whole LNs were added to 96-well round bottom microwell plates with 100 μ g/mL OVA in 200 μ L of media. Plates were incubated at 37°C in 5% CO₂ between four and five days at which time cytokine production was analyzed.

In Vitro Chemotaxis Assays

Chemotaxis of sorted CD11c⁺CD11b⁺CD301b⁺ (CD11c⁺) DCs and CD11c⁺CD11b⁺CD301b⁺ DCs was performed using 5- μ m pore size Transwell membranes (Neuro Probe). Briefly, 29 μ L of diluted chemokines in media, 0.5% (wt/vol) BSA in RPMI, or media alone were added to the lower chamber. To the upper chamber, 25,000 cells were added in triplicate in 50 μ L of media with or without chemokine. Plates were incubated for 2 hr at 37°C in 5% CO₂ after which point the upper membrane was washed with PBS. A brief spin was performed to collect any migrated adherent cells, the upper chamber was removed, and migrated cells were counted. When applicable, cells were pretreated for 30 min with 10 μ M of the Src inhibitor PP2 and chemotaxis was performed in the presence of this or DMSO as a control.

RNA Purification and qPCR Analysis

Total RNA was collected from whole LN or skin using a Trizol (Invitrogen) reagent protocol in accordance with the manufacturer. Total RNA from sorted cells was collected using the QIAGEN RNeasy Micro or Mini Kit, based on the manufacturer's protocol. For both methods, RNA was diluted in RNase-free water and stored at -80°C until prepared for cDNA using reverse transcription. 50 μ L of cDNA per reaction was generated using random hexamers, Oligio(dT), magnesium chloride, dNTPs (10mM), Reverse Transcriptase, and RNase Inhibitor (all from Fisher). A Roche LightCycler 96 Real-Time PCR System was utilized to quantitate gene expression using SYBR Green Master Mix (Roche). The reaction cycles were carried out in the following manner: 95°C for 600 s, then 95°C, 60°C, and 72°C for 10 s each for 45 total cycles, followed by 95°C for 10 s, 65°C for 60 s, and finally 97°C for 1 s. Fluorescence was measured during each amplification step and Roche LightCycler 96 Software 1.1 was utilized to determine quantification cycle (Cq) values for each sample. In Excel, copy values were determined based off of the Cq values which were then divided by values from the housekeeping genes β 2-microglobulin or Gapdh to generate ratios.

Confocal Immunofluorescence

Lymph nodes were fixed at room temperature for 2-3 hr in periodate-lysine-paraformaldehyde fixative. Organs then were placed sequentially in 10%, 20% and 30% sucrose (wt/vol) in PBS for cryoprotection, then embedded in optimum cutting temperature (OCT) compound (Tissue-Tek) and frozen in 2-methylbutane over liquid nitrogen. A Cryostat (Microm HM 505 E, GMI) was used to generate 6-9 μ m sections, which were kept at -20°C. On the day of staining, sections were thawed, rehydrated in PBS for 20 min at room temperature, blocked in PBS containing 0.1% (vol/vol) Tween-20 and 5% (vol/vol) Normal Goat Serum (Jackson ImmunoResearch), and were stained with biotinylated anti-Siglec1 or anti-SIGN-R1 (both from R&D Systems) or fluorochrome-conjugated anti-mouse CD11c (BD Biosciences), anti-mouse LYVE-1 (eBioscience), anti-mouse CD301b, anti-mouse podoplanin, anti-mouse CD169, or anti-mouse F4/80 (all from BioLegend). Streptavidin-Alexa Fluor 647 or Alexa Fluor 594 (both from BioLegend) were used as secondary antibodies. Secondary antibodies were allowed to incubate for 1 h at room temperature. Sections were also stained with biotinylated anti-mouse MCP-2/CCL8 or CCL1 (both from R&D Systems) which were blocked using a streptavidin/biotin blocking kit (Vector Laboratories) to block endogenous biotin. All dilutions were made in PBS containing 0.1% (wt/vol) Saponin and 5% rabbit serum (vol/vol), with the exception of primary antibody which was diluted in PBS containing 0.1% (w/v) Saponin only. Primary antibody was allowed to incubate overnight at 4°C. A biotinylated anti-goat IgG secondary antibody (Vector Laboratories) incubated at room temperature for 30 min followed by addition of fluorescein streptavidin (1:200) for 15 min at 4°C. All slides were mounted in Prolong Gold to prevent fading of staining. A Zeiss LSM confocal microscope was used to acquire images that were generated by collecting consecutive scans with 1 laser line active per scan to prevent excitation crosstalk.

Kaede Photoconversion

All Kaede transgenic mice were anesthetized to shave a $\sim 2 \times 2$ cm patch of skin above the base of tail. A chemical depilatory agent (Veet) was applied to the shaved patch for 1 min, followed by multiple washings with PBS. To perform photoconversion, the exposed skin was subjected to violet light (420 nm) using a Bluewave LED visible light curing unit (Dymax) that expressed a 420 nm bandpass filter (Andover Corp). Skin was exposed to this wavelength with the light source at a maximum power, approximately 7.5 cm away from the skin for 5 min. Following photoconversion, mice were immunized subcutaneously in the shaved region as described above. 24 hr following immunization, dLNs were isolated and analyzed for skin DC migration using flow cytometry.

Clodronate-Mediated Cell Depletion

Mice were injected subcutaneously in the rear footpads with 50 μ L of either Clodrosome Liposomal Clodronate or Encapsome Control Liposome (Encapsula Nano Sciences). Footpad immunizations were performed four days following liposome administration, as described above, and draining lymph nodes were isolated 24 hr following immunization.

Immunoblotting

4DE4 cells stably transfected with human CCR8 as previously described were serum starved for 4 hr and then stimulated with CCL8 (Islam et al., 2011). Cells were lysed and lysates were immunoblotted for presence of phosphorylated Src (Tyr416) and SHP2 (Tyr542) (Cell Signaling).

Ca²⁺ Flux Assay

To access intracellular Ca²⁺ flux in primary dendritic cells, MACS isolated CD11c positive cells from the dLN of Th2-immunized mice were washed with PBS (with Ca/Mg) supplemented with 1% FCS. Indo-1, AM (Invitrogen) was prepared immediately before use by dissolving each vial according to the manufacturer's recommendations. 5 μ L of freshly diluted Indo-1 was added to 500 μ L of PBS 1% FCS (with Ca/Mg) per every two million cells. Cells were allowed to incubate for 45 min at 37°C in the dark and then washed with cold PBS (with Ca/Mg) and re-suspended in 500 μ L cold PBS (with Ca/Mg). Ca²⁺ flux was then assessed by flow cytometry measuring Indo-1 ratio over a time course of 90 s following chemokine stimulation.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses, as specified in figure legends, were performed with Prism 7 (GraphPad software). Data were analyzed with the non-paired Student's t test. A p value < 0.05 was considered significant (*p < 0.05, **p < 0.01, ***p < 0.001).

Immunity, Volume 49

Supplemental Information

**The Chemokine Receptor CCR8 Promotes the Migration
of Dendritic Cells into the Lymph Node Parenchyma
to Initiate the Allergic Immune Response**

Caroline L. Sokol, Ryan B. Camire, Michael C. Jones, and Andrew D. Luster

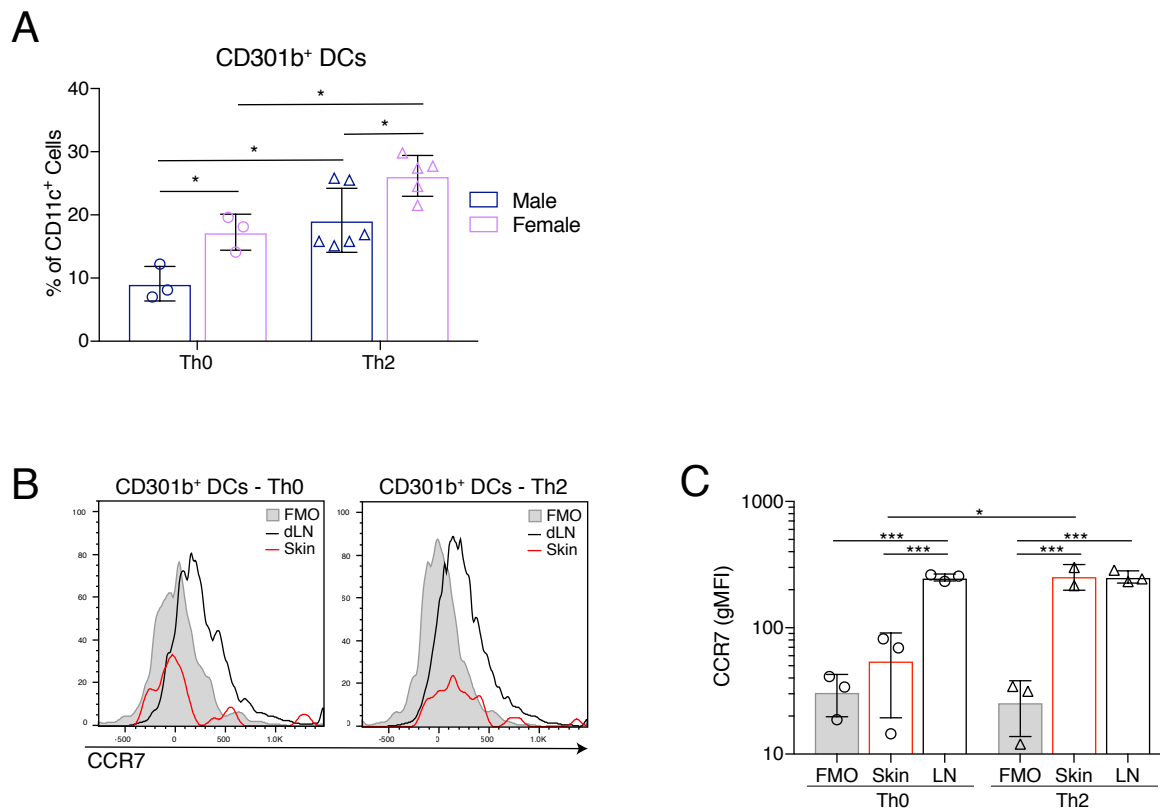


Figure S1 | Related to Figure 1

(A) CD301b⁺ DCs as a percentage of total viable CD11c⁺ cells from the popliteal LN 24 hrs after Th0 or Th2-skewing immunization in male or female mice. (B & C) CCR7 expression compared to fluorescence minus one (FMO) on viable CD45⁺CD11c⁺CD301b⁺ cells from the immunization site (skin) or popliteal LN (dLN) 24 hrs after the indicated immunization. Bar graphs represent mean value of indicated data points, error bars represent SEM. Data shown is representative of 3 independent experiments with 2-6 individual mice per condition per experiment. * p<0.05, ** p<0.01, *** p<0.001, Student's T test.

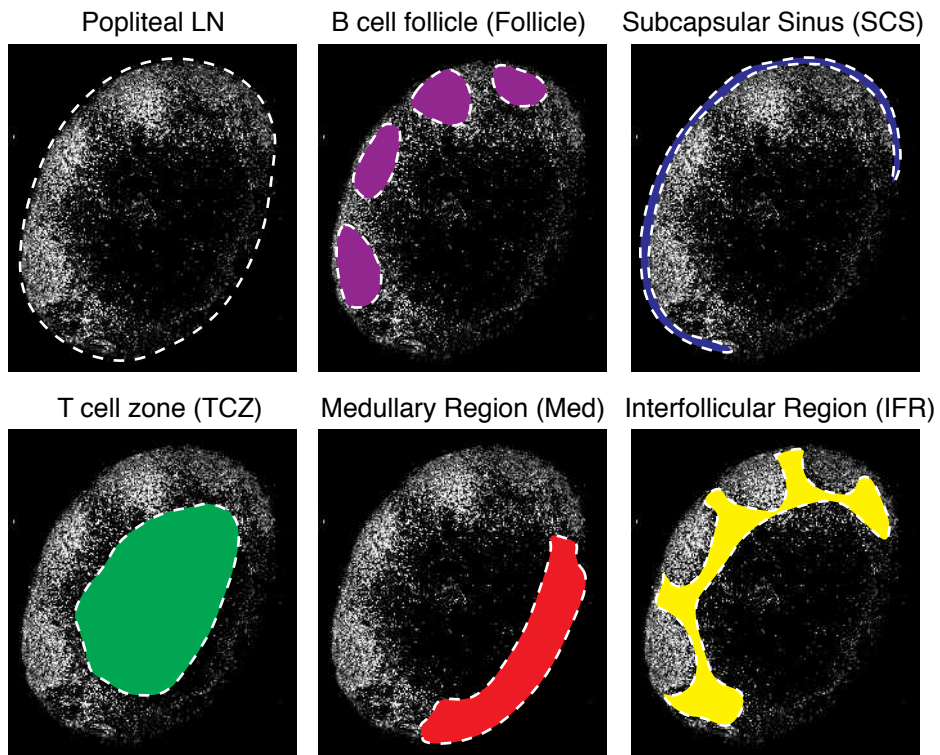


Figure S2 | Related to Figure 2.

Representative section of draining popliteal LN is shown (top left). Color regions indicated LN areas analyzed as determined by B220 staining (white) and LN architecture. Scale bars = 200 μ m.

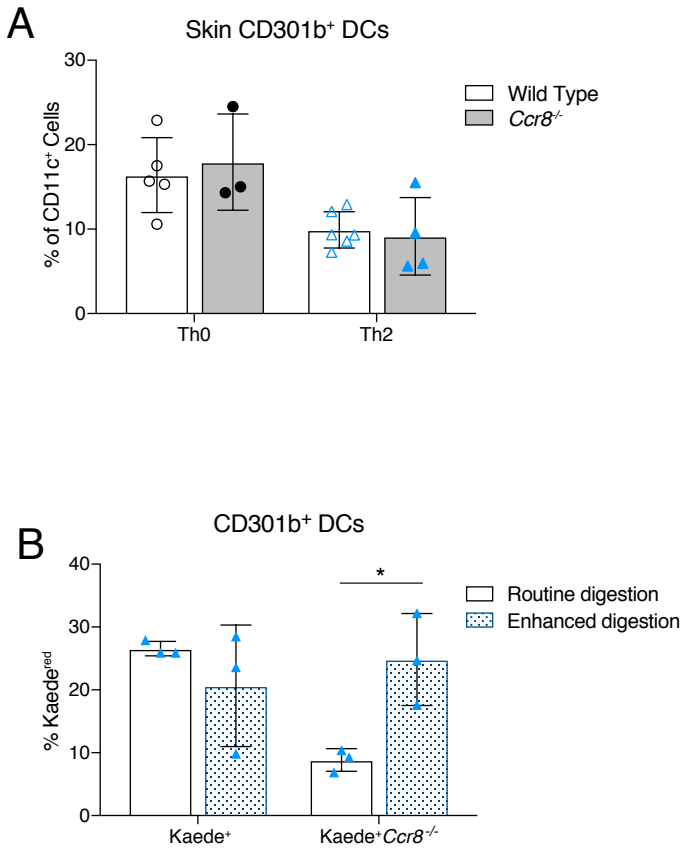


Figure S3 | Related to Figure 3.

(A) CD301b⁺ DCs as a percentage of total viable CD45⁺CD11c⁺ cells from the cutaneous immunization site (skin, footpad) 24 hrs after the indicated immunization of Wild Type or *Ccr8*^{-/-} mice. (B) Percentage of Kaede^{Red} cells out of total CD11c⁺CD301b⁺ cells from the dLN 24 hours after photoconversion and Th2-skewing immunization of Kaede⁺ or Kaede⁺*Ccr8*^{-/-} mice. dLNs were harvested and divided for either routine enzymatic digestion (clear black bar) or enhanced enzymatic digestion (dotted blue bar). Bar graphs represent mean value of indicated data points, error bars represent SEM. Data shown is representative of 2 independent experiments with 2-3 individual mice per condition per experiment. * p<0.05, ** p<0.01, *** p<0.001, Student's T test.

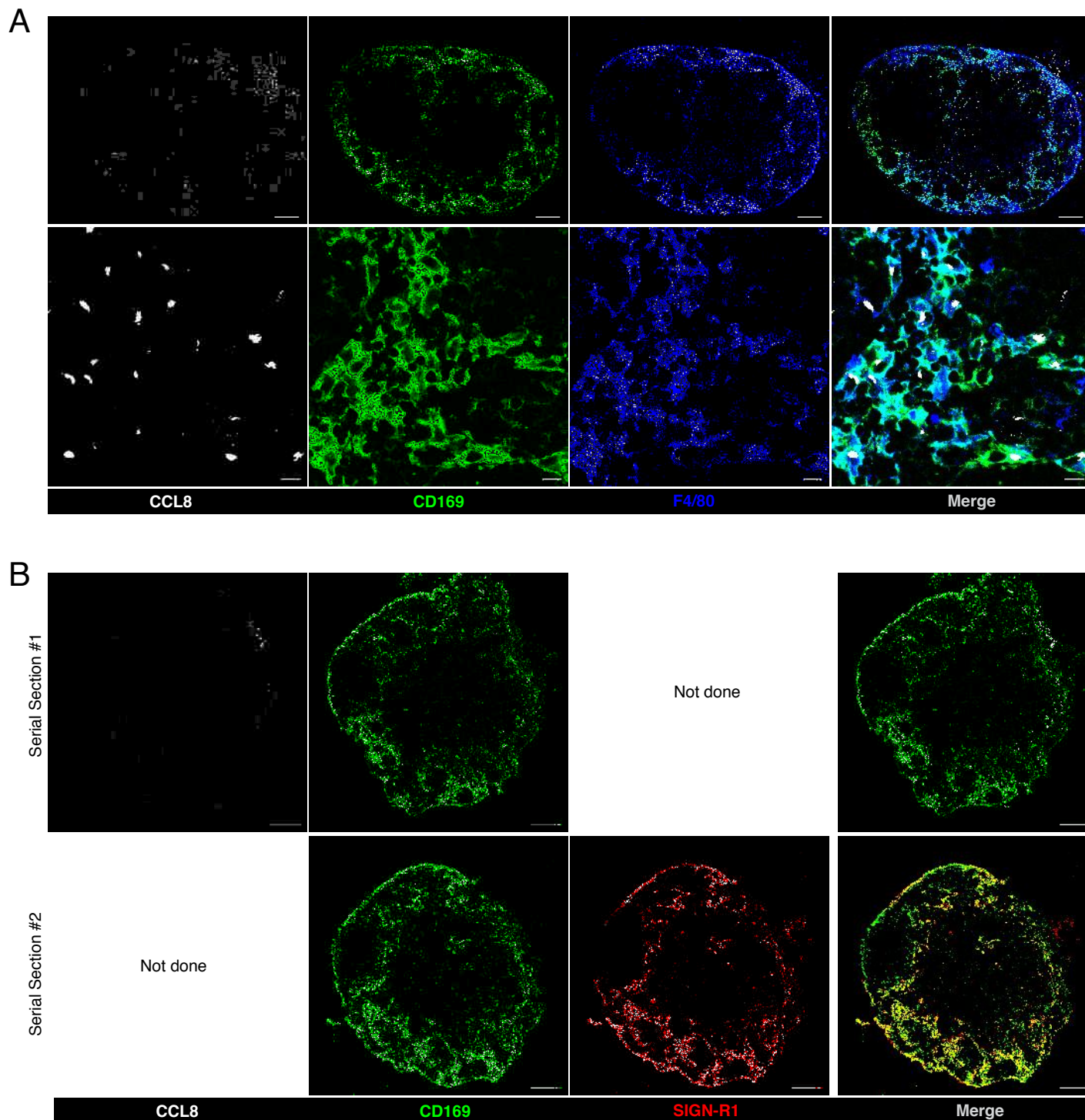


Figure S4 | Related to Figure 4.

(A) CCL8⁺ cells co-express CD169 and F4/80 on their surfaces. Top panels show CCL8 (white), CD169 (green) and F4/80 (blue) staining of entire LN, scale bar = 200 μ m. Bottom panels show zoom in of LN area of interest, scale bar = 10 μ m. (B) CD169⁺CCL8⁺ cells are colocalized with CD169⁺SIGNR1⁺ cells in serial sections. Top panels show CD169 (green) and CCL8 (white) in dLN 24 hrs after Th2-skewing immunization. Bottom panels are of serial section showing CD169 (green) and SIGN-R1 (red) staining in the same dLN. SIGN-R1 and CCL8 could not be done in the same sections due to antibody cross-reactivity and so are labeled “Not done” where appropriate. Scale bars = 200 μ m. Images shown are representative of two independent experiments with 3 mice in each experiment and images are one of six sections per mouse.

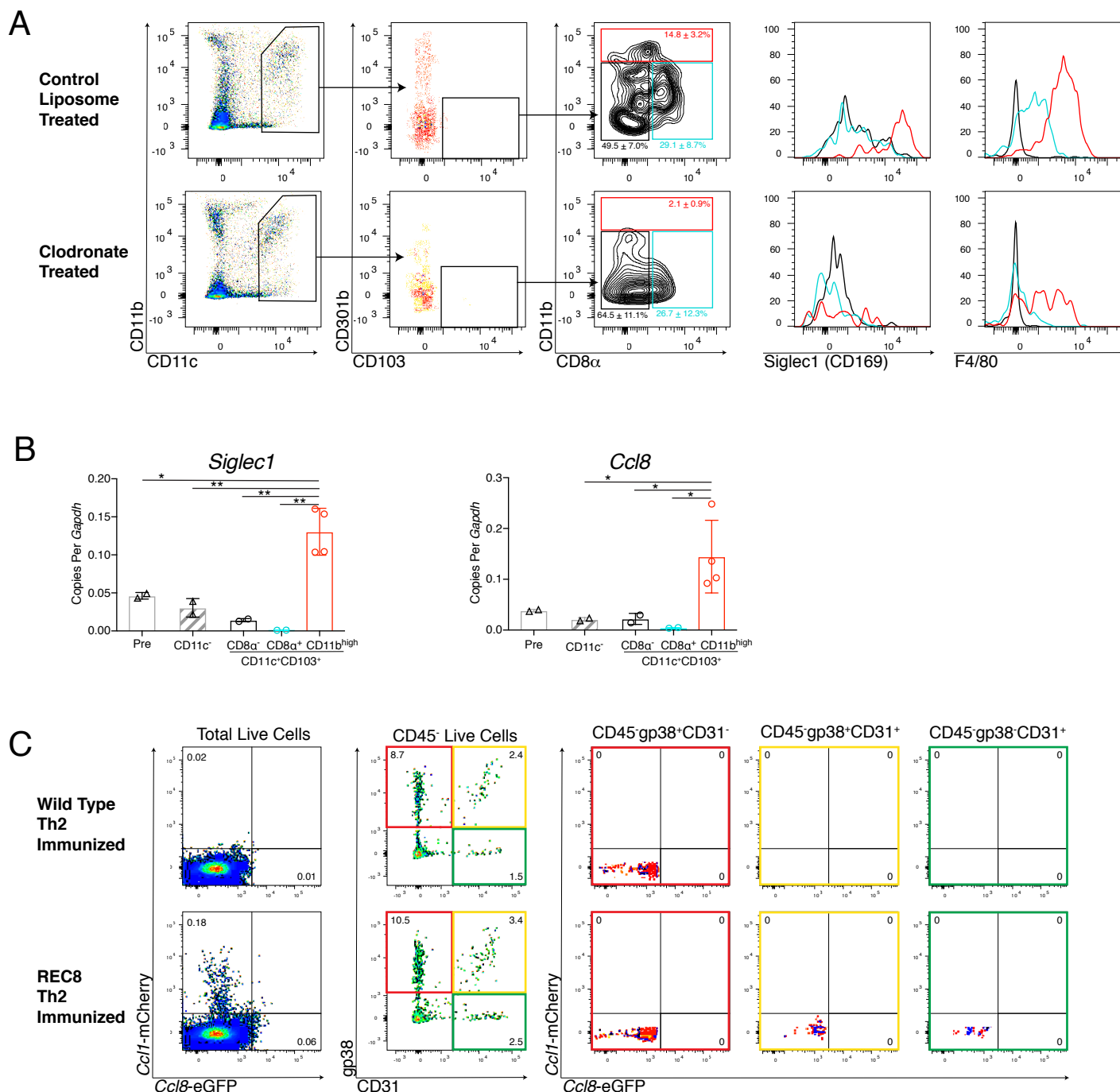


Figure S5 | Related to Figures 4 and 5.

