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Mouse CCL8, a CCR8 agonist, promotes atopic dermatitis by recruiting IL-5$^+$ T_H2 cells

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Abstract

Mouse CCL8 is a CC chemokine of the monocyte chemoattractant protein (MCP) family whose biological activity and receptor usage have remained elusive. Here we show that CCL8 is highly expressed in the skin, where it serves as an agonist for the chemokine receptor CCR8 but not for CCR2. This distinguishes CCL8 from all other MCP chemokines. CCL8 responsiveness defined a population of highly differentiated, CCR8-expressing inflammatory T helper type 2 (T_H2) cells enriched for interleukin (IL)-5. *Ccr8*- and *Ccl8*-deficient mice had markedly less eosinophilic inflammation than wild-type or *Ccr4*-deficient mice in a model of chronic atopic dermatitis. Adoptive transfer studies established CCR8 as a key regulator of T_H2 cell recruitment into allergen-inflamed skin. In humans, CCR8 expression also defined an IL-5–enriched T_H2 cell subset. The CCL8-CCR8 chemokine axis is therefore a crucial regulator of T_H2 cell homing that drives IL-5–mediated chronic allergic inflammation.

Monocyte chemoattractant proteins (MCP) comprise one of two major CC-chemokine families encoded in the genome as closely linked gene clusters. ‘Cluster’ chemokines tend to be inflammatory—in contrast to noncluster chemokines, which tend to be homeostatic—although there is some overlap of function[1]. Cluster chemokines often do not correspond exactly between vertebrate species, whereas noncluster and homeostatic chemokines are better conserved across species[1]. Multispecies comparative genomic and phylogenetic analyses of the MCP-encoding cluster suggest that gene duplication and modification

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AUTHOR CONTRIBUTIONS

A.D.L. screened the EST database to identify mouse *Ccl8*, designed the construct to express functional recombinant mouse CCL8 protein, conducted the RNA blot analysis, designed experiments and wrote the paper. D.S.C. did mouse experiments, immunohistochemistry and QPCR of skin RNA preps. R.A.C. helped devise a strategy to clone *Ccr8* and helped generate *Ccr2* transient transfectants. M.H.B. helped clone *Ccr8*. M.L.M. and B.M. generated and provided the biotinylated monoclonal antibody to human CCR8. S.A.L. provided the $Ccr8^{-/-}$ mice, and I.F.C. provided the $Ccl8^{-/-}$ $Ccl12^{-/-}$ and $Ccl12^{-/-}$ mice. S.A.I. carried out all other *in vitro* and *in vivo* experiments, designed research, analyzed data and wrote the paper.

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occurred within this gene cluster after the branching of rodents and primates[1,2]. The orthologous relationships between mouse and human MCP-cluster chemokines are therefore uncertain[1]. For example, human MCP-4 has no mouse ortholog, and mouse MCP-5 has no human ortholog. The only MCP chemokine not yet functionally characterized is mouse CCL8 (also known as MCP-2 (A001485)).

Chemokines and their receptors, in conjunction with adhesion molecules and their ligands, provide cues to enable tissue-specific homing of T cells in the steady state and during inflammation[3,4]. CCR7 and L-selectin provide T cells with the ability to home to secondary lymphoid organs, CCR9 and integrin $\alpha_4\beta_7$ enhance the ability of T cells to home to the gut, and CCR10 and cutaneous lymphocyte antigen direct T cells to the skin[3,5]. CCR4 also mediates skin-specific recruitment of T cells in the steady state and during inflammation, in addition to directing T cell recruitment to other organs[3,5].

Recent studies on cells isolated from human skin have identified CCR8 (A000631) as an additional CC-chemokine receptor that mediates skin-tropic homing of memory $CD4^+$, $CD8^+$ and γδ T cells in the steady state[6,7] and skin-infiltrating leukocytes during atopic inflammation[8]. CCR8 is expressed by regulatory T helper cells[9], $CD4^+$ thymocytes[10], a subset of dendritic cells and macrophages[11,12], and T_H2 (but not T_H1) cells[13,14]. The role of CCR4 and CCR10 in regulating T cell recruitment to skin is well established[3,5]. However, whether there is a unique role for CCR8-mediated recruitment of T cells to skin in the steady state or during inflammation has not been established by either *in vivo* models of inflammation or *in vivo* trafficking studies. Notably, the skin-tropic viruses molluscum contagiosum and Kaposi's sarcoma–associated herpesvirus (human herpesvirus 8) encode a CCR8 antagonist and agonist, respectively[15,16].

Here we define mouse CCL8 as a second mammalian agonist for the chemokine receptor CCR8. We also show that human CCL8, a known CCR2 agonist[1,17], does not activate CCR8. Mouse CCL8 specifically induces migration of a population of recently activated, highly differentiated T_H2 cells enriched in interleukin 5 (IL-5) and the IL-25 receptor (IL-25R), cytokines implicated in eosinophilic inflammation[18-20]; as well as tumor necrosis factor (TNF) and OX40, molecules associated with inflammatory T_H2 cells[21]. Using a model of atopic dermatitis, we demonstrate a pathologic role for CCR8 and mouse CCL8, but not for the T_H2-associated chemokine receptor CCR4 or the previously known mouse CCR8 chemokine ligand mouse CCL1 (TCA3), in mediating chronic cutaneous allergic inflammation. Using competitive *in vivo* homing experiments, we show that CCR8 and mouse CCL8 direct T_H2 cell recruitment into allergen-inflamed skin and draining lymph nodes (DLNs). Finally, in humans, we show that peripheral blood $CCR8^+$ $CD4^+$ T cells are enriched for IL-5, whereas $CCR4^+$ $CD4^+$ T cells are enriched for IL-4. We thus define mouse CCL8 as a new CCR8 ligand and demonstrate a role for this chemokine in mediating migration of an IL-5–enriched T_H2 cell population into the skin, driving eosinophilic inflammation during chronic allergen exposure.

RESULTS

Gene and protein characterization of mouse CCL8

The human and mouse MCP gene clusters both contain six genes (Fig. 1a), yet CCL12 (MCP-5) lacks a human ortholog, and CCL13 (MCP-4) lacks a mouse ortholog. In the mouse genome, *Ccl8* is positioned between *Ccl1*, which encodes the only known CCR8 ligand, and *Ccl12*, encoding a CCR2 ligand. In contrast, in the human genome, *CCL8* is positioned between *CCL13*, encoding a pleiotropic ligand for CCR2, CCR3 and CCR5 receptors, and *CCL11* (eotaxin-1), encoding a CCR3 ligand. To search for mouse genes related to human *CCL13*, we screened an expressed sequence tag (EST) database. The

closest hit was the clone identified as mouse *Ccl8*, which was previously designated as such owing to its sequence homology to human *CCL8* (Fig. 1b). With regard to function, CCL11 recruits eosinophils exclusively through CCR3, and CCL2 recruits monocytes exclusively through CCR2. The other characterized MCP chemokines activate monocytes through CCR2 (refs. 1,17). CCL7 (MCP-3), CCL13 and human CCL8 also activate eosinophils through CCR3 (ref. 1).

Mouse *Ccl8* cDNA was 492 base pairs long, with an open reading frame that encoded 97 amino acids (Supplementary Fig. 1). The 5′ region of *Ccl8* cDNA encoded a 23-amino-acid hydrophobic leader sequence and had a predicted cleavage site at a position similar to that of other MCP-cluster chemokines. The N terminus of mature mouse CCL8 was predicted to be glycine, a feature shared with CCL12 and human CCL11 but not with other MCP proteins (Supplementary Fig. 1). The mature N-terminal amino acid is important for biological activity and leukocyte selectivity of MCP and eotaxin proteins[22]. Notably, the N terminus of mouse CCL8 was shorter than that of other MCP chemokines by two amino acids but was identical in length to the N terminus of mouse and human CCL11.

We compared mRNA expression of mouse *Ccl8* to that of other mouse MCP chemokines and the closely related CC chemokine *Ccl11* in normal mouse tissue using RNA hybridization analysis (Fig. 1c). *Ccl8* was the only MCP-family chemokine that had high constitutive expression in skin. *Ccl8* mRNA was also detected in thymus, lymph node and breast tissue. Immunofluorescence staining confirmed the presence of mouse CCL8 protein in normal mouse skin (Fig. 1d), mainly in epidermal keratinocytes.

Screen for mouse CCL8–responsive leukocyte populations

We first observed that bone marrow–derived macrophages (BMDMs) did not migrate in response to mouse CCL8 in *in vitro* chemotaxis assays, but did migrate to mouse CCL2, which confirmed CCR2 expression on these cells (Fig. 2a). This finding was unexpected, as all other MCP chemokines activate monocyte lineage cells through CCR2. Baf/3 cells transfected with mouse CCR2 receptor did not migrate to mouse CCL8 *in vitro*, although they migrated to CCL2 and CCL12 (Supplementary Fig. 2), confirming that mouse CCL8 is not a CCR2 agonist. We next assayed mouse eosinophils, which migrate in response to CCL11, for migration to mouse CCL8 because of similarities in the N-terminal structures of CCL8 and CCL11. Mouse CCL8 induced neither migration nor calcium flux of eosinophils isolated from the spleens of *Il5*-transgenic mice (data not shown), making it unlikely that mouse CCL8 is a CCR3 agonist. We therefore turned to other potential target leukocyte populations to discern the biological activity of mouse CCL8. We assessed the ability of mouse CCL8 to induce the chemotaxis of natural killer (NK) cells, which express functional CCR2 and CCR5. NK cells migrated in response to the CCR2-specific agonist CCL2 and the CCR5-specific agonist CCL4 (MIP-1β), but they did not migrate in response to mouse CCL8, making it unlikely that mouse CCL8 is a CCR5 or CCR2 agonist (Fig. 2b).

Freshly isolated lymph node $CD4^+$ and $CD8^+$ T cells also did not respond to mouse CCL8 but migrated to the positive control CXCL12 (SDF-1), a CXCR4 agonist (Fig. 2c). Similarly, T_H1 and T_H2 cells generated by one round of *in vitro* differentiation, henceforth referred to as T_H1-R1 and T_H2-R1, respectively, did not migrate to mouse CCL8. However, T_H1-R1 cells did migrate to CXCL11 (ITAC; Fig. 2d), a CXCR3 agonist, and T_H2-R1 did migrate to CCL22 (MDC; Fig. 2e), a CCR4 agonist, as positive controls. T_H1-R1 and T_H2-R1 cells also migrated to CXCL12 and leukotriene B_4 (Fig. 2d,e and Supplementary Fig. 3a,b). T_H1-R1 and T_H2-R1 cells were thus capable of migrating to multiple chemoattractants but specifically could not migrate to mouse CCL8.

A subset of T_H2 cells respond to mouse CCL8

More highly differentiated T_H2 cells, generated by multiple rounds of *in vitro* polarization followed by T cell receptor (TCR) re-activation, migrate differently than do T_H2-R1 cells[13,14]. We thus generated such cells, termed T_H2-R2A cells, by *in vitro* differentiation of T_H2 cells through two rounds of polarization (T_H2-R2), followed by brief TCR reactivation (T_H2-R2A). T_H2-R2A cells migrated potently to mouse CCL8 in a dose-dependent, bell-shaped migration curve (Fig. 2f). This mouse CCL8–induced chemotaxis was inhibited by pertussis toxin (PTX), an inhibitor of $G\alpha_i$, indicating that a $G\alpha_i$-coupled chemo-attractant receptor[23] mediates mouse CCL8–induced chemotaxis of T_H2-R2A cells. T_H2-R2A cells also showed robust calcium flux to mouse CCL8 (Fig. 2g). Dose-response studies revealed that increasing concentrations of mouse CCL8 elicited incremental increases in the magnitude of the calcium flux (Fig. 2h).

CCR8 mediates mouse CCL8–induced T_H2 cell migration

The migration of T_H2-R2A cells in response to mouse CCL8 provided a tool to identify potential mouse CCL8 receptors. When we assayed CC-chemokine receptor mRNA abundance in mouse CCL8–responsive cells, *Ccr8* was the most highly enriched receptor (Fig. 3a). Signals regulating polarized T helper subset differentiation are tightly linked to expression of chemokine receptors, and CCR8 is expressed on cells of T_H2 (but not T_H1) lineage[3,4,13,14]. However, assaying for CCR8 function in T_H2 cells is confounded by the following factors (Fig. 3b): CCR8 induction at levels high enough to mediate agonist function is evident only in T_H2 cells generated by multiple rounds of polarization followed by additional TCR activation[13,14]; TCR ligation leads to transient induction of CCR8 expression[13,14]; and TCR ligation induces abundant autocrine CCL1 production[24], which promotes CCR8 receptor desensitization *in vitro*.

Ccr8 mRNA levels were highest in T_H2-R2A cells compared with T_H2-R1 or T_H2-R2 cells, BMDMs, blood monocytes, NK cells or T_H1 cells (Fig. 3c). Further, T_H1 cells generated by two rounds of polarization or T_H2 cells generated by one round of polarization that underwent additional TCR activation did not migrate to mouse CCL8 (Supplementary Fig. 3c,d). These data confirm the specificity of CCR8 expression in T_H2-R2A cells. By comparing migration of wild-type and *Ccr8*-, *Ccr2*- or *Ccr5*-deficient T_H2-R2A cells in response to mouse CCL8, we determined that only deficiency of *Ccr8* abrogated migration to mouse CCL8 and the known mouse CCR8 agonist mouse CCL1 (Fig. 3d). A blocking antibody to mouse CCR8 specifically inhibited migration of wild-type T_H2-R2A cells to mouse CCL8 (Fig. 3e). In contrast, *Ccr8* deficiency had no effect on T_H2-R2A cell migration to human CCL8 (Supplementary Fig. 4a), consistent with human CCL8 being an agonist for CCR2 (refs. 1,16,17) but not for CCR8.

Mouse CCL8 is a CCR8 agonist

Baf/3 cells transfected with mouse *Ccr8* showed similar peak migration to mouse CCL8 and CCL1 (Fig. 4a). Mouse *Ccr8* transfectants showed robust and specific calcium flux to mouse CCL8, in a dose-dependent manner (Fig. 4b,c). Signaling induced by mouse CCL8 inhibited subsequent signaling by mouse CCL1, reflecting mouse CCL8–induced desensitization to mouse CCR8 receptor (Fig. 4d). Likewise, stimulation of mouse *Ccr8*–transfected Baf/3 cells with mouse CCL1 inhibited subsequent mouse CCL8 signaling. Thus, mouse CCL1 and CCL8 are specific mouse CCR8 agonists. Human *CCR8* transfectants migrated to mouse CCL8, albeit more weakly than to human CCL1 (I-309) (Fig. 4e). Mouse CCL8 induced specific calcium flux in human *CCR8*–transfected cells and inhibited subsequent signaling by mouse CCL8 (Fig. 4f). In contrast, human CCL8 did not induce calcium flux or migration of human *CCR8*–transfected cells (Fig. 4g and Supplementary Fig. 4b). Human CCL8 did not inhibit signaling by either mouse CCL8 or human CCL1 (Fig. 4g), confirming

that human CCL8 was not a human CCR8 agonist, consistent with a prior study[15]. Finally, mouse CCL8 and human CCL1 cross-inhibited signaling in human *CCR8*–transfected cells (Fig. 4h). Collectively, these data establish mouse CCL8 as a specific agonist of mouse and human CCR8.

Ccr8 is required for a model of atopic dermatitis

We next addressed the *in vivo* relevance of the mouse CCL8-CCR8 pathway in a model of chronic cutaneous T_H2 inflammation in which mice underwent three 1-week periods of epicutaneous sensitization with ovalbumin (OVA) at 2-week intervals for a total 7-week period of sensitization[25,26] (Fig. 5). Gentle tape stripping at the onset of each 1-week round of sensitization induces cutaneous T_H2 inflammation in this model[25,26]. OVA-sensitized wild-type mice developed peak inflammation at day 50, 1 d after completion of the third round of topical sensitization, when they manifested cutaneous hyperplasia characterized by a leukocytic infiltrate consisting of eosinophils and $CD3^+$ T cells[25,26] (Fig. 5a,d). In contrast, *Ccr8*$^{-/-}$ mice were protected from chronic allergic skin inflammation (Fig. 5a,d). *Ccl8*$^{-/-}$*Ccl12*$^{-/-}$ mice were also protected from chronic allergic skin inflammation (Fig. 5b,e), whereas *Ccl12*$^{-/-}$ mice were not (Fig. 5c,e), suggesting a crucial role for the CCR8 ligand CCL8 in this model. Additionally, *Ccr4*$^{-/-}$ mice developed atopic dermatitis similar to wild-type mice (Fig. 5c,e), and a monoclonal antibody blockade of the other CCR8 ligand CCL1 during the final week of OVA sensitization did not abrogate cutaneous atopic inflammation (Fig. 5f,g). Thus, the mouse CCL8-CCR8 pathway is essential for promoting chronic cutaneous eosinophilic T_H2 inflammation.

CCR8 regulates eosinophil chemokine expression in skin

Less *Ccl11* (eotaxin-1) expression was induced in the skin of allergen-sensitized *Ccr8*$^{-/-}$ mice compared to wild-type mice (Fig. 6a,b), and the induction of *Ccl24* (eotaxin-2) expression seen in wild-type mice was abolished in *Ccr8*$^{-/-}$ mice (Fig. 6a,b). Locally recruited T_H2 cells are the source of T_H2 cytokines in this model[25]. *Il4* and *Il13* expression were similarly induced in OVA-sensitized skin of wild-type and *Ccr8*$^{-/-}$ mice relative to PBS-sensitized controls, as were interferon (IFN)-γ (*Ifng*) and *Il17a* (Fig. 6c and Supplementary Fig. 5). However, *Il5* induction was significantly decreased in *Ccr8*$^{-/-}$ mice ($P = 0.006$). IL-5 is crucial for eosinophil survival and recruitment to peripheral tissue[19]. Fewer infiltrating $CD3^+$ T cells were evident in the skin of OVA-sensitized *Ccr8*$^{-/-}$ mice compared to wild-type mice (Fig. 5d), but these were sufficient for local *Il4* induction. OVA-specific IgE induction is IL-4-dependent in this model[25], and IgE induction was intact in *Ccr8*$^{-/-}$ mice (Fig. 6d). However, OVA-specific IgG_1 antibody production was impaired in *Ccr8*$^{-/-}$ mice, consistent with reports of IL-5–dependent IgG_1 class-switching[19]. Thus, the absence of CCR8 led to impaired local recruitment of a subset of IL-5–producing T_H2 cells after allergen rechallenge. The specific defect in eosinophil recruitment and IL-5 induction in sensitized *Ccr8*$^{-/-}$ mice also led us to measure local IL-25 production (Fig. 6c). IL-25 is an IL-17–family cytokine important in T_H2 inflammation[18,20]. Indeed, *Il25* was induced in OVA-sensitized skin of wild-type but not *Ccr8*$^{-/-}$ mice (Fig. 6c).

Topical allergen induces local expression of mouse CCL8

We next compared expression of CCR8 ligands in atopic skin to that of chemokine ligands implicated in T cell migration to the skin through the receptors CCR4 and CCR10 (Fig. 6b,e–h). Mouse CCL8 was the only T cell–attracting chemokine induced in allergen-sensitized skin recovered from both wild-type and *Ccr8*$^{-/-}$ mice (Fig. 6b,e–h). We confirmed the induction of mouse CCL8 protein by immunofluorescence staining. More mouse CCL8 protein was detected in epidermal keratinocytes of wild-type mice compared to *Ccr8*$^{-/-}$ mice (Fig. 6f). Levels of transcripts of the known CCR8 ligand mouse CCL1

(Fig. 6e), the CCR4 ligands CCL17 and CCL22 (Fig. 6g), and the CCR10 ligand CCL27 (Fig. 6h) obtained at the same time were not increased, consistent with published reports[26].

OVA-specific IL-5$^+$ T cells accumulate in *Ccr8*$^{-/-}$ DLNs

We obtained DLN cells on day 50 at the time of peak skin inflammation. T cells obtained from *Ccr8*$^{-/-}$ DLNs produced more IL-5 but equivalent IL-4 after OVA stimulation *ex vivo* compared to T cells obtained from wild-type DLNs (Fig. 6i). In contrast, IL-5 levels were equivalent after anti-CD3 activation of DLN T cells. On day 50, after completion of the third round of antigen sensitization, activated and differentiated effector T cells should have acquired migratory capabilities enabling exit from DLNs and entry into inflamed skin. Thus, antigen-specific effector T cells producing IL-5, but not IL-4, accumulated in the DLNs of *Ccr8*$^{-/-}$ mice, with a corollary reduction in skin inflammation compared to wild-type mice.

CCR8 and mouse CCL8 mediate T_H2 cell homing *in vivo*

We compared the *in vivo* trafficking of adoptively transferred antigen-specific wild-type and *Ccr8*$^{-/-}$ T_H2 cells into OVA-sensitized skin and DLNs (Fig. 7a,b). We detected more wild-type T_H2 cells than *Ccr8*$^{-/-}$ T_H2 cells in skin and DLNs 4 d after topical OVA rechallenge. This was reflected by homing indices of 2.8 ± 0.32 and 2.2 ± 0.29 for wild-type T_H2 cells to skin and DLNs, respectively. In contrast, proliferation of antigen-specific wild-type and *Ccr8*$^{-/-}$ T_H2 cells *in vivo* was similar, as measured by bromodeoxyuridine (BrdU) uptake (Fig. 7c). Because mouse CCL8 was abundantly detected in sensitized skin (Fig. 6) and DLNs (Fig. 7d) and the other CCR8 ligand CCL1 was not, we concluded that mouse CCL8 is the relevant CCR8 ligand mediating the trafficking advantage of wild-type T_H2 cells *in vivo*. We saw no differences in recruitment to spleens in sensitized mice, where we did not detect mouse CCL8 after sensitization or at baseline (Fig. 7d). In contrast to T_H2 cells, CCR8 in T_H1 cells did not have a role in recruitment to the skin or DLNs (Fig. 7a,b), indicating specificity of CCR8 trafficking for T_H2 cells. Notably, we did not see a CCR8-dependent difference in the trafficking of CD11c$^+$ major histocompatibility complex class II$^+$ antigen-presenting cells to skin-draining lymph nodes after tape-stripping and epicutaneous sensitization (Supplementary Fig. 6). We also did not see a difference in baseline frequencies of Langerhans cells or γδ T cells in the epidermis, or myeloid or plasmacytoid DCs in the total skin, of *Ccr8*$^{-/-}$ or *Ccl8*$^{-/-}$*Ccl12*$^{-/-}$ mice compared to wild-type mice (Supplementary Fig. 7).

Mouse CCL8 is abundant in skin lymph nodes

We compared baseline expression of the two CCR8 ligands in lymph nodes from various organs (Fig. 7e). Mouse *Ccl1* was not detected in any lymph nodes examined, whereas *Ccl8* was present in cervical, mediastinal and skin-draining lymph nodes at concentrations comparable to that of the CCR7 ligand *Ccl21*, which is known to be important for lymph node homing. Notably, *Ccl8* was not detected in mesenteric or gastric lymph nodes, indicating organ specificity of the mouse CCL8-CCR8 lymphoid homing axis. Lastly, we were unable to detect *Ccl1* mRNA in normal mouse tissue by quantitative real-time PCR (QPCR), in contrast to the detectable amounts of *Ccl8*, which was found primarily in the skin and lymphoid tissue outside the gastrointestinal tract (Fig. 7e,f).

Mouse CCL8-responsive T_H2 cells are enriched for IL-5

Intracytoplasmic cytokine staining (ICS) revealed that wild-type T_H2-R2A cells that expressed CCR8 and responded to mouse CCL8 produced abundant IL-5 compared to IL-4 (Fig. 8a). We observed a similar profile of T_H2 cytokine production in *Ccr8*-deficient T_H2-R2A cells (Supplementary Fig. 8). Transcripts of the T_H2-regulatory transcription factor *Gata3* were more abundant in T_H2-R2 cells than in T_H2-R1 cells (Fig. 8b). Differential

GATA3 levels provide a rationale for the known intrinsic properties of more-differentiated T_H2 cells, such as ease of effector cytokine production[27] and, we propose, ease of CCR8 expression.

We examined T_H2-R2A cells for expression of mRNA encoding transcription factors and markers of T_H2 differentiation. We observed a correlation between expression of *Ccr8* and *Tnfrsf4* (OX40; Fig. 8c). OX40L-expressing dendritic cells induce the generation of TNF-producing inflammatory T_H2 cells[21]. T_H2-R2A cells produced high levels of TNF but not other T_H2- associated cytokines, such as IL-9 (Fig. 8d). Notably, mouse CCL8–responsive T_H2-R2A cells were enriched for *Il5*, *Il17rb* (IL-25R), *Tnfrsf4* (OX40), *Tnf* and *Il1rl1* (interleukin-1 receptor-like 1, also known as IL-33R and T1/ST2) mRNA compared to CXCL12- responsive T_H2 cells in Transwell migration assays (Fig. 8e). Further supporting these *in vitro* observations, significantly less *Tnf* and *Tnfrsf4* was evident in OVA-sensitized skin of *Ccr8*-deficient mice relative to wild-type mice in the model of chronic atopic dermatitis (Fig. 8f).

We also examined fresh human peripheral blood $CD4^+$ T cells from healthy individuals for T_H2 cytokine production by ICS. $CCR8^+CD4^+$ T cells produced markedly more IL-5 than did total bulk $CD4^+$ T cells (Fig. 8g). Circulating steady-state $CCR8^+CD4^+$ T cells also produced more IL-5 than IL-4, a pattern shared by IL-25R– and IL1RL1-expressing $CD4^+$ T cell subsets and opposite to the IL-4–predominant T_H2 cytokine expression profiles of $CCR4^+$ and $CRTH2^+CD4^+$ T cell subsets (Fig. 8h). Moreover, very little IL-5 was produced *ex vivo* by $CCR4^+CD4^+$ T cell subsets. Of note, in healthy individuals, $CCR8^+IL\text{-}5^+CD4^+$ T cells also did not produce IL-13 (data not shown).

DISCUSSION

We have described here the functional characterization of mouse CCL8 and established it as a second mammalian agonist for the chemokine receptor CCR8. We showed that mouse CCL8 was constitutively expressed in the skin and lymph nodes of normal mice in a pattern distinct from that of other related chemokines, reflecting its unique role in regulating immune responses. We determined that recombinant mouse CCL8 was chemotactic for activated, highly differentiated T_H2 (T_H2-R2A) cells specifically through CCR8, and that these cells were enriched for IL-5 and IL-25R, cytokines implicated in eosinophilic inflammation. $CCR8^+$ T_H2 cells were also enriched for OX40 and TNF. The absence of CCR8 or mouse CCL8 was protective, whereas the absence of CCR4 had no impact in a model of chronic allergic dermatitis. Adoptive transfer studies indicated that the mouse CCL8-CCR8 chemokine pathway mediates T_H2 cell recruitment to inflamed atopic skin *in vivo*. Examination of CCR8 on peripheral blood T cells in healthy humans showed that $CCR8^+CD4^+$ T cells were enriched in IL-5 in contrast to $CCR4^+CD4^+$ T cells, which were enriched in IL-4 but not IL-5.

The restricted pattern of CCR8 expression in antigen-activated, highly differentiated T_H2 cells, which abundantly produce IL-5 compared to less differentiated T_H2 cells, may be a result of coordinate epigenetic modification of gene loci in these cells. Multiple rounds of polarization enable dual TCR- and IL-4R–activated transcriptional networks to induce epigenetic modifications of the *Il4* locus, allowing rapid transcription of effector cytokines upon antigen rechallenge[27]. Epigenetic modifications of the adjacent *Il4* and *Il13* genes occur simultaneously; in contrast, the *Il5* gene is located further away and transcribed in an opposite orientation and is thus regulated differently by the transcription factor GATA3 (refs. 28,29). CD28 co-stimulation also regulates epigenetic modification and enhanced induction of IL-5 (refs. 28,30), but not IL-4 or IL-13. Transcriptional networks that regulate epigenetic modification of *Il5* may regulate the loci encoding CCR8 and IL-25R. Supporting

this hypothesis, a recent study using chromatin immunoprecipitation sequencing to study the genome-wide epigenetic effects of STAT4 and STAT6 transcription factor binding during T helper cell differentiation found that *Ccr8* is in a rare category of genes containing both STAT4-repressive and STAT6-activating binding sites[31]. Thus, successive rounds of polarization would enable removal of the inhibitory regulation of STAT4 on *Ccr8* expression, enabling easier STAT6 induction of *Ccr8* expression in more terminally differentiated T_H2 cells.

The role of CCR8 in T_H2 cell migration has been controversial. Our study sheds light on this controversy by highlighting stringent requirements for functional CCR8 expression on T_H2 cells *in vitro*. *In vivo* models have also yielded conflicting results regarding the importance of CCR8 in acute models of allergy and T_H2 airway inflammation[32-34]. However, CCR8-expressing effector T_H2 cells were shown to have a role in a 46-d mast cell–dependent model of chronic allergic airway inflammation[35]. The relative importance of CCR8-regulated responses in different models of allergic airway inflammation may depend on the kinetics of *in vivo* antigen-specific memory T cell generation[36]. Shorter-term acute models may not capture the differentiation and reactivation of $CCR8^{hi}$ highly differentiated T_H2 cells.

Our findings point to a link between eosinophils and $CCR8^+$ T_H2 cells in chronic T_H2 inflammation, as sensitized $Ccr8^{-/-}$ mice have fewer local eosinophils and less local induction of IL-5, IL-25 and the chemokines CCL11 and CCL24. A role for eosinophils in the activation and proliferation of antigen-specific T cells in secondary but not primary immune responses has been observed[37]. A recent study proposed that eosinophils critically regulate T_H2 responses during pulmonary allergen challenge[38]. Eosinophil-derived IL-25 also promotes IL-4–independent activation and effector functions of IL-25R–expressing memory and effector T_H2 cells[20]. IL-25R expression is also enhanced in CD3- and CD28-activated, antigen-experienced human memory T_H2 cells compared to *in vitro* T_H2 effector cells generated by one round of polarization[20]. Our findings lead us to propose that eosinophil-derived IL-25 and IL-25R–expressing $CCR8^+$ T_H2 cell–derived IL-5 enable reciprocal cross-regulation of eosinophils and T_H2 cells to amplify local secondary allergic immune responses.

Our studies suggest that $CCR8^+$ T_H2 cells, by producing TNF, are strongly proinflammatory. TNF-expressing T_H2 cells have been called ‘inflammatory’ T_H2 cells and are generated by OX40L-expressing dendritic cells[21]. Mouse CCL8–responsive and CCR8-expressing T_H2 cells in our study were enriched for OX40. OX40-expressing $CCR8^+$ allergen-specific T_H2 cells may be more likely to persist and promote recurrent disease, as OX40 signaling prevents apoptosis and promotes long-term survival of activated T helper cells[39]. This survival advantage may be reinforced by a second anti-apoptotic pathway involving the autocrine production of CCL1 by $CCR8^+$ activated T_H2 cells[40].

As in many other chemokine systems, the relative role of two CCR8 ligands must now be considered when interpreting *in vivo* models of inflammation and disease, and their respective roles are likely to depend on differential regulation *in vivo*. In the model of atopic dermatitis studied here, mouse CCL8 was induced in the skin to levels 2 logs higher than that of mouse CCL1, which was not induced. Moreover, mouse *Ccl8* deficient–deficient mice mirrored the defects evident in *Ccr8*-deficient mice, suggesting that mouse CCL8 was the relevant CCR8 ligand. This conclusion was confirmed by our studies using a CCL1-blocking antibody, which had no influence on the model. In a model of chronic allergic pulmonary inflammation, a neutralizing antibody to mouse CCL1 produced a partial reduction of airway hyperresponsiveness, leading the authors to speculate on the existence of an unidentified CCR8 ligand[35]. In fact, considerable effort has been expended to identify

a second CCR8 chemokine ligand. Putative CCR8 ligand assignments have been made, only to be refuted in follow-up studies[41,42]. Our data convincingly show that mouse CCL8 is the long-sought-after second mouse CCR8 chemokine ligand and is the crucial ligand that mediates CCR8-dependent T_H2 cell trafficking into skin to drive allergic skin inflammation.

The MCP chemokine cluster is not orthologous across the human and mouse genomes. Although human CCL8 is a CCR2 agonist like the other MCPs, we have found that mouse CCL8 is not a CCR2 agonist. Mouse CCL8 did not induce chemotactic activity in BMDMs, CCR2 transfectants or NK cells, all of which responded to the CCR2 agonist CCL2. We also found that human CCL8, in contrast to mouse CCL8, is not a functional CCR8 ligand. Thus, although mouse CCL8 was given the designation orthologous to human CCL8 in the database, our findings establish that they are not orthologous chemokines. However, the mouse and human CCR8 receptors are orthologs, and our findings do not rule out the possibility that there is a second human CCR8 ligand.

Our study expands the current paradigm of T_H2 inflammation by showing that T_H2 cells can be further stratified based on production of IL-5 and IL-4. We propose that $CCR8^+$ T_H2 cells are an IL-5–enriched subset associated with chronic allergic eosinophilic inflammation, whereas $CCR4^+$ T_H2 cells are an IL-4–enriched subset associated with acute IgE-driven allergic inflammation. In support of this concept, we found that blood $CCR8^+CD4^+$ T cells were enriched in IL-5, whereas $CCR4^+CD4^+$ T cells produced abundant IL-4 and very little IL-5. *Ccr8*$^{-/-}$ mice had diminished IL-5–driven inflammation, independent of IgE production. Two types of allergic inflammation have been described in humans and in mice: an IL-4– and IgE-dependent inflammation, and an IL-5– and eosinophil-dependent inflammation[43]. Notably, a recent human study found that anaphylactic peanut allergy is associated with a dominant $IL\text{-}5^-IL\text{-}4^+$ allergen-specific T_H2 cell type associated with IgE responses[44]. Conversely, a dominant $IL\text{-}5^+IL\text{-}4^+$ food allergen–specific T_H2 cell type is associated with allergic eosinophilic gastroenteritis that is less IgE dependent[44]. Our study provides a relevant parallel to these human studies and provides functional relevance for the reported observation in humans that more CCR8-expressing cells are found in the inflamed skin of individuals with active atopic dermatitis relative to normal skin of healthy individuals[8].

Our findings establish that the diminished atopic inflammation in the skin of *Ccr8*-deficient mice is a result of a trafficking defect and not the result of decreased functionality of *Ccr8*-deficient T cells or compromised trafficking or functionality of antigen-presenting cells. Our *in vivo* and *in vitro* data show that differentiation and effector function of *Ccr8*-deficient T_H2 cells were equivalent to wild-type T_H2 cells. In competitive *in vivo* homing assays, we detected fewer *Ccr8*-deficient T_H2 cells compared to wild-type T_H2 cells in inflamed skin and DLNs, and we determined that these cells had an equivalent *in vivo* expansion capacity. Thus, the mouse CCL8-CCR8 homing axis directs T_H2 cell migration to skin and DLNs during cutaneous allergic inflammation.

In conclusion, we define mouse CCL8 as an agonist of CCR8 and show a mouse CCL8-CCR8–directed pathway of T_H2 cell trafficking to the skin in chronic allergic inflammation. We propose a model in which IL-5– and IL-25R–enriched, CCR8-expressing, highly differentiated inflammatory T_H2 cells synergize with locally recruited eosinophils to amplify chronic T_H2 inflammation.

ONLINE METHODS

Mice

C57BL/6 mice congenic for Thy1.2 and Thy1.1 were from the National Cancer Institute and The Jackson Laboratory, respectively. *Ccr4*$^{-/-}$ and *Ccr5*$^{-/-}$ mice on a C57BL/6 background were from The Jackson Laboratory. *Ccr8*$^{-/-}$, *Ccl12*$^{-/-}$, *Ccl8*$^{-/-}$ *Ccl12*$^{-/-}$ and *Ccr2*$^{-/-}$ mice were generated and backcrossed onto the C57BL/6 background as described and bred in our facility[32,45]. Thy1.1 and Thy1.2 OTII mice on a C57BL/6 background transgenic for the TCR recognizing the OVA(323–339) peptide and *Il5*-transgenic mice were gifts from P. Shrikant, L. Lefrancois and C. Sanderson, respectively. *Ccr8*$^{-/-}$ OTII and Thy1.1^{+} × Thy1.2^{+} mice were bred in our laboratory[34,46]. BALB/c mice and DO11 mice transgenic for the TCR recognizing OVA(323–339) on a BALB/c background were from The Jackson Laboratory. Mice were housed under specific pathogen-free conditions. All protocols were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Human studies

Peripheral blood was obtained from healthy volunteers, and fresh buffy coats were obtained from the Massachusetts General Hospital blood bank. All human subject protocols were approved by the Institutional Review Board. Informed consent was obtained from all study participants.

EST database search for mouse *Ccl8* and *Escherichia coli* expression system

An amino acid sequence BLAST search was conducted to identify sequences homologous to human CCL13. The putative *Ccl8* sequence was identified as an EST encoding a mouse CC chemokine with homology to the MCP genes. The predicted mouse *Ccl8* open reading frame with the N-terminal glycine was expressed and purified from *E. coli* (PeproTech).

RNA hybridization and QPCR analysis

RNA hybridization and QPCR analysis were done as described[46]. Sequences of all validated QPCR primers used are provided in Supplementary Table 1. QPCR data (with the exception of Fig. 3a) were normalized with β2-microglobulin expression defined as 100.

Preparation of primary BMDMs, eosinophils, NK cells and lymph node T cells

Bone marrow cells were cultured in medium supplemented with mouse macrophage colony-stimulating factor to generate BMDMs. Eosinophils were isolated from the spleens of *Il5*-transgenic mice by negative selection (Miltenyi). Spleen NK and lymph node T cells were obtained by magnetic bead purification (Miltenyi).

Generation of T_H1-R1, T_H2-R1, T_H2-R2 and Th2-R2A cells

T_H1-R1 and T_H2-R1 cells were generated from CD4^{+} and CD4^{+}CD62L^{+} T cells isolated from spleens and lymph nodes of mice as described[46]. T_H2-R2 cells were generated by repolarizing T_H2-R1 cells with irradiated splenocytes, OVA(323–339) peptide, or antibodies to CD3, CD28, IL-4 or IFN-γ. T_H1-R1, T_H2-R1 and T_H2-R2 cells were rested for 4 h in low–IL-2 medium before migration assays. T_H2-R2A cells were generated from T_H2-R2 cells by activating with 2 μg/ml plate-bound antibody to CD3 (145.2C11; eBioscience) and 1 μg/ml soluble antibody to CD28 (37.51; eBioscience) for 24 h. Cells were then washed, replated and rested for 5–6 h in low–IL-2 medium for migration or IL-2–free medium for calcium flux. For *in vivo* homing, naive cells from *Ccr8*$^{-/-}$ OTII and Thy1.1 OTII mice were differentiated under T_H1- and T_H2-polarizing conditions as above.

Chemotaxis assays

BMDMs, T cells, NK cells, *Ccr8*- and *Ccr2*-transfected cells, and nontransfected cells (2.5×10^4 per well) and eosinophils (1×10^6 per well) in RPMI containing 0.5% BSA were placed on the top of a 96-well ChemoTx chemotaxis apparatus with 5-μm pores (Neuro Probe) as described[46]. Mouse CCL8, mouse CCL2, mouse CCL22, mouse CXCL12 and human CCL8 were obtained from PeproTech; mouse CCL1 and human CCL1 were obtained from R&D Systems. The number of cells migrating at each concentration of chemokine was normalized to the number of cells migrating in the presence of medium alone (chemokinesis) to calculate the chemotactic index for each leukocyte type. PTX (Sigma) or polyclonal goat antibody to mouse CCR8 (Abcam) were used in some assays. For some experiments, 5×10^5 T_H2-R2A cells were placed in the upper chambers of Transwell plates (polycarbonate membrane with 5-μm pore size and 12-mm diameter; Costar), and 600 μl of chemokine or medium was placed in the lower chamber.

Calcium flux assays

Calcium flux was analyzed on a UV laser-equipped 13-color LSR II flow cytometer as described[47]. Indo-1 fluorescence was analyzed with the UV-A detector at 530/30 and UV-B detector at 440/40 for free and bound probe with Indo-1 (blue) and Indo-1 (violet), respectively. Data were analyzed with standardized settings using FlowJo 8.8.

Intracytoplasmic cytokine staining

Mouse intracytoplasmic staining was done using a standard protocol[46]. Cells were stained with FITC-conjugated antibody to IFN-γ (XMG1.2; BD Pharmingen) and either phycoerythrin (PE)- or allophycocyanin (APC)-conjugated antibody to IL-4 (BVD4-1D11; BD Pharmingen), FITC-conjugated antibody to IL-4 (BVD6-24G2; eBioscience), APC-conjugated antibody to IL-5 (TRFK5; eBioscience), PE-conjugated antibody to TNF (TN3-19.12; BioLegend), or PE-conjugated antibody to IL-9 (RM9A4; BioLegend). All cells were also stained with isotype-control fluorochrome-conjugated IgG_1 (R3-34; BD Pharmingen). Cells were acquired on a FACSCalibur and analyzed with FlowJo 8.8.

Chemokine receptor transfectants

The IMAGE cDNA clone for mouse *Ccr8* (clone id 40044993, accession no. BC103566) was obtained from Open Biosystems. cDNA was amplified using forward primer 5′-CGCAATAAGCTTATGGATTACACGATGGAGCCCAACGG-3′ (HindIII site underlined) and reverse primer 5′-GCGCGCGAATTCTTACAAGATGTCATCCAGGGTGGAAGAATGGG-3′ (Eco R1 site underlined) and subcloned into pcDNA 3.1. Baf/3 mouse pro–B cell lymphoma cells were transfected using the Amaxa nucleofection system (Lonza) to generate stable *Ccr8*-transfected cells. *Ccr2* receptor cloned into pcDNA3 (provided by I.F.C.) was transiently transfected into Baf/3 cells.

Mouse model of atopic dermatitis

Atopic dermatitis was induced in mice as described[25]. Briefly, shaved back skin was stripped with 3M scotch tape and epicutaneously treated with 100 μg of OVA (grade V; Sigma) or PBS placed on a 1×1 cm^2 patch of sterile gauze secured with a bio-occlusive dressing (Tegaderm). Each mouse received three 1-week periods of epicutaneous sensitization at 2-week intervals for a total 7-week period of sensitization. Specimens were obtained on day 50 for histology, ELISA and RNA analysis 24 h after the patch from the third sensitization was removed.

In vivo competitive homing

OVA-sensitized recipient Thy1.1 × Thy1.2 mice received OVA(323–339)-activated OTII Thy1.1 and *Ccr8*$^{-/-}$ OTII Thy1.2 T cells (1.5×10^7 and 1.5×10^7, respectively) on day 41. Twenty-four hours later, recipients were topically sensitized with OVA for 96 h. Skin, DLNs and spleens were then collected to quantify *in vivo* homing as described[34,46]. Cells were stained with FITC-conjugated antibody to Thy-1.1(OX-7), APC-conjugated antibody to Thy-1.2 (30-H12), PE-conjugated antibody to mouse CD4 (GK1.5) and the dead cell marker 7-aminactinomycin D (all from BD Pharmingen). *In vivo* proliferation of adoptively transferred cells was assayed by injecting mice with 2 mg of BrdU intraperitoneally 24 h before collection. Cells were subsequently stained with antibody to BrdU (BD Biosciences) after isolation for analysis.

In vivo CCL1 antibody inhibition

The monoclonal antibody to CCL1 (148113) and isotype control (54447) were obtained from R&D Systems. The antibody or isotype control (100 μg per mouse) was given intravenously twice during the final week (days 42 and 46) of epicutaneous sensitization, based on published studies[35,48,49].

Immunohistochemistry

H&E and immunofluorescence staining with polyclonal goat antibody to mouse CCL8 (R&D Systems) was done as described[7].

Characterization of human T_H2 subsets

Ex vivo characterization of cytokine production by human $CD4^+$ T cells was done as described[47]. Monoclonal antibody to human CCR8 (ref. 50) was purified from 433H hybridoma (ATCC) supernatants and biotinylated according to the protocol outlined by M. Roederer (http://www.drmr.com/abcon/). Antibodies to CCR4 (205410), CXCR5 (51505), CCR7 (150503) and IL-25R (170220) were from R&D Systems; antibody to T1/ST2 (DJ8) was from MD Biosciences; antibody to CRTh2 (BM16) was from Miltenyi; and FITC-conjugated antibody to human IL-4 (MP4-25D2) and APC-conjugated antibody to IL-5 (TRFK5) were from eBioscience.

ELISA

Day 50 DLN cells were resuspended at 1×10^6/ml and stimulated with OVA (100 μg/ml) or plate-bound antibody to CD3 (1 μg/ml) for 96 h. IL-5 and IL-4 concentrations in the supernatant were measured with a sandwich ELISA from BioLegend.

Statistics

Statistical analysis was carried out using Student's two-tailed *t*-test (unpaired) for means. $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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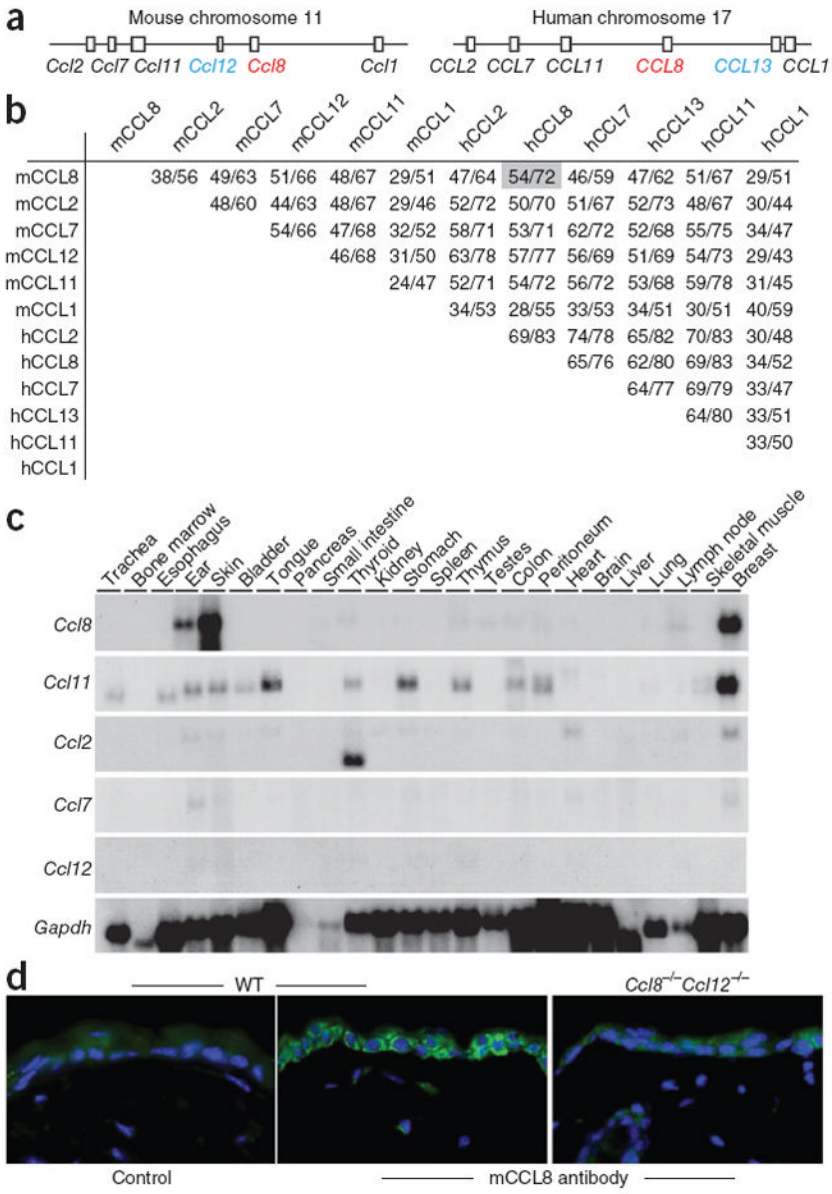

Figure 1.

Mouse CCL8 RNA and protein are detected in normal mouse skin. (**a**) Relative positioning of the six MCP-cluster chemokine genes on human chromosome 17 and mouse chromosome 11, modified from the Ensembl site. Black, known orthologs; blue, non-orthologs; red, presumed orthologs (focus of our study here). (**b**) Percent identity/percent similarity of amino acids using the GenBank sequence extending from the start codon to the stop codon, calculated using the EMBOSS Needleman-Wunsch algorithm. m, Mouse; h, human. (**c**) RNA hybridization blot comparing mRNA expression of MCP-family chemokines and CCL11 (eotaxin-1) in pooled organs of normal BALB/c mice, conducted once. (**d**) Representative immunofluorescence staining of normal wild-type (WT) C57BL/6 mouse skin and *Ccl8*$^{-/-}$*Ccl12*$^{-/-}$ C57BL/6 mouse skin with a primary polyclonal antibody to mouse CCL8 and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Data are reflective of at least six independent experiments from three or more mice.

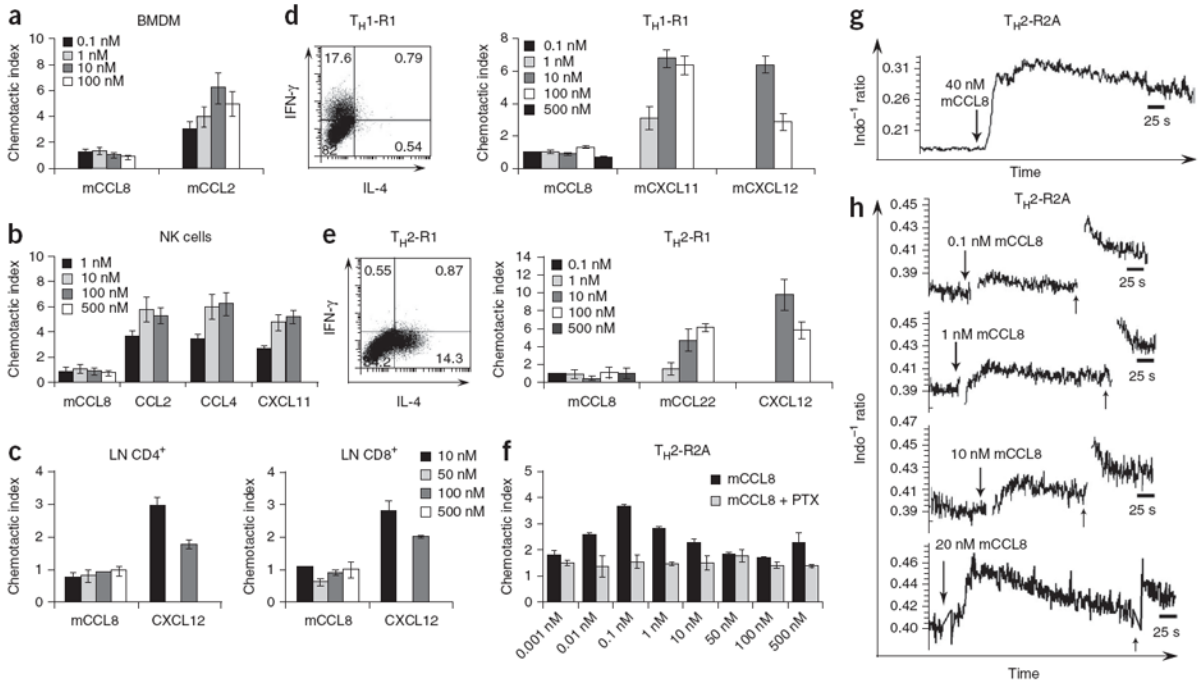

Figure 2.

Mouse CCL8 induces migration and calcium flux in T_H2-R2A cells. (**a**) Migration of mouse BMDMs to mouse CCL8 and CCL2. (**b**) Migration of NK cells to mouse CCL8, CCL2, CCL4 and CXCL11. (**c**) Migration of lymph node $CD4^+$ and $CD8^+$ T cells to mouse CCL8 and CXCL12. (**d**) Representative cytokine profiles and assays of T_H1-R1 cell migration to mouse CCL8. Migration to mouse CXCL11 and CXCL12 was a positive control. (**e**) Representative cytokine profiles and assays of T_H2-R1 cell migration to mouse CCL8. Migration to mouse CCL22 and CXCL12 was a positive control. Data in **a–e** are representative of three or more experiments. (**f**) Dose-response migration of T_H2-R2A cells to mouse CCL8 (representative of more than 10 experiments) and PTX-mediated inhibition of mouse CCL8–induced migration (one of three independent experiments shown). Results in **a–f** are shown as mean ± s.e.m. (**g**) Analysis of T_H2-R2A cells for calcium flux to 40 nM mouse CCL8 (downward arrow indicates time of addition); representative of eight separate experiments. (**h**) Analysis of dose-response of T_H2-R2A cells to mouse CCL8 in calcium flux assays; representative of two independent experiments. Arrows pointing up indicate onset of control calcium flux response to ionomycin (1 μg/ml) at each concentration of chemokine.

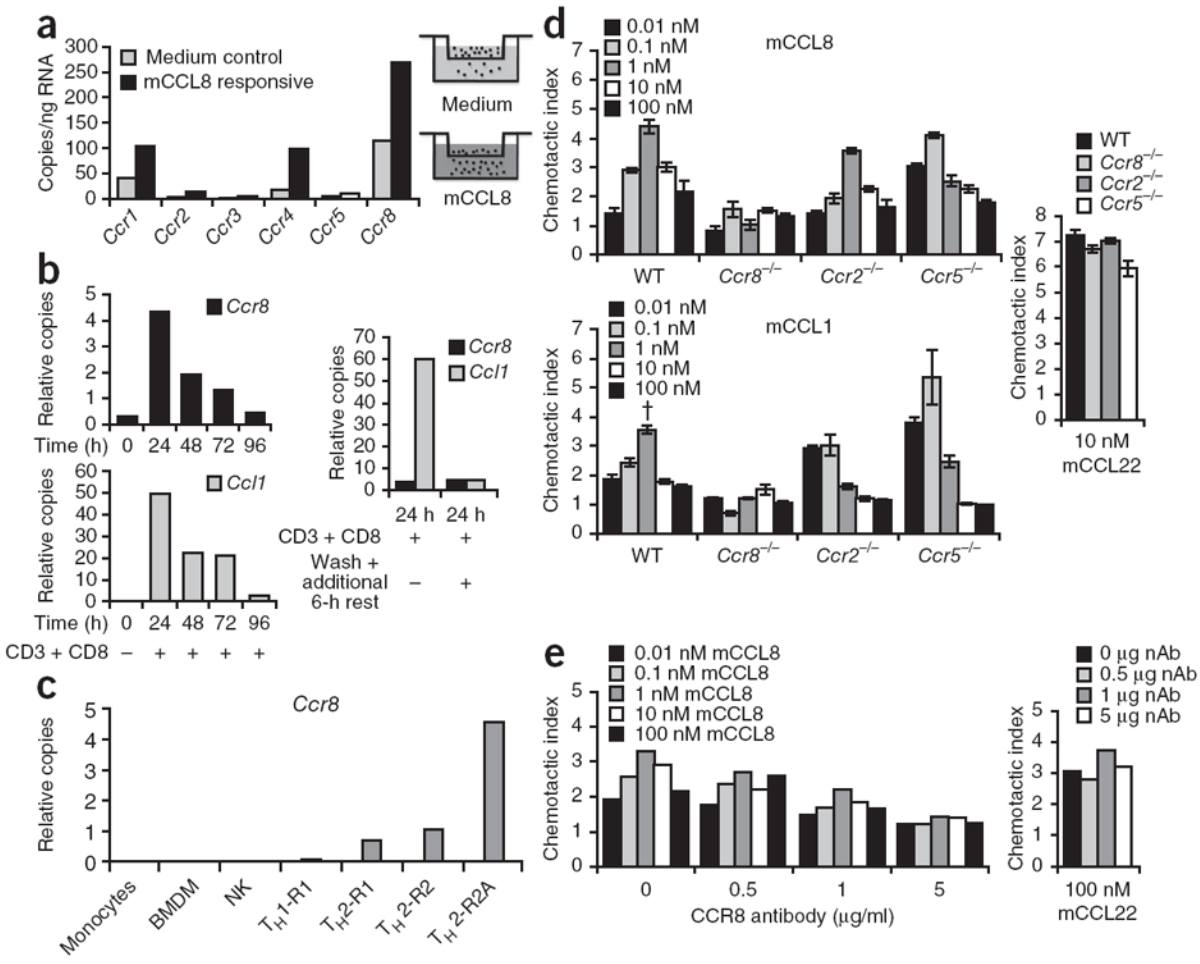

Figure 3.

CCR8 is required for mouse CCL8-induced T_H2-R2A cell migration. (**a**) CC-chemokine receptor mRNA enrichment measured by QPCR in T_H2-R2A cells that migrated to mouse CCL8 in Transwell assays relative to medium alone; representative of three experiments. (**b**) Kinetics of mouse *Ccr8* and *Ccl1* mRNA induction in T_H2-R2A cells generated by repeat rounds of polarization and subsequent activation with antibodies to CD3 and CD28. Data are normalized to β2-microglobulin and presented on a scale of 0 to 100; representative of three experiments. (**c**) Comparison of *Ccr8* mRNA expression in leukocyte subsets assayed for mouse CCL8 migration; representative of two to five experiments. (**d**) Comparison of chemotaxis of wild-type (WT), *Ccr8*-, *Ccr2*- and *Ccr5*-deficient T_H2-R2A cells to mouse CCL8 or mouse CCL1; control migration to the CCR4 agonist CCL22 is also shown. One of three experiments is shown. (**e**) Dose-dependent inhibition of wild-type T_H2-R2A cell migration to mouse CCL8 using a neutralizing polyclonal mouse CCR8 antibody (nAb), and migration to control CCL22 chemokine at various concentrations of antibody. One of two experiments is shown. Results in **d** and **e** are shown as mean ± s.e.m.

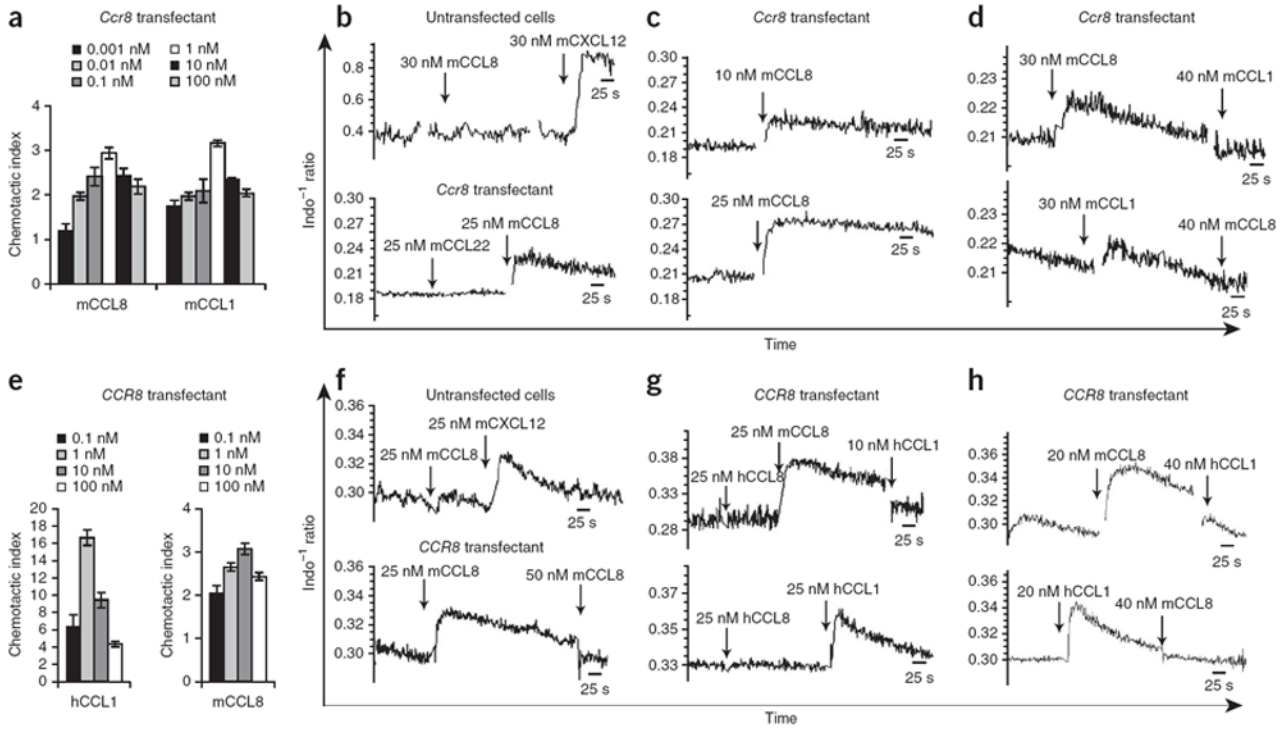

Figure 4.

Mouse CCL8 is a specific agonist of mouse and human CCR8. (**a**) Dose-response chemotaxis assay of mouse *Ccr8*–transfected Baf/3 cells to mouse CCL8 and CCL1. Untransfected Baf/3 cells migrated only to CXCL12 (data not shown). (**b**) Calcium flux of mouse *Ccr8*–transfected cells to mouse CCL8 but not to the CCR4 agonist mouse CCL22. Mouse CCL8 did not induce calcium flux of untransfected Baf/3 cells, but positive control CXCL12 did. (**c**) Dose-response calcium flux of mouse *Ccr8*–transfected cells to mouse CCL8. (**d**) Cross-desensitization of mouse *Ccr8*–transfected cells to mouse CCL8 and CCL1. (**e**) Dose-response chemotaxis assay of human *CCR8* receptor–transfected 4DE4 cells to mouse CCL8 and CCL1. Nontransfected 4DE4 cells did not migrate to either ligand (data not shown). (**f**) Specificity of mouse CCL8–induced calcium flux signaling in human *CCR8*–transfected cells, as shown by desensitization to repeat signaling by mouse CCL8; lack of flux of untransfected 4DE4 cells to mouse CCL8. (**g**) Human CCL8 did not induce calcium flux in *CCR8*-transfected cells. (**h**) Cross-desensitization of human *CCR8*–transfected cells by mouse CCL8 and human CCL1. Data are representative of at least three independent experiments (error bars (**a–e**), s.e.m).

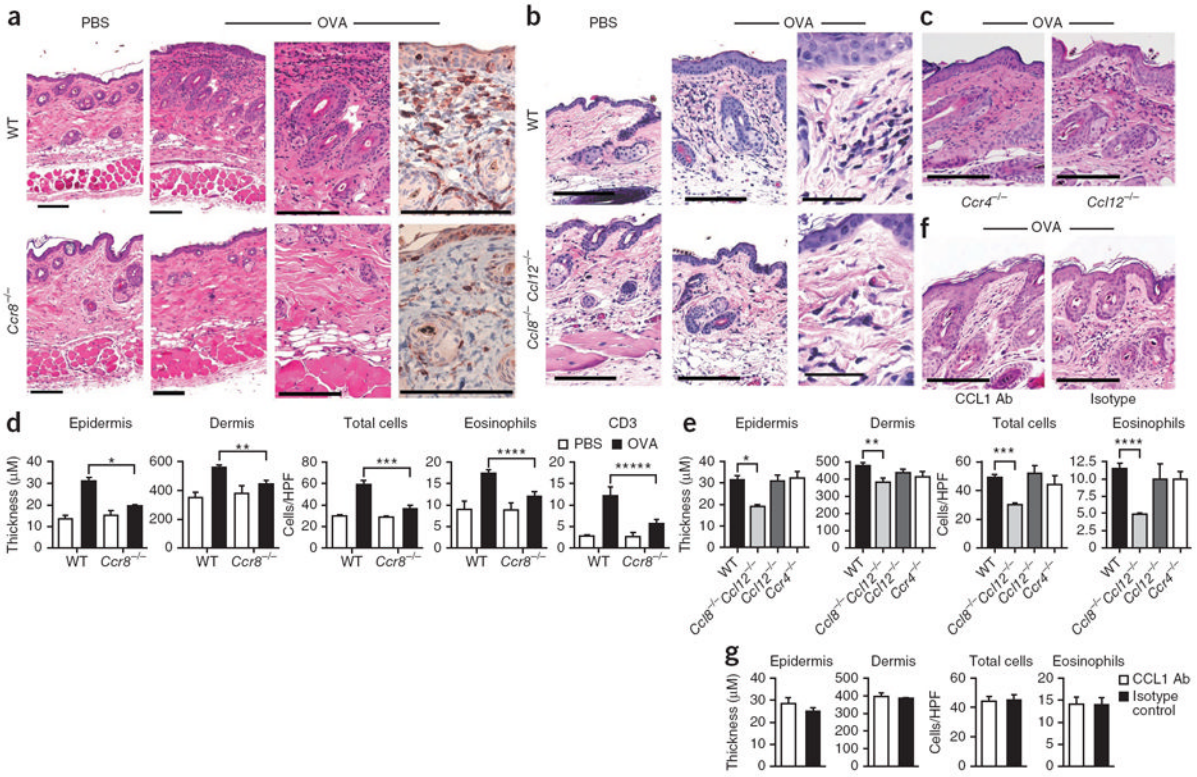

Figure 5.

Ccr8$^{-/-}$ and *Ccl8*$^{-/-}$*Ccl12*$^{-/-}$ mice have decreased skin inflammation in a model of chronic atopic dermatitis. Histological analysis of skin was done on day 50, 24 h after the last of three 1-week rounds of topical sensitization with PBS or OVA. (**a**) Hematoxylin and eosin (H&E) staining of wild-type (WT) and *Ccr8*$^{-/-}$ mice sensitized with PBS or OVA. Far right, immunohistochemical analysis of CD3^{+} T cells in sensitized skin of wild-type and *Ccr8*$^{-/-}$ mice. Data are representative of three to seven experiments. (**b**) H&E staining of wild-type and *Ccl8*$^{-/-}$*Ccl12*$^{-/-}$ mice sensitized with PBS or OVA. Far-right panels are at higher magnification, showing eosinophils and characteristic spongiosis of keratinocytes in wild-type sensitized mice; representative of three experiments. (**c**) H&E staining of OVA-sensitized *Ccr4*$^{-/-}$ and *Ccl12*$^{-/-}$ mice; representative of two experiments. (**d**) Skin thickness and leukocyte counts in wild-type and *Ccr8*$^{-/-}$ mice shown as mean ± s.e.m.; n = 6–8 mice per group. *P < 0.00001, **P = 0.004, ***P = 0.002, ****P = 0.02 and *****P = 0.005 for OVA-sensitized *Ccr8*$^{-/-}$ versus wild-type mice. HPF, high-powered field. (**e**) Skin thickness and leukocyte counts in OVA-sensitized wild-type, *Ccl8*$^{-/-}$*Ccl12*$^{-/-}$, *Ccl12*$^{-/-}$ and *Ccr4*$^{-/-}$ mice. *P = 0.0004, **P = 0.02, ***P < 0.00001 and ****P < 0.00001 for gene-deficient versus wild-type mice. Data are shown as mean ± s.e.m.; n = 6–9 mice per group. (**f**) H&E-stain of OVA-sensitized wild-type mice treated with CCL1-neutralizing antibody (left) or isotype control (right) during last week of epicutaneous sensitization. (**g**) Skin thickness and leukocyte counts in mice from **f**; n = 4–5 mice per group in two independent experiments. Scale bar = 100 μm in all photomicrographs except for far-right panels in **b**, where scale bar = 20 μm.

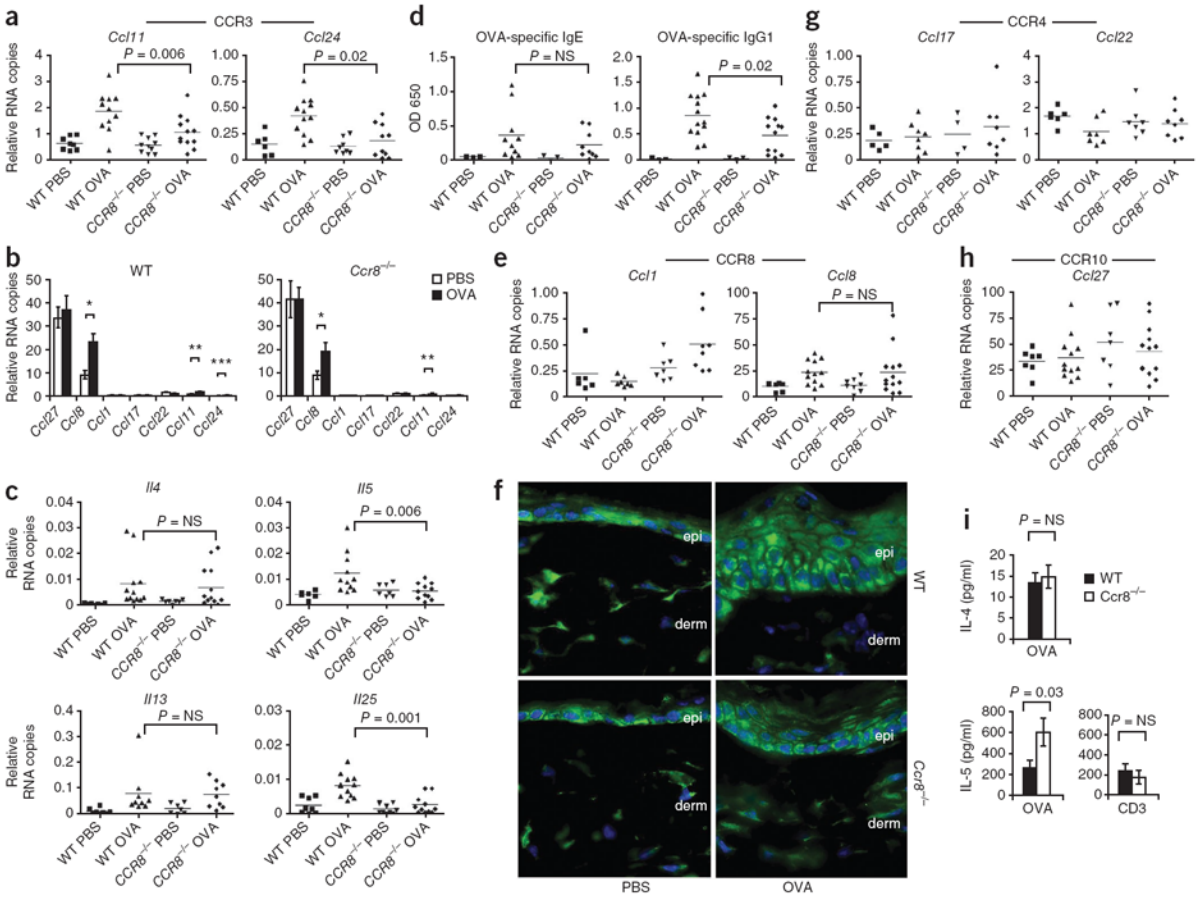

Figure 6.

Ccr8$^{-/-}$ mice have decreased production of IL-5, IL-25 and eosinophil-active chemokines in allergen-sensitized skin. (**a**) QPCR measurements of transcripts for eosinophil-attracting chemokines. (**b**) Summary of QPCR measurements of chemokine mRNA expression in skin biopsies of wild-type (WT; **P* = 0.01, ***P* = 0.0004 and ****P* = 0.003 for PBS versus OVA) and *Ccr8*$^{-/-}$ (**P* = 0.04 and ***P* = 0.03 for PBS versus OVA) mice after topical sensitization. (**c**) QPCR measurements of transcripts for T_H2 cytokines. NS, not significant. (**d**) Serum OVA-specific IgE and IgG_1 concentrations measured by enzyme-linked immunosorbent assay (ELISA) in sensitized mice. (**e**) QPCR of transcripts for CCR8 ligands. (**f**) Representative immunofluorescence analysis of mouse CCL8 protein expression in PBS-sensitized epidermis (epi) and dermis (derm) and OVA-sensitized epidermis and dermis of wild-type and *Ccr8*$^{-/-}$ mice from two experiments; *n* = 4 mice per group. (**g, h**) QPCR measurements of CCR4 and CCR10 ligands in skin biopsies of wild-type and *Ccr8*$^{-/-}$ mice after topical sensitization. (**i**) T_H2 cytokine production by DLN cells after *ex vivo* stimulation with OVA protein and CD3, measured by ELISA (*n* = 8–10 mice per group). Pooled data from three experiments are shown. Results in **b** and **i** are shown as mean ± s.e.m. All data were obtained 50 d after the initiation of sensitization and are reflective of at least three experiments, except for **f**.

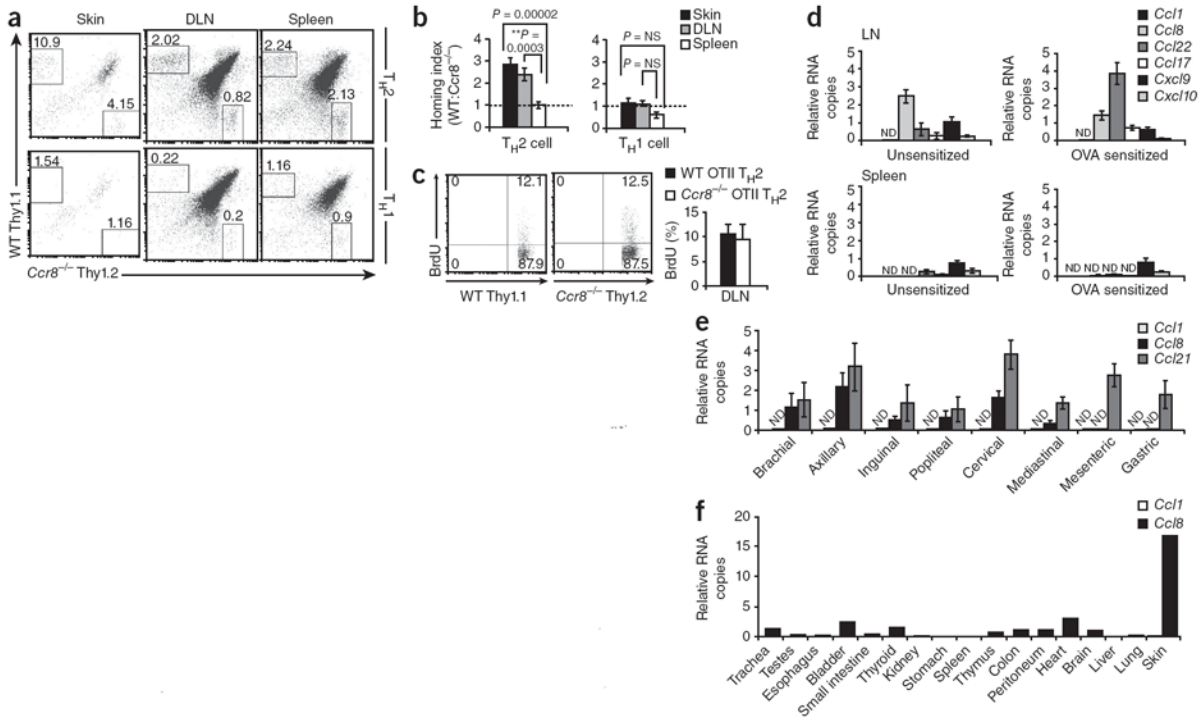

Figure 7.

Competitive *in vivo* homing of adoptively transferred OVA-specific wild-type and *Ccr8*$^{-/-}$ T_H2 and T_H1 cells in OVA-sensitized mice. OVA(323–339) peptide–specific TCR-transgenic (OTII) Thy1.1 and *Ccr8*$^{-/-}$ OTII Thy1.2 T_H2 cells were transferred into OVA-sensitized Thy1.1 × Thy1.2 mice on day 41. Separate mice received OTII Thy1.1 T_H1 cells and *Ccr8*$^{-/-}$ OTII Thy1.2 T_H1 cells. Twenty-four hours later, recipient mice underwent topical sensitization with OVA for 96 h, and sensitized skin, DLN and spleen cells were analyzed by flow cytometry. (**a**) $CD4^+$ T cells recovered from organs at time of collection. Top, mice that received T_H2 cells; bottom, mice that received T_H1 cells. (**b**) Homing index was calculated as the ratio of wild-type Thy1.1 to *Ccr8*$^{-/-}$ Thy1.2 cells, corrected for input ratio at time of transfer. Summary of competitive *in vivo* homing of wild-type and *Ccr8*$^{-/-}$ T_H2 (left; n = 13–16 mice) and T_H1 (right; n = 6 mice) cells from three or more experiments. (**c**) *In vivo* proliferation of transferred OTII Thy1.1 wild-type and OTII Thy1.2 *Ccr8*$^{-/-}$ T_H2 cells in response to antigen was assessed by injecting sensitized recipient mice with BrdU intraperitoneally 24 h before organ collection in experiments set up as above. Left and middle, representative BrdU staining of wild-type and *Ccr8*$^{-/-}$ T cells isolated from lymph nodes. Right, summary of three experiments comparing *in vivo* proliferation of wild-type and *Ccr8*$^{-/-}$ T cells measured by BrdU uptake; n = 12 mice. (**d**) Amounts of T_H1 cell–active (*Cxcl9* and *Cxcl10*) and T_H2–cell active (*Ccl1*, *Ccl8*, *Ccl17* and *Ccl22*) chemokine mRNA in unsensitized and OVA-sensitized wild-type day-50 skin DLNs and spleen; n = 6 mice. ND, not detected. (**e**) Comparison of steady-state mRNA expression of *Ccl1*, *Ccl8* and *Ccl21* in lymph nodes draining various organs in normal 6- to 8-week-old mice; pooled data from n = 3–7 mice are shown as mean ± s.e.m. (**f**) *Ccl1* and *Ccl8* mRNA expression in organ library of representative C57BL/6 mouse.

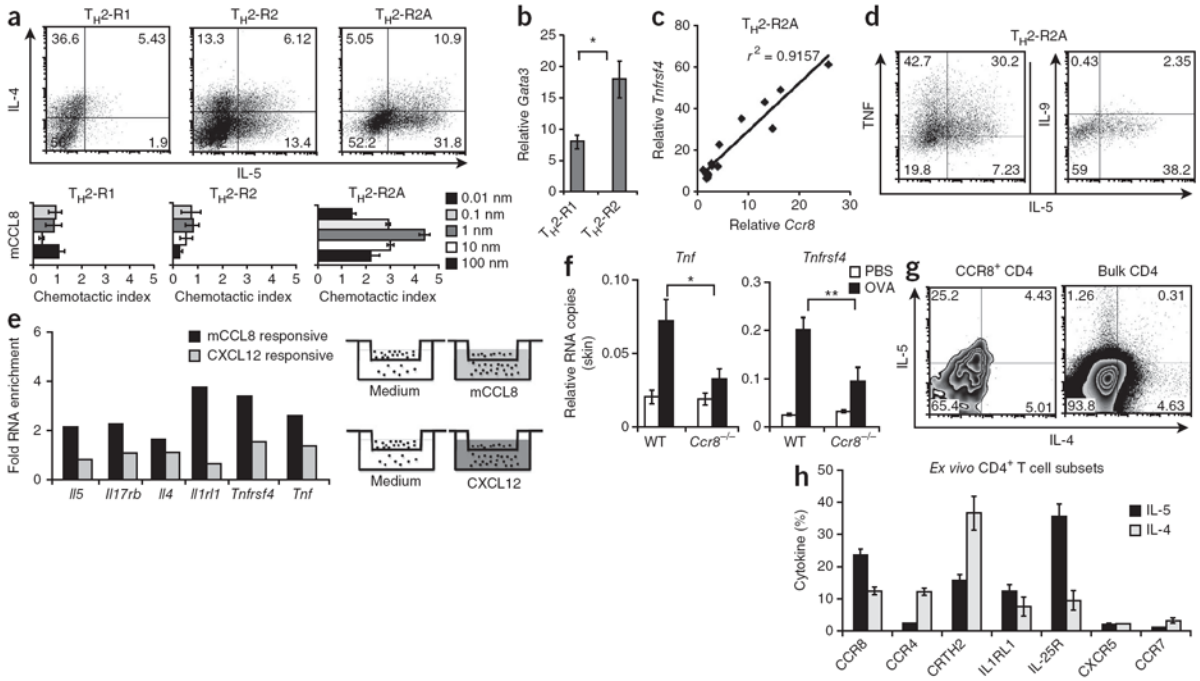

Figure 8.

Mouse CCL8–responsive T_H2-R2A cells are enriched for IL-5, IL-25R, TNF and OX40. (**a**) Flow cytometry of IL-4 and IL-5 by ICS in T_H2-R1, T_H2-R2, and T_H2-R2A cells; representative of six experiments. Below each plot is the chemotactic index of the various T_H2 cells to mouse CCL8 compared to medium alone in migration assays. (**b**) *Gata3* mRNA expression in paired T_H2-R1 and T_H2-R2 cells from four independent experiments; **P* = 0.02. (**c**) Correlation of *Ccr8* and *Tnfrsf4* (OX40) mRNA expression in T_H2-R2A cells. (**d**) Flow cytometry of TNF and IL-9 by ICS in T_H2-R2A cells; representative of three to six experiments. (**e**) Enrichment of T_H2-associated cytokines and receptors in T_H2-R2A cells that migrated to mouse CCL8 and CXCL12 relative to medium in Transwell assay; schematic of assay shown on right. Representative of at least three experiments. (**f**) QPCR analysis of *Tnf* and *Tnfrsf4* mRNA expression in skin biopsies of wildtype and $Ccr8^{-/-}$ mice after topical sensitization. Pooled data from two to three independent experiments with *n* = 5–9 mice per group are shown; **P* = 0.03 and ***P* = 0.03 in OVA-sensitized wild-type versus $Ccr8^{-/-}$ mice. (**g**) Flow cytometry of IL-5 and IL-4 by ICS after phorbol myristate acetate–ionomycin activation of peripheral blood $CD4^+$ T cells from healthy human donors. Data from same sample were gated on $CCR8^+CD4^+$ T cells (left) and bulk total $CD4^+$ T cells (right); reflective of data from seven donors. (**h**) Summary of IL-5 and IL-4 cytokine production by circulating fresh $CD4^+$ T cell subsets from healthy human donors. Results are shown as mean ± s.e.m., reflecting data from *n* = 3–7 donors.