

Absence of CCR4 Exacerbates Skin Inflammation in an Oxazolone-Induced Contact Hypersensitivity Model

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Chemokine receptor CCR4 is expressed by Th2 cells and is involved in the recruitment of inflammatory cells into the skin. We studied the effects of CCR4 deficiency in the murine model of oxazolone-induced contact hypersensitivity in CCR4^{−/−} and wild-type (WT) mice. The inflammatory response in the skin at 24 hours post-elicitation was stronger in CCR4^{−/−} mice compared with WT, evidenced by increased ear swelling and inflammatory cell infiltration. In addition, the mRNA expression levels of several cytokines, chemokines, chemokine receptors, and selectins in the skin of CCR4^{−/−} mice were significantly elevated compared with WT mice. Time kinetic experiments during the sensitization and elicitation phases revealed that the number of CD3⁺ CD4⁺ cells in CCR4^{−/−} mice remained high longer during the sensitization phase and increased more rapidly during the elicitation phase compared with WT mice. These data demonstrate that the absence of CCR4 results in enhanced secondary immune response during allergic skin inflammation.

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INTRODUCTION

Contact hypersensitivity (CHS) is an immunological response against a small chemical hapten. During the sensitization phase, dermal dendritic cells and Langerhans cells take up the hapten peptide conjugate and process it and migrate to the draining lymph node (LN) (Kaplan *et al.*, 2008), where they present the antigen to naive T cells, leading to the formation of antigen-specific memory T cells. Subsequent contact with the same chemical results in the activation of memory T cells and the recruitment of inflammatory cells to the site of allergen exposure. This elicitation response is manifested as skin inflammation, which in a clinical context is called allergic contact dermatitis (Saint-Mezard *et al.*, 2004).

The recruitment of inflammatory cells from the circulation to the site of inflammation is a multifaceted process. It involves the expression of E-selectin and P-selectin, adhesion

molecules important for tethering and rolling of leukocytes on the vascular endothelium (Harari *et al.*, 1999) and is essential for the development of skin inflammation in CHS (Catalina *et al.*, 1999; Fujita *et al.*, 2007; Alban *et al.*, 2009). In addition, a chemotactic stimulus on the endothelial cell surface is required for firm attachment of the leukocytes onto the endothelial cells and migration into the tissue (Vestweber and Blanks, 1999).

CCR4 is a chemokine receptor responding to chemokines CCL17 and CCL22 (Godiska *et al.*, 1997; Imai *et al.*, 1997, 1998) and is expressed by the majority of skin-homing CD4⁺ CLA⁺ cells (Andrew *et al.*, 2001). CCR4 is involved in antigen-mediated recruitment of CD4 T cells into the skin and is important in the formation of a mature circulating cutaneous memory Th-cell population (Andrew *et al.*, 2001; Reiss *et al.*, 2001; Baekkevold *et al.*, 2005; Campbell *et al.*, 2007). Although CCR4 cells have traditionally been associated with Th2 cells (Imai *et al.*, 1999), Kusumoto *et al.* (2007) reported elevated expression of CCR4, CCL17, and CCL22 in the skin of mice sensitized with oxazolone (OXA), which is known to induce a Th1-dominated response (Dearman *et al.*, 1994). Also, it has been observed in various CHS models that, together with CCR10, CCR4 is an important receptor for recruiting inflammatory cells to the site of inflammation (Reiss *et al.*, 2001; Mirshahpanah *et al.*, 2008). In addition, CCR4 has been shown to be important for the function of regulatory T cells (Tregs), and more than 80% of human peripheral blood CD25⁺ T cells express CCR4 (Iellem *et al.*, 2001, 2003; Baatar *et al.*, 2007; Yuan *et al.*, 2007). Furthermore, mice that lack CCR4 in their Tregs develop spontaneous inflammation in the skin (Sather *et al.*, 2007).

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Abbreviations: CHS, contact hypersensitivity; LN, lymph node; OXA, oxazolone; Treg, regulatory T cell; WT, wild type

