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This information is current as of July 4, 2013.

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J Immunol published online 24 June 2013

<http://www.jimmunol.org/content/early/2013/06/22/jimmunol.1203089>

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Distinct Chemokine Receptor Axes Regulate Th9 Cell Trafficking to Allergic and Autoimmune Inflammatory Sites

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Migration of Th cells to peripheral sites of inflammation is essential for execution of their effector function. The recently described Th9 subset characteristically produces IL-9 and has been implicated in both allergy and autoimmunity. Despite this, the migratory properties of Th9 cells remain enigmatic. In this study, we examined chemokine receptor usage by Th9 cells and demonstrate, in models of allergy and autoimmunity, that these cells express functional CCR3, CCR6, and CXCR3, chemokine receptors commonly associated with other, functionally opposed effector Th subsets. Most Th9 cells that express CCR3 also express CXCR3 and CCR6, and expression of these receptors appears to account for the recruitment of Th9 cells to disparate inflammatory sites. During allergic inflammation, Th9 cells use CCR3 and CCR6, but not CXCR3, to home to the peritoneal cavity, whereas Th9 homing to the CNS during experimental autoimmune encephalomyelitis involves CXCR3 and CCR6 but not CCR3. To our knowledge, these data provide the first insights into regulation of Th9 cell trafficking in allergy and autoimmunity. *The Journal of Immunology*, 2013, 191: 000–000.

Thelper 9 cells are the most recently described Th cell subset, but their *in vivo* function is incompletely understood, and their homing capacity remains unknown. Th9 differentiation is dependent on TGF- β and IL-4, and these cells express the pleiotropic cytokine IL-9 but no other Th lineage-specific cytokine or transcription factor (1, 2). Th9 cells are described to be functionally dynamic, with reports that they participate in mechanistically disparate forms of inflammation, such as allergy and autoimmunity, once thought to be restricted to the functions of Th2 and Th1/Th17 cells, respectively (3).

The role of IL-9 in adaptive immunity has been associated most closely with type 2 inflammatory settings, including antiparasitic and allergic inflammation. Despite one study that reported normal development of allergic inflammation in *IL9*^{-/-} mice using a sensitization-challenge model of allergy (4), several studies demonstrated a protective effect of IL-9 neutralization/blockade that is characterized by reduced lung eosinophilia, serum IgE, and airway epithelial damage, using similar models (5–7). Following

reports that T cells were major cellular sources of IL-9 (8), as well as detection of IL-9 production from T cells in *Leishmania major*-infected BALB/c mice, an infection model that elicits a strong Th2-driven inflammatory response (9), IL-9 was originally classified as a Th2-derived cytokine. However, owing to a lack of suitable mAb to IL-9 at the time of these studies, reliable flow cytometric analyses of IL-4/IL-9 coexpression by Th2 cells on a single-cell level did not exist. More recently, two independent laboratories demonstrated that the presence of IL-4 and TGF- β during TCR-mediated activation drove differentiation of a CD4⁺ T cell subset that lacked expression of T-bet, GATA-3, Foxp3, and ROR γ t, preferentially produced IL-9, but not IL-4, and was subsequently designated Th9 (1, 2). Since this discovery, other studies exemplified the importance of Th9-derived IL-9 in promoting allergic inflammation. In a T cell-transfer model of allergic airway disease, adoptive transfer of *in vitro*-generated Th9 or Th2 cells into *Rag2*-deficient recipients led to development of allergic pulmonary inflammation characterized by increased airway reactivity to methacholine and augmented eosinophil recruitment following airway challenge (10). Coadministration of IL-9-neutralization Ab profoundly ameliorated Th9 cell-induced asthma, but had little effect in mice that received Th2 cells (10). Furthermore, a role for Th9 cells in promoting allergic inflammation is supported by a recent study demonstrating a requirement for T cell expression of the Th9-promoting transcription factor PU.1 in experimental asthma (11). Mice with a T cell-specific deletion of PU.1 failed to generate Th9 cells and were resistant to IL-9-dependent allergic inflammation, despite the generation of a normal Th2 response, clearly establishing PU.1 as a master regulator of Th9 development and Th9 cells as important mediators of allergy (11). Collectively, these studies demonstrate that Th9 cells, in concert with Th2 cells, drive the pathogenesis of type 2 inflammatory disease.

Numerous studies also demonstrated the proinflammatory nature of Th9 cells in autoimmune inflammatory settings. In a T cell-transfer model of colitis, adoptive transfer of *in vitro*-generated Th9 cells into *Rag1*-deficient recipient mice led to significant weight loss that was associated with the induction of colitis and peripheral neuritis (2). Moreover, cotransfer of Th9 cells with

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Received for publication November 7, 2012. Accepted for publication May 22, 2013.

This work was supported by funding from the National Health and Medical Research Council. I.C. is a recipient of funding from Multiple Sclerosis Research Australia. T.M.H. is supported by funds from the National Institutes of Health (R01 AI37113).

E.E.K. designed and performed research, analyzed data, and wrote the manuscript; I.C. designed and supervised the study, performed research, and wrote the manuscript; C.R.B., K.A.F., and W.L. performed research; T.M.H. provided key reagents and expertise and edited the manuscript; and S.R.M. designed and supervised the study and wrote the manuscript.

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Abbreviations used in this article: EAE, experimental autoimmune encephalomyelitis; iTreg, inducible regulatory T cell; LN, lymph node; PLP, proteolipid protein; Treg, regulatory T cell.

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CD45RB^{hi}CD4⁺ effector T cells resulted in heightened pathology relative to mice that received CD45RB^{hi}CD4⁺ effector T cells alone (2). The pathogenic effector functions of Th9 cells were further explored more recently in the context of experimental autoimmune encephalomyelitis (EAE). Adoptive transfer of in vitro-generated encephalitogenic Th9 cells into naive hosts led

to development of neuroinflammatory CNS lesions and induction of severe EAE (12). Consistent with the results of these studies, inhibition of IL-9, using IL-9-deficient mice or Ab-mediated neutralization of IL-9 or its receptor, was shown to ameliorate EAE (13–15), although, in most studies, the cellular source of IL-9 was not specifically addressed. Furthermore, adoptive transfer of

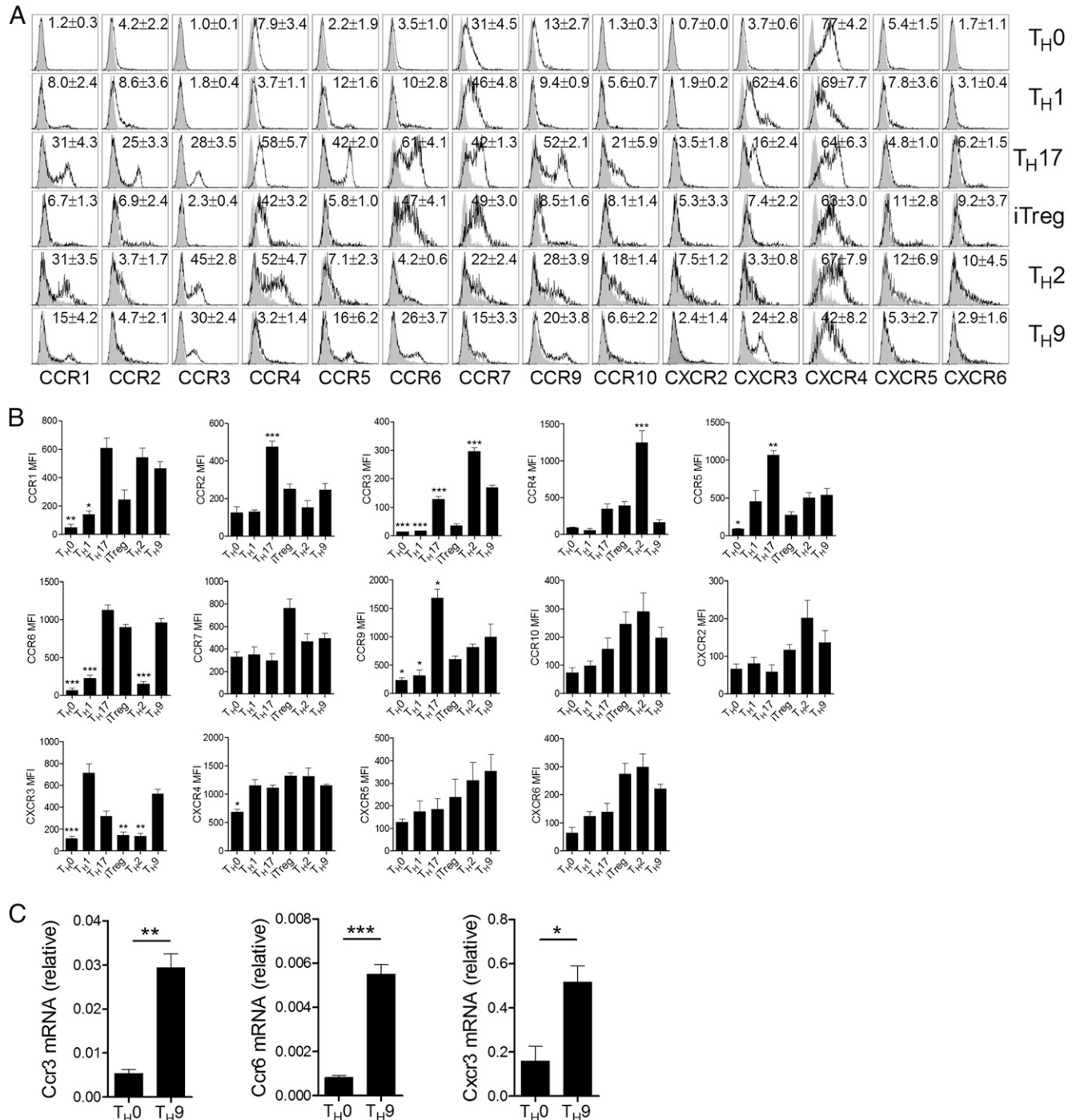


FIGURE 1. In vitro-generated Th9 cells express chemokine receptors associated with other effector Th cell subsets. **(A)** Representative histograms of chemokine receptor expression on in vitro-generated Th cell subsets gating on lineage-specific cytokine-positive (Th1, Th17, Th2, and Th9) or Foxp3-positive (iTreg) CD4⁺ T cells. Mean $\pm$ SEM of three independent experiments is indicated within each panel. Filled histogram: isotype control; open histogram: anti-CCR staining. **(B)** Flow cytometric analyses of chemokine receptor mean fluorescence intensity (MFI) on in vitro-generated Th9 cells compared with Th0, Th1, Th17, iTreg, and Th2 cells. Chemokine receptor MFI was calculated by subtracting respective isotype-matched negative-control staining from anti-CCR staining MFI. Data are mean $\pm$ SEM ($n = 3$ independent experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Th9 cells versus other Th subsets, one-way ANOVA with Bonferroni multiple-comparison posttest. **(C)** Quantitative PCR analysis of *Ccr3*, *Ccr6*, and *Cxcr3* mRNA expression by Th0 and Th9 cells generated from FACS-purified naive CD4⁺ T cell precursors, presented relative to the housekeeping gene *Rplp0*. Data are mean $\pm$ SD and are representative of one of two independent experiments performed with similar results. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, unpaired two-tailed Student t test.

hen egg lysozyme-specific Th9 cells into recipient mice that express a hen egg lysozyme transgene in the eye led to induction of moderate ocular inflammation, exemplifying the proinflammatory nature of this subset in the context of autoimmunity (16). Collectively, these studies suggest that Th9 cells can contribute to the pathogenesis of autoimmunity in multiple organ systems.

Together, these observations raise an important question regarding Th9 biology: how do Th9 cells migrate to diverse sites of inflammation that normally differentially recruit Th1/Th17 and Th2 cells? It is known that stimuli encountered during T cell priming sets up a transcriptional program in CD4⁺ T cells, tailoring their function to combat the initiating stimulus while imprinting a lineage-specific and tissue-tropic chemokine receptor-expression profile that facilitates migration to defined inflammatory sites (17). Chemokine receptor usage by naive, Th1, Th2, and Th17 cells, as well as regulatory T cells (Tregs), is well documented (17, 18), whereas the migratory capacity of the functionally dynamic Th9 subset remains unknown. Therefore, in the current study, we examined chemokine receptor usage by Th9 cells and determined the homing receptors involved in their recruitment to sites of allergic and autoimmune inflammation.

Materials and Methods

Mice, immunization strategies, and in vivo chemokine receptor antagonism

Eight- to ten-week-old C57BL/6, BALB/c, and SJL/j mice were obtained from the University of Adelaide Animal House. To generate Th9 cells under allergic conditions, BALB/c mice were immunized s.c. in the hind flanks with 100 μ g type IV OVA in 100 μ l AIOH gel (both from Sigma) on days 0, 3, and 7, as previously described (19). Seven days following final sensitization, mice were administered chemokine receptor antagonists (in 250 μ l endotoxin-free PBS) i.v.: 250 μ g misfolded chemokine peptide (MCP_{ala/ala}; scrambled peptide control) (20, 21), 100 μ g CCL20₆₋₇₀ (CCR6 antagonist) (21), 250 μ g CXCL11₄₋₇₉ (CXCR3 antagonist) (20), 250 μ g SB-328457 (CCR3 antagonist; Tocris Bioscience) (22), or PBS + 0.01% Tween 80 (vehicle for SB-328457). One hour following treatment, mice were challenged i.p. with 1 mg OVA in 250 μ l endotoxin-free PBS. Six hours postchallenge, draining lymph nodes (LNs), spleen, peripheral blood, and four peritoneal washes (1 ml PBS each) were harvested, and cells were analyzed by flow cytometry.

For generation of Th9 cells under autoimmune conditions, SJL/j mice were immunized s.c. in the hind flanks with 100 μ g proteolipid protein (PLP)₁₃₉₋₁₅₁ in 100 μ l CFA, as previously described (21). On days 0 and 2, mice received 300 ng pertussis toxin (List Biological Laboratories) in 250 μ l endotoxin-free PBS i.v. Chemokine receptor antagonists were administered i.p. every 48 h beginning 8 d post-EAE induction (as above). Fifteen days postinduction, draining LNs, spleen, peripheral blood, and CNS (spinal cord and brain) were harvested and processed, as previously described (21), and assessed by flow cytometry. The University of Adelaide institutional animal ethics committee approved all experimentation involving the use of animals.

In vitro T cell differentiation

Erythrocyte-lysed splenocytes were cultured in complete IMDM at 1×10^6 cells/ml in the presence of plate-bound anti-CD3 (10 μ g/ml) and soluble anti-CD28 (1 μ g/ml). The following cytokines were used for Th1 and Th17 differentiation, as described (23): for inducible Tregs (iTregs): TGF- β (10 ng/ml), anti-IFN- γ (10 μ g/ml), and anti-IL-4 (10 μ g/ml); for Th9: TGF- β (10 ng/ml), IL-4 (10 ng/ml), IL-2 (1 U/ml), and anti-IFN- γ (10 μ g/ml); for Th2: IL-4 (10 ng/ml), IL-2 (1 U/ml), and anti-IFN- γ (10 μ g/ml); and for Th0: anti-IFN- γ (10 μ g/ml) and anti-IL-4 (10 μ g/ml). Cytokines and neutralizing Abs were purchased from R&D Systems and BD, respectively. Cells were cultured for 3 d and then restimulated for 4 h with PMA, ionomycin, and GolgiStop (BD) in complete IMDM before surface and intracellular staining.

Naive T cell sorting, RNA extraction, and RT-PCR

Naive CD4⁺ T cells (CD4⁺CD8⁻CD44^{lo/-}CD25⁻) were sorted from erythrocyte-lysed splenocyte suspensions using a FACSAria (BD) and differentiated into Th0 and Th9 cells, as described above. Total RNA was extracted from these cells using the RNeasy Micro Kit (QIAGEN), as per

the manufacturer's instructions, with on-column RNase-free DNase I treatment (QIAGEN) to remove contaminating genomic DNA. cDNA was synthesized from RNA using the Transcriptor First Strand cDNA Synthesis Kit and used as a template in reactions using LightCycler 480 SYBR Green I Master Mix (both from Roche), according to the manufacturer's instructions. Primers used in this study were as follows: CCR3: 5'-TTGCCTACACCCACTGCTGTAT-3' (forward) and 5'-TTTCCGGAACCTCTCACCAA-3' (reverse), CCR6: 5'-TCTGCACTAGTGAGAGTGTG-3' (forward) and 5'-GTCATCACCACCATAATGTTG-3' (reverse), and CXCR3: 5'-GTGCTAGATGCCTCGGACTT-3' (forward) and 5'-GAGGCGCTGATCGTAGTTGG-3' (reverse). Primers for the reference gene *RPLP0* were 5'-AGATGCAGCAGATCCGCAT-3' (forward) and 5'-GGATGGCCTTGCGCA-3' (reverse). The relative level of chemokine receptor mRNA was calculated as $1/2^{\Delta\text{CT}}$ (ΔCT = CT of chemokine receptor - CT of *RPLP0*).

In vitro chemotaxis

Chemokines diluted in 150 μ l chemotaxis buffer (RPMI 1640 supplemented with 0.5% BSA/20 mM HEPES) were added to lower chambers of 96-well Transwell chemotaxis plates (5- μ m pores; Corning). In some experiments, chemokine receptor antagonists were included in the bottom chamber. For naive T cell migration, splenocyte preparations were cultured overnight in complete IMDM. For effector T cells, Th subsets were differentiated as described above. Cells were washed in chemotaxis buffer, loaded into upper chambers (5×10^5 cells in 50 μ l chemotaxis buffer/well), and incubated for 3 h at 37°C. To enumerate Th cell migration, migrated cells were harvested from the bottom chamber and restimulated as described above prior to flow cytometric analyses. Migrated cells of interest were enumerated by flow cytometry using CalibRITE beads (BD) as an internal reference.

Abs and flow cytometry

Cells were stained as previously described (23). For intracellular staining, the Cytofix/Cytoperm Kit (BD) was used as per the manufacturer's instructions. In certain experiments, a LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Invitrogen) was used to exclude dead cells. Cells were acquired using either a FACSCanto or LSR II (BD) and analyzed using FlowJo software (TreeStar).

Results

In vitro-generated Th9 cells express functional CCR3, CCR6, and CXCR3

To assess the migratory capabilities of Th9 cells, chemokine receptor expression by in vitro-generated Th9 cells was examined.

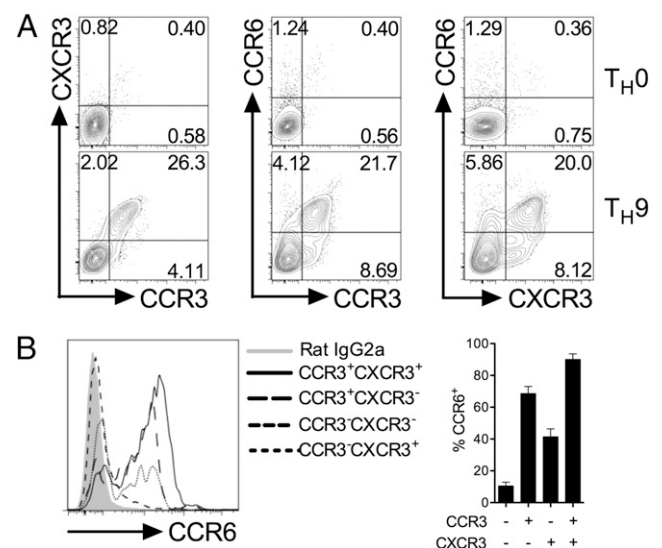
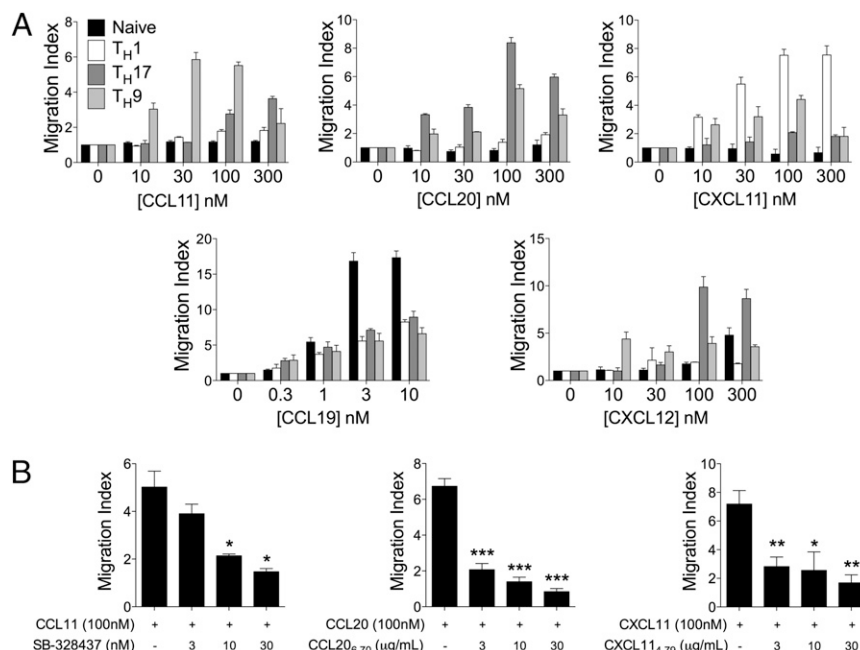


FIGURE 2. In vitro-generated Th9 cells coexpress CCR3, CCR6, and CXCR3. **(A)** Representative FACS contour plots of dual chemokine receptor expression on Th0 and Th9 cells generated in vitro. **(B)** CCR6 expression on CCR3⁻CXCR3⁻, CCR3⁺CXCR3⁻, CCR3⁻CXCR3⁺, and CCR3⁺CXCR3⁺ Th9 cell populations. Quantitation is shown. Data are mean $\pm$ SEM ($n = 3$ independent experiments).

FIGURE 3. Functional expression of CCR3, CCR6, and CXCR3 on Th9 cells. **(A)** Transwell chemotaxis of Th9 cells relative to naive, Th1, and Th17 cells. Migration index was calculated by dividing the number of positive events in test wells by the number of positive events in which no chemokine was added to the bottom chamber. Cells were enumerated as follows: naive CD4⁺; CD4⁺CD8[−]CD62L⁺CD44^{lo}; Th1: CD4⁺CD8[−]IFN- γ ⁺IL-17A[−]; Th17: CD4⁺CD8[−]IFN- γ [−]IL-17A⁺; and Th9: CD4⁺CD8[−]IFN- γ [−]IL-9⁺. Data are mean $\pm$ SEM ($n = 3$ independent experiments). **(B)** Inhibition of Th9 cell migration to CCL11, CCL20, and CXCL11 using chemokine receptor antagonists. Migration assays were performed as in (A), with the inclusion of the indicated chemokine receptor antagonist in the lower chamber. Data are mean $\pm$ SEM ($n = 2$ independent experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Flow cytometric profiling of Th9 cells revealed protein expression of chemokine receptors characteristically associated with Th1 (CXCR3) (24), Th2 (CCR3) (24, 25), and Th17/iTreg (CCR6) (26) subsets, as well as those expressed by naive and memory T cells (CCR7 and CXCR4) (Fig. 1A, 1B) (17). CCR6, CXCR3, and CCR3 also were elevated on Th9 cells relative to Th0 cells when mRNA levels were assessed using RT-PCR (Fig. 1C). Th9 cells in these systems lacked detectable expression of CCR2, CCR4, CCR10, CXCR2, CXCR5, and CXCR6 (Fig. 1A, 1B).

Multiple chemokine receptors coordinate the migration of effector Th cells, and prior studies indicate that effector Th subsets coexpress chemokine receptors with overlapping tissue-homing function (17). Thus, to determine whether chemokine receptors were coexpressed or confined to distinct subsets of Th9 cells, a dual chemokine receptor–staining strategy was used. These experiments revealed that ~95% of CCR3⁺ Th9 cells coexpressed CXCR3, whereas >75% of CCR3⁺ Th9 cells were positive for CCR6 (Fig. 2A). Triple chemokine receptor staining demonstrated that the majority of CCR6⁺ Th9 cells coexpressed CCR3 and CXCR3 (Fig. 2B). Therefore, among the effector CD4⁺ T cell subsets, Th9 cells carry a unique chemokine receptor surface phenotype that may explain their participation in both autoimmunity and allergy.

In vitro–migration assays were performed to determine whether CCR3, CCR6, and CXCR3 on Th9 cells were functionally active. Consistent with the high levels of surface expression of these receptors, efficient migration of Th9 cells toward CCL11, CCL20, and CXCL11 were measured (Fig. 3A). Migratory responses of Th9 cells to CCL19 and CXCL12 were also observed; however, these cells were substantially less responsive than were naive CD4⁺ T cells (to CCL19) and Th17 cells (to CXCL12) (Fig. 3A). The specificity of these migratory responses of Th9 cells to CCR3, CCR6, and CXCR3 was confirmed using specific antagonists targeting these receptors. Treatment with the small-molecule CCR3 antagonist SB-328437 inhibited Th9 migration toward CCL11, whereas the peptide antagonists CCL20₆₋₇₀ and CXCL11₄₋₇₉ efficiently prevented Th9 migration to CCL20 and CXCL11, respectively (Fig. 3B).

Reports that chemokines promote proliferation and subsequent secretion of IFN- γ or IL-4 demonstrated that chemokines may

play important roles in T cell costimulation (27). Therefore, the possible effects of these chemokines on Th9 differentiation were also examined. However, the addition of CCL11, CCL20, or CXCL11 (or combinations of these chemokines), at concentrations known to activate their cognate receptors, to Th9-polarizing T cell cultures did not alter Th9 differentiation in vitro (data not shown).

Collectively, these data indicate that Th9 differentiation is coupled with the induction of a functional chemokine receptor repertoire that supports migration to chemokines commonly associated with type-1, -2 and -17 inflammation.

In vivo–generated Th9 cells express CCR3, CCR6, and CXCR3 in allergic and autoimmune inflammatory models

As Th9 cells are able to participate both in allergic (10, 11) and autoimmune inflammation (12), it was hypothesized that these cells differentially use chemokine receptors to migrate to allergic

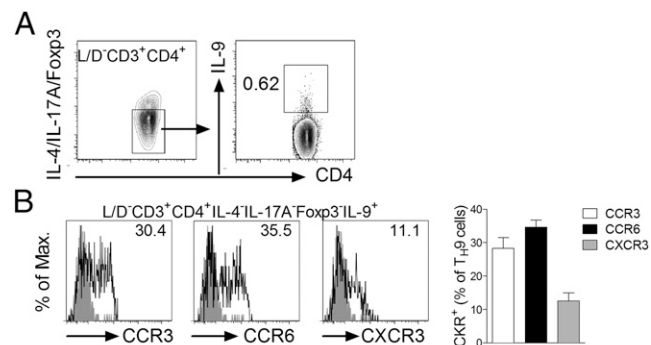


FIGURE 4. Th9 cells generated in vivo during allergic inflammation express CCR3, CCR6, and CXCR3. **(A)** Representative FACS contour plots of Th9 cells generated in a type 2 immunization strategy. BALB/c mice were sensitized with OVA/AIOH gel on days 0, 3, and 7; inguinal LNs were collected 7 d later; and Th9 cells were identified in flow cytometry as live (L/D[−]) CD3⁺CD4⁺, IL-4/IL-17A/Foxp3[−] (bottom gate, left panel), IL-9⁺ (gated in right panel). **(B)** Representative FACS graphs of CCR3, CCR6, and CXCR3 expression on Th9 cells gated in (A) (left panels), with the percentage of chemokine receptor–positive Th9 cells indicated (right panel). Filled histogram: isotype control; open histogram: anti-CCR staining. Quantitation is shown ($n = 5$ mice).

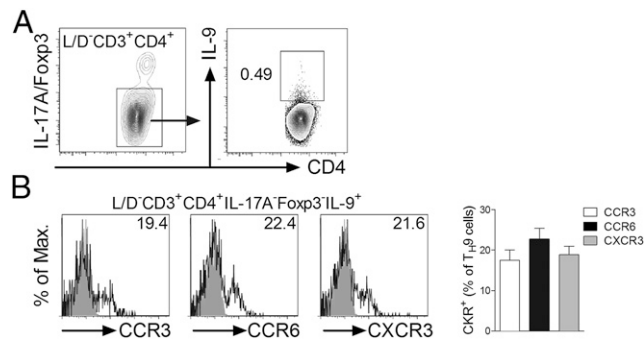


FIGURE 5. Th9 cells generated in vivo during PLP-induced EAE express CCR3, CCR6, and CXCR3. **(A)** Representative FACS contour plots of Th9 cells generated in a type 1/type 17 immunization strategy. SJL/j mice were immunized with PLP_{139–151}/CFA, inguinal LNs were collected 10 d later, and Th9 cells were identified in flow cytometry as live CD3⁺CD4⁺, IL-17A/Foxp3⁻ (bottom gate, left panel), and IL-9⁺ (gated in right panel). **(B)** Representative graphs of CCR3, CCR6, and CXCR3 expression on Th9 cells gated in (A) (left panels), with the percentage of chemokine receptor-positive Th9 cells indicated (right panel). Filled histogram: isotype control; open histogram: anti-CCR staining. Quantitation is shown ($n = 5$ mice).

and autoimmune sites. Therefore, chemokine receptor expression on Th9 cells generated during models of experimental allergy and autoimmunity was investigated. Previous reports indicated that IL-9 can also be elicited, to some extent, from other Th subsets, in-

cluding iTregs, Th17, and Th2 cells (28). Therefore, to study bona fide Th9 cells in vivo, IL-9 was stained against a dump gate excluding IL-17A, Foxp3, and IL-4. Consistent with a previous study (19), multiple s.c. OVA/alum immunizations in BALB/c mice, a classic type 2 priming model that leads to the formation of Th2 cells and IgE (19), also led to the generation of Th9 cells (CD3⁺CD4⁺IL-4⁻IL-17A⁻Foxp3⁻IL-9⁺) that could be detected in draining LNs (Fig. 4A). Analysis of chemokine receptor expression on these Th9 cells supported in vitro observations as Th9 cells generated in this model expressed CCR3, CCR6, and CXCR3 (Fig. 4B).

Assessment of Th9 chemokine receptor expression generated under type 1/type 17 autoimmune conditions was initially examined using the MOG_{35–55}-induced model of EAE in C57BL/6 mice. However, in this model, IL-9⁺CD4⁺ T cells were not detected in draining LNs or spleens on days 5, 9, or 12 post-immunization (data not shown). Conversely, immunization of SJL/j mice for PLP_{139–151}-induced EAE, another type 1/type 17 priming model, led to the generation of IL-9⁺CD4⁺ T cells that lacked IL-4, IL-17A, and Foxp3 expression (Fig. 5A, data not shown). Chemokine receptor-expression profiling of Th9 cells generated under these conditions revealed that these cells also expressed CCR3, CCR6, and CXCR3 (Fig. 5B).

Differential chemokine receptor axes mediate recruitment of Th9 cells in allergic and autoimmune inflammatory settings

To investigate the contribution of these receptors to Th9 cell trafficking in vivo, chemokine receptor antagonists were admin-

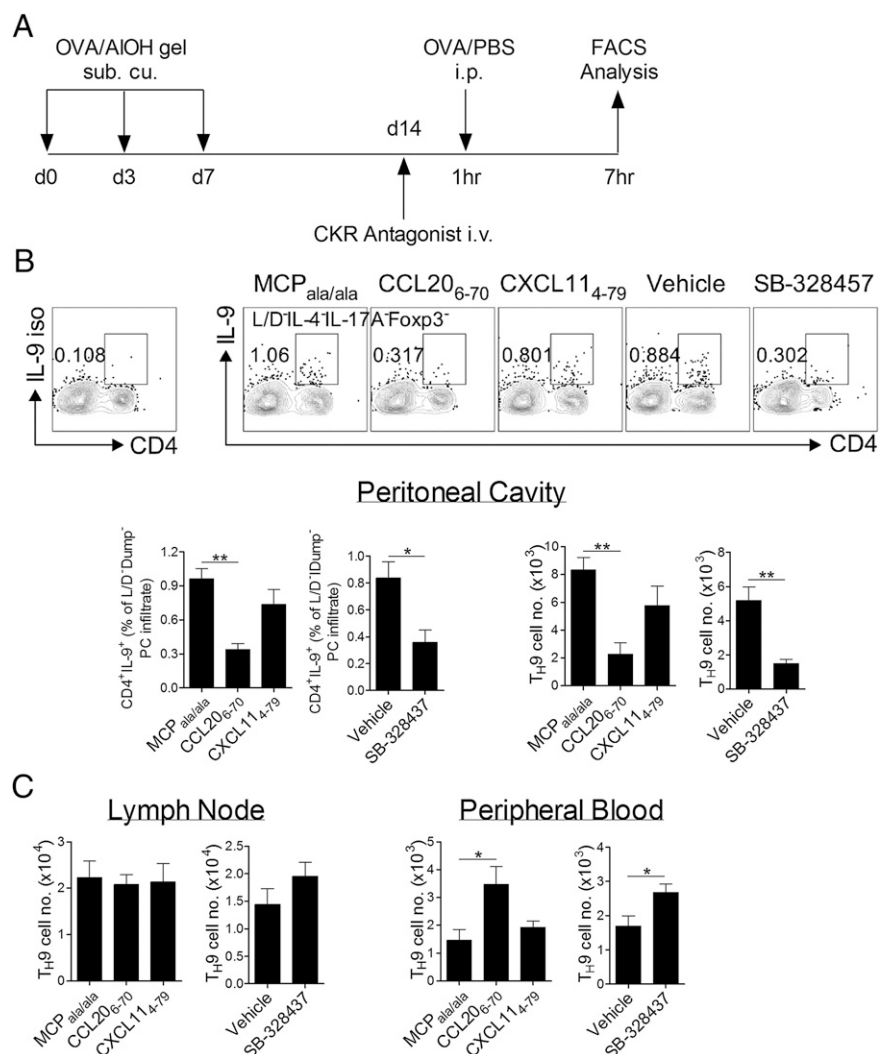


FIGURE 6. CCR3 and CCR6 mediate recruitment of Th9 cells to an allergic microenvironment. **(A)** Schematic diagram of experimental strategy. **(B)** Representative FACS contour plots of peritoneal cavity-infiltrated Th9 cells (CD4⁺IL-9⁺) 6 h post-OVA challenge (upper panels). Plots are gated on live IL-4/IL-17A/Foxp3⁻ lymphocytes. Number and proportion of Th9 cells in the peritoneal cavity 6 h post-OVA challenge (lower panels). **(C)** Number and proportion of Th9 cells 6 h post-OVA challenge in LN and peripheral blood (live IL-4/IL-17A/Foxp3⁻ lymphocytes that are CD4⁺IL-9⁺). Data are mean ± SEM ($n = 4$ mice/group). * $p < 0.05$, ** $p < 0.01$, unpaired two-tailed Student t test.

istered during the effector phase of these models. Importantly, these receptor antagonists were shown to inhibit pathogenesis in animal models of allergic (29) and autoimmune (20, 21) diseases. Following OVA/alum sensitization and subsequent i.p. OVA/PBS challenge in a type 2 priming model (Fig. 6A), antagonism of CCR3 or CCR6, using SB-328437 or CCL20₆₋₇₀, respectively, led to reduced frequencies of Th9 cells in the peritoneal cavity relative to control mice that were administered vehicle only or treated with MCP_{ala/ala} (Fig. 6B). In addition, treatment with the CCR3 and CCR6 antagonists resulted in accumulation of Th9 cells in the peripheral blood but not the draining LNs (Fig. 6C). No effect of CXCR3 antagonism using CXCL11₄₋₇₉ was observed in this model with regard to recruitment of Th9 cells to the peritoneal cavity or accumulation of Th9 cells in the peripheral blood or the draining LNs in response to OVA challenge (Fig. 6B, 6C). The reduced frequency of Th9 cells in the peritoneal cavity and the increase in these cells in peripheral blood when CCR6 or CCR3 are antagonized strongly implicate these receptors as functioning in blood-to-effector-site trafficking in allergic inflammation. In these settings, CXCR3 does not appear to be of critical importance to Th9 homing. These results are in keeping with previous findings that CCR6 and CCR3 ligands, but not CXCR3 ligands, are abundantly expressed at type 2 inflammatory sites (30, 31).

Conversely, chemokine receptor antagonism during PLP₁₃₉₋₁₅₁-induced EAE (Fig. 7A) revealed that antagonism of CXCR3 led to reduced recruitment of these cells to the CNS compared with

control-treated mice with a concomitant accumulation of Th9 cells in LNs (Fig. 7B, 7C). A similar effect was observed for CCR6 antagonism, which also led to reduced recruitment of Th9 cells to the CNS and accumulation of Th9 cells in LNs (Fig. 7B, 7C). In contrast to allergic inflammation, no effect of CCR3 antagonism was apparent on Th9 cell trafficking in the EAE model (Fig. 7B, 7C). These data indicate that CCR6 and CXCR3 are likely to be involved in Th9 egress from LNs during EAE and are important for recruitment of these cells to the CNS. These findings are consistent with previous reports demonstrating that both CCR6 and CXCR3 ligands are induced in the secondary lymphoid organs and CNS in EAE (32, 33).

Discussion

Th9 cells are the most recently described and least well understood subset of effector Th cells. In the current study, we observed that in vitro and in vivo generation of Th9 cells is coupled with the upregulation of functional chemokine receptors commonly associated with other effector Th subsets, including CCR3 (Th2) (24, 25), CCR6 (Th17/Tregs) (26), and CXCR3 (Th1) (24). Using chemokine receptor antagonists, we also demonstrated that Th9 cells use CCR3 and CCR6 to traffic to an allergic inflammatory site, whereas migration of these cells to an autoimmune effector site is CCR6 and CXCR3 dependent.

Disrupting pathogenic effector Th cell migration in certain CD4⁺ T cell-driven pathologies through antagonism/neutralization of

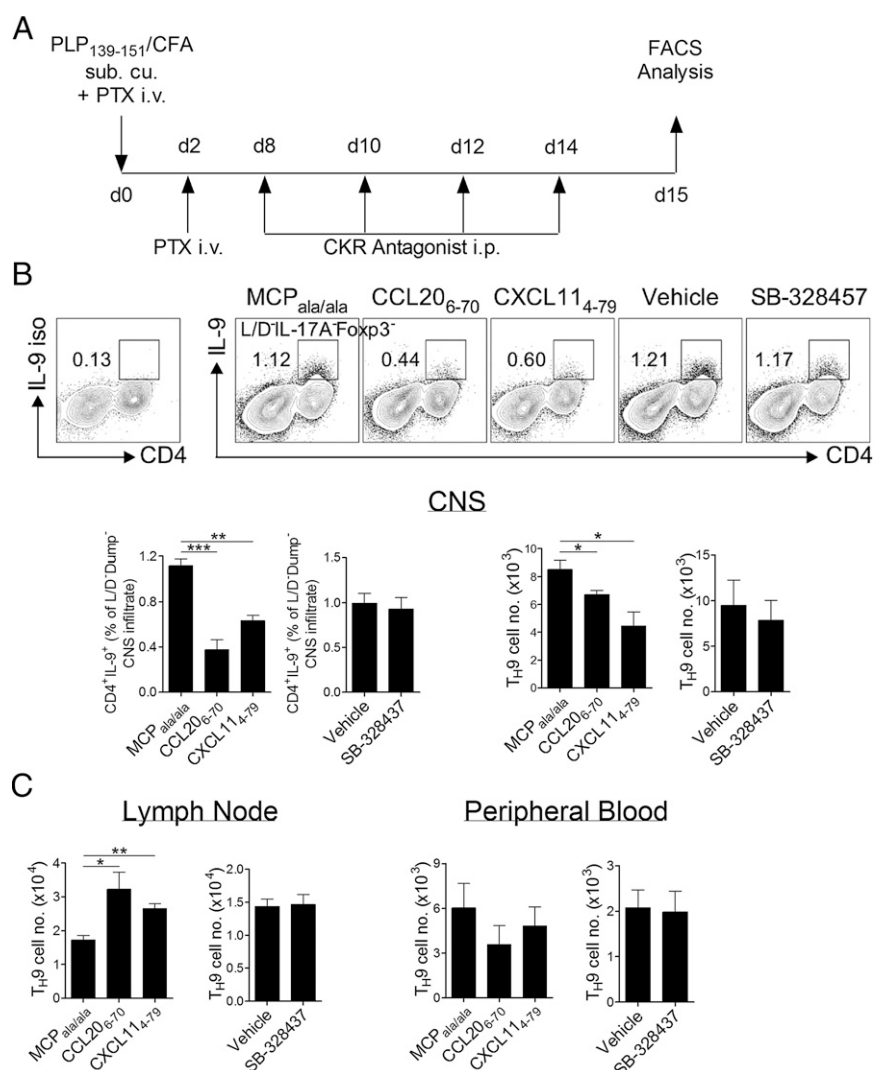


FIGURE 7. CCR6 and CXCR3 mediate recruitment of Th9 cells to an autoimmune microenvironment. **(A)** Schematic diagram of experimental strategy. **(B)** Representative FACS contour plots of CNS-infiltrated Th9 cells (CD4⁺IL-9⁺) on day 15 post-EAE induction (upper panels). Plots are pregated on live IL-17A/Foxp3⁻ (Dump)⁻. Number and proportion of Th9 cells in CNS on day 15 post-EAE induction (lower panels). **(C)** Number and proportion of Th9 cells in LN and peripheral blood (live IL-4/IL-17A/Foxp3⁻ [Dump]⁻ lymphocytes that are CD4⁺IL-9⁺). Data are mean ± SEM (n = 3–4 mice/group). *p < 0.05, **p < 0.01, ***p < 0.001, unpaired two-tailed Student t test.

specific chemokine receptors holds much promise for the treatment of human disease (34). Central to the success of this approach is a greater understanding of the subsets of effector Th cells responsible for driving particular pathologies and identifying the unique homing signals used by these cells to infiltrate tissues. Previous studies identified Th1 cells as carrying a CXCR3⁺CCR5⁺ phenotype (24, 35), with subpopulations also positive for CCR1 and/or CCR2 (24, 36). Characteristically, Th2 cells do not share any of these Th1-homing receptors and instead variously express CCR3, CCR4, and CCR8 on their cell surface (25, 37). These distinct chemokine receptor repertoires are thought to largely account for exclusive homing of these functionally antagonistic effector Th subsets to initiate macrophage-rich or eosinophil-rich inflammatory lesions, respectively. In contrast, the apparently functionally opposing Th17 and Treg subsets share expression of the chemokine receptor CCR6 (26). This receptor facilitates recruitment of these cells to sites of CCL20 production, a chemokine that is expressed constitutively and whose expression is strongly upregulated during acute inflammation at epithelial and mucosal sites (38). Subsets of Th17 cells were also variously reported to express CCR2, CCR4, CXCR4, and CXCR6 (39), whereas Treg function appears to additionally depend on CCR7 (40). It was postulated that the balance between inflammatory Th17 cells and Tregs infiltrating a given inflammatory site dictates whether prolonged and perhaps pathological inflammation or response resolution occurs (38). The results of the current study indicate that, in contrast to these other Th subsets, Th9 cells do not express chemokine receptors that are unique to the subset; instead, they share chemokine receptors with other known subsets of Th cells.

Our results reveal several unique aspects of Th9 cells that may provide key information for understanding the migratory properties of these cells, as well as their functional significance. Given that Th9 cells are equipped with trafficking receptors that facilitate migration to virtually any inflammatory lesion, we propose that these cells may contribute to cell-mediated, humoral and acute inflammatory responses. Indeed, IL-9 production was detected in various human inflammatory settings, including allergy (reviewed in Ref. 41); IL-9 is also present in classic Th1 responses, such as during *Mycobacterium tuberculosis* (42) and respiratory syncytial virus infection (43); and it can be detected in murine models of Th1/Th17-driven human autoimmune disease, such as EAE (13, 14). IL-9 was shown to have pleiotropic effector functions at these inflammatory lesions, including the induction of various chemokines, such as CCL20 from astrocytes during EAE (44), CCL11 in smooth muscle cells (45) and, in synergy with TNF- α , CXCL8 in the lung (46). Moreover, Th9 cells themselves were shown to produce the CCR4 ligands CCL17 and CCL22 (11). Therefore, it is possible that Th9 cells may contribute to these various forms of inflammation by providing an adaptive immune source of IL-9, a cytokine that amplifies the chemotactic potential of an inflammatory site, facilitating the subsequent homing of innate effector cells and other effector T cell subsets. However, these hypotheses remain to be tested. Indeed, the functional significance of the Th9 subset has yet to be determined in detail, and most data have come from studies prior to the demonstration that these cells were a distinct differentiation lineage or in which IL-9 blockade/deletion in all compartments was used. More refined studies taking advantage of T cell-specific PU.1-knockout mice (11) or the recently engineered IL-9 fate-mapping reporter mouse (47) will be required to address these issues in various inflammatory settings. Moreover, the question of plasticity of this subset remains. Recent studies using an IL-17 fate-mapping strategy revealed that Th17 cells convert to Th1 cells in vivo during the progression of EAE

(48), which presumably allows them to contribute to the early Ag-specific adaptive response through inflammation and recruitment of neutrophils, as well as to macrophage activation via the production of IFN- γ . A similar approach using the IL-9 fate-mapping reporter mouse could be used to delineate the fate of Th9 cells in different types of immune responses.

Several other key findings related to Th9 cells are apparent from this study. It is of note that CCR6 and CXCR3 antagonism during EAE appeared to prevent Th9 cell egress from draining LNs into the peripheral blood, thereby inhibiting recruitment of the cells into the CNS. Although a clear role for chemokine receptors in the promotion of egress of effector T cells from secondary lymphoid organs has yet to be formally demonstrated, these results are in keeping with our previous observations that CCR6 antagonism and neutralization of CCL20 increase the frequency of effector T cells in draining LNs during EAE and reduce their abundance in the circulation (21, 33). Egress of T cells from LNs is considered to be largely controlled by relative expression of the sphingosine-1-phosphate receptor S1P₁, which attracts cells into the peripheral blood, and CCR7, which retains cells in the LN (49). Our results suggest that inflammatory chemokine receptors, such as CXCR3 and CCR6, can also contribute to Th cell egress from LNs. However, these effects appear to be model dependent, because no effect of CCR6 or CXCR3 antagonism on Th9 cell accumulation in LNs was apparent following immune priming in the OVA/alum model. Dissecting out the precise chemokine signals that promote effector Th cell egress from secondary lymphoid tissues in specific inflammatory settings may be a promising avenue of investigation given the therapeutic effectiveness, as well as the generalized immune suppression, of S1P₁ antagonism in humans (50).

In summary, to our knowledge, the data presented in this article are the first to define the trafficking of Th9 cells and provide an explanation for the appearance and involvement of these cells in disparate forms of inflammation. Th9 cells express a broader range of functional chemokine receptors than do other Th subsets, allowing migration to types 1, 2, and 17 inflammatory sites, where they have been implicated in T cell-driven pathology. These findings will be of importance to the design of therapeutic strategies to limit detrimental inflammation driven by pathogenic Th9 and other effector Th cells. However, further investigations using model systems in which these chemokine receptors have been specifically knocked out on Th9 cells will be required to unequivocally prove whether recruitment of Th9 cells by these specific receptors is required for disease pathogenesis.

Acknowledgments

We thank the late Prof. Ian Clark-Lewis for various chemokines and chemokine receptor antagonists.

Disclosures

The authors have no financial conflict of interests.

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