(A) Flow cytometry showing gating scheme to identify CD11c⁺CD103⁺CD11b^{hi}CD169⁺ cells out of total viable singlets from the dLN of control liposome treated or macrophage-depleted, clodronate-treated mice. Colored histograms correspond to colored gates. Data are representative graphs, with percentages and standard deviations derived from 3-5 mice per group, one of four independent experiments shown. (B) QPCR analysis of *Siglec1* and *Ccl8* mRNA from total LN cells (Pre), CD11c⁻ cells, as well as CD11c⁺CD103⁺ cells from the three populations indicated in (A). (C) Flow cytometry of dLN from WT and REC8 mice revealed *Ccl1*-mCherry and *Ccl8*-eGFP expression in total live cells but not in CD45-gp38⁺CD31⁻ (red gate and plots, fibroblastic reticular cells), CD45-gp38⁺CD31⁺ (yellow gate and plots, lymphatic endothelial cells), and CD45-gp38⁻CD31⁺ (green gate and plots, blood endothelial cells). Values are percentages in gate taken from 2-4 independent replicates, one of three independent experiments shown. QPCR data presented as copies of indicated transcript over *Gapdh*. Bar graphs represent mean value of indicated data points, error bars represent SEM. * p<0.05, ** p<0.01, Student's T test.