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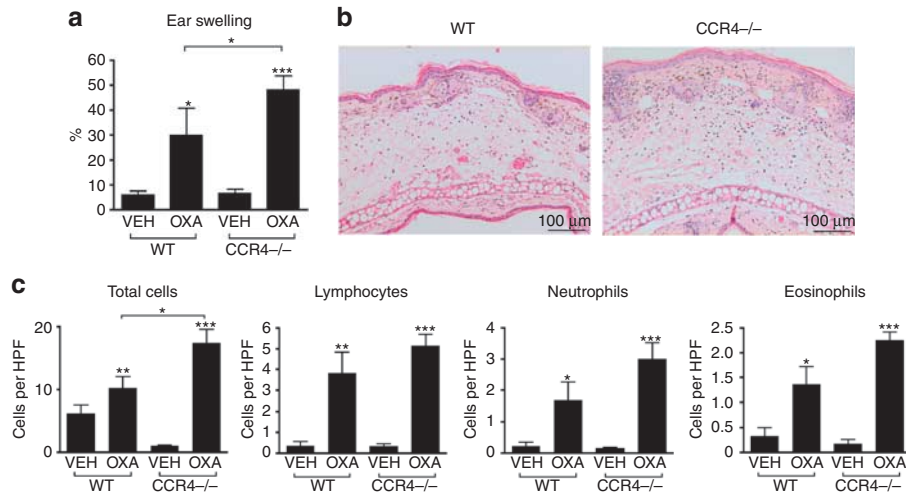


Figure 1. Ear tissue from vehicle (VEH) and oxazolone (OXA)-treated mice 24 hours after OXA elicitation. (a) Ear swelling is significantly increased in OXA-treated CCR4^{-/-} mice compared with wild-type (WT). (b) Hematoxylin-eosin staining of ear tissue from OXA-treated WT and CCR4^{-/-} mice reveals increased edema and inflammatory cell infiltration in CCR4^{-/-} mice compared with WT mice. Bar = 100 μ m. (c) Number of total inflammatory cells, lymphocytes, eosinophils, and neutrophils in the ear tissue of VEH-treated and OXA-treated mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; bars represent mean $\pm$ SEM, $n = 8$. HPF, high power field.

Because CCR4 is involved in both promoting and suppressing immune responses, and has a crucial function in CHS, we studied the effects of OXA sensitization in CCR4^{-/-} mice. Compared with wild-type (WT) mice, CCR4^{-/-} mice mounted a more severe inflammatory response in the skin.

RESULTS

OXA-treated CCR4^{-/-} mice mount more vigorous inflammation

To study the role of CCR4 in OXA-induced CHS, we sensitized WT mice and CCR4^{-/-} mice with OXA or vehicle and challenged all the mice 1 week later with OXA on both ears. At 24 hours post-OXA exposure, the swelling of the ears was significantly increased in the CCR4^{-/-} mice compared with WT mice (Figure 1a). Histological samples show that the increase in ear thickness in the knockout mice correlates well with increased edema and cell infiltration (Figure 1b). The CCR4^{-/-} mice had significantly elevated numbers of total inflammatory cells and slightly elevated numbers of lymphocytes, neutrophils, and eosinophils (Figure 1c). According to immunohistochemical stainings, significantly more CD4⁺ cells had infiltrated the skin in CCR4^{-/-} mice compared with WT, although there were no differences in the number of CD8⁺ cells (Figure 2a). The ratio of CD4⁺/CD8⁺ cells in the skin was elevated in CCR4^{-/-} mice compared with WT (Figure 2b), which was confirmed also with flow cytometric analyses (Figure 2c). The number of mast cells was similar in both OXA-sensitized groups (data not shown).

Upregulated mRNA expression of inflammatory mediators in OXA-treated CCR4^{-/-} mice compared with the WT

Next, we compared the mRNA expression levels of inflammatory mediators from the ear tissue of the WT and CCR4^{-/-}

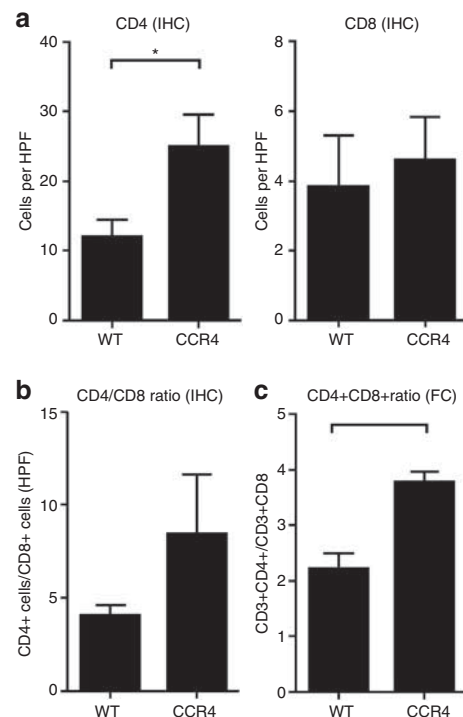


Figure 2. CD4⁺ and CD8⁺ cells in the skin. (a) According to immunohistochemical stainings, increased numbers of CD4⁺ cells have infiltrated the skin of oxazolone-sensitized and challenged CCR4^{-/-} mice compared with wild-type (WT) mice, although there are no differences in the number of CD8⁺ cells. Ratio of CD4⁺/CD8⁺ cells is increased in CCR4^{-/-} mice compared with WT as evidenced by (b) immunohistochemistry (IHC) stainings and (c) flow cytometric (FC) analysis. * $P < 0.05$; bars represent mean $\pm$ SEM, $n = 4-6$. HPF, high power field.