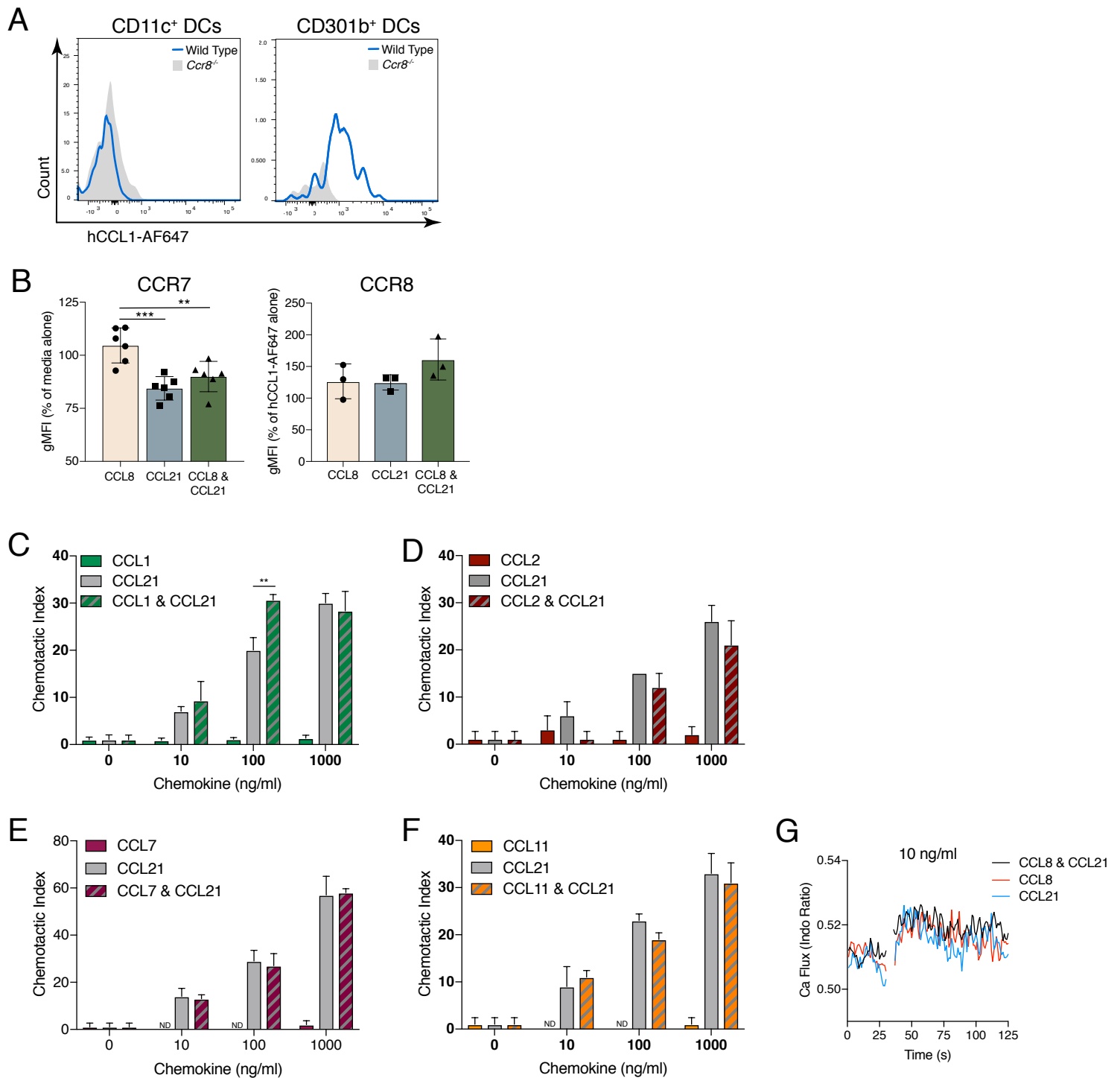


Figure S6 | Related to Figure 6.

(A) CCR8 expression as a measure of hCCL1-AF647 binding after overnight exposure of wild type (blue histogram) or *Ccr8*^{-/-} (shaded gray histogram) CD11c⁺ DCs or CD301b⁺ DCs to hCCL1-AF647. (B) CCR7 expression and hCCL1-AF647 binding of ex vivo CD301b⁺ DCs after 2 hours stimulation with the specified chemokine over that of the media control (gray shaded histogram). gMFI data shown is normalized to that of the media control. Transwell chemotaxis of CD301b⁺ DCs to (C) CCL1 (green bar), (D) CCL2 (red bar), (E) CCL7 (magenta bar), (F) CCL11 (orange bar), (C-F) CCL21 (gray bar), or combined respective chemokines (hatched bars). (G) Ca²⁺ flux expressed as the Indo-1 AM ratio from CD301b⁺ DCs stimulated ex vivo as indicated. In all cases cells were harvested from dLNs of mice 24 hrs after Th2-immunization. Representative graphs are shown from one of three (A, B, C) or one of two (D-G) independent experiments. Cells in (C-D) were pooled from 50 mice and cells in (G) were pooled from 12 mice. Bar graphs represent mean value of 2-3 data points/replicates per bar, error bars represent SEM. ** p < 0.01, Student's T test.

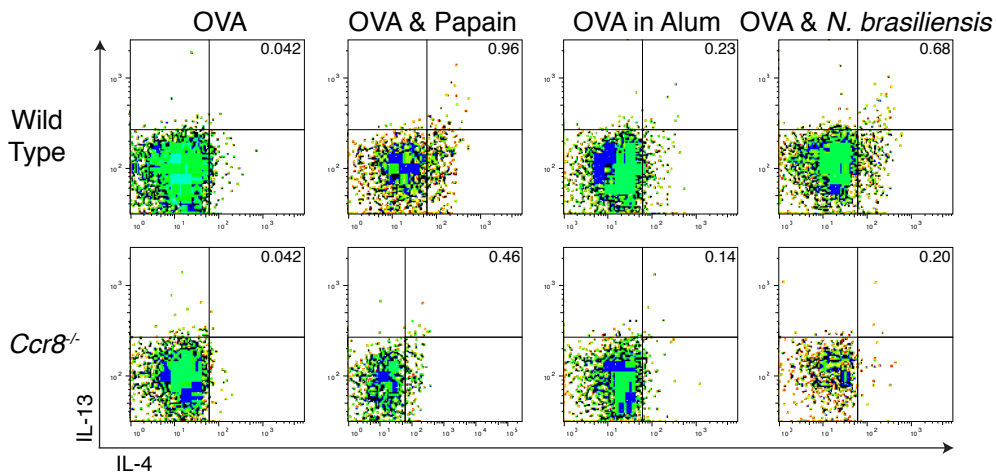


Figure S7 | Related to Figure 7.

Intracellular IL-4 and IL-13 staining of CD4⁺Thy1.1⁺ viable cells 4 days after OVA, OVA & Papain, or OVA in Alum immunization or 5 days after OVA & *N. brasiliensis* infection. Mice received 2.5-5x10⁵ naïve CD4⁺Thy1.1⁺ OTII cells prior to immunization. Representative plots are shown with percentage of cells in quadrants indicated. Data shown is representative of 3 independent experiments.