mice. The expression levels of proinflammatory cytokines such as tumor necrosis factor- α and IL-6 were elevated in both OXA-treated groups compared with vehicle-treated

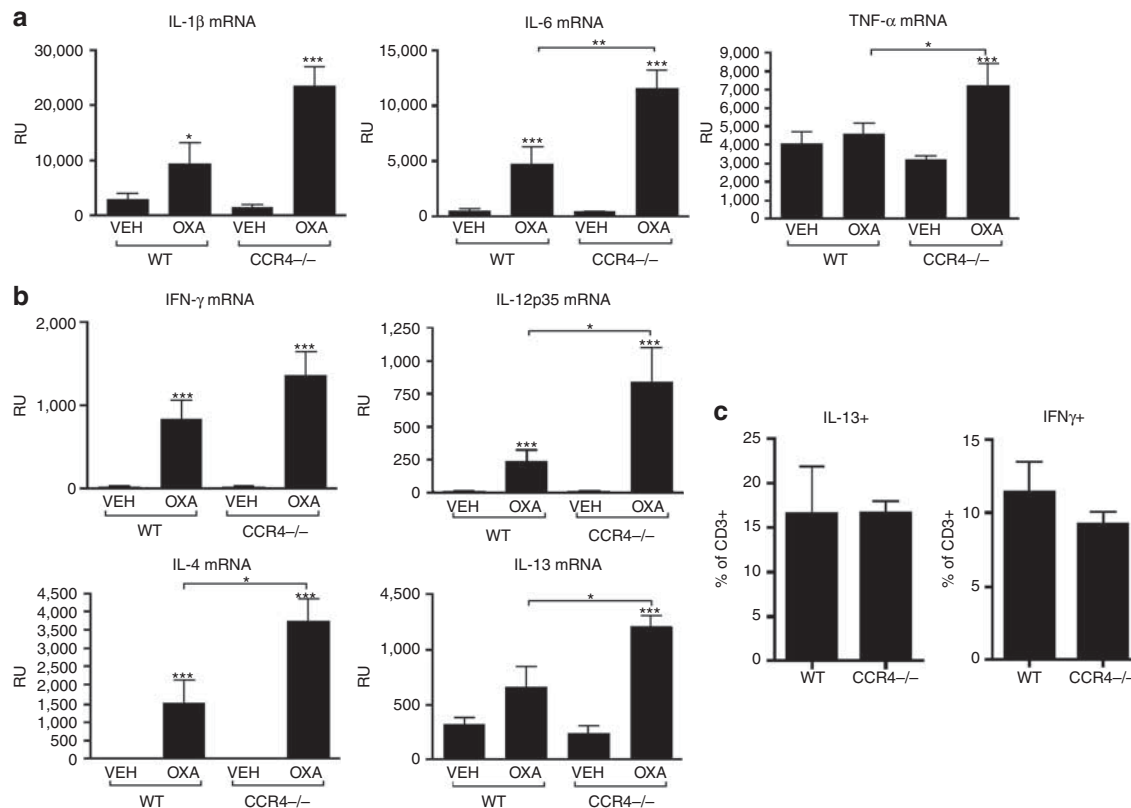


Figure 3. Cytokine expression in the skin. (a) Real-time PCR analysis of ear tissue 24 hours after elicitation with oxazolone. Significantly elevated mRNA expression of proinflammatory cytokines IL-1 β , IL-6, and tumor necrosis factor (TNF)- α in oxazolone (OXA)-treated groups versus vehicle-treated mice. In addition, IL-6 and TNF- α are significantly more expressed in the CCR4^{-/-} OXA group compared with the WT (wild-type) OXA group. (b) The mRNA expression of Th1 (IL-12p35) and Th2 (IL-4, IL-13) cytokines shows a similar pattern to proinflammatory cytokines, $n = 8$. (c) Similar percentages of CD3⁺ cells express IL-13 and IFN- γ in phorbol myristate acetate and ionomycin-stimulated skin cells from OXA-sensitized and challenged WT and CCR4^{-/-} mice, $n = 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; bars represent mean + SEM. RU, relative units.

mice, but significantly more in CCR4^{-/-} than in WT. Also, IL-1 β expression was slightly elevated in CCR4^{-/-} mice compared with WT (Figure 3a). In addition, mRNA expression levels of the Th1-type cytokine IL-12p35 and Th2-type cytokines IL-4 and IL-13 were significantly elevated in OXA-sensitized CCR4^{-/-} mice compared with the WT. The expression level of IFN- γ was only slightly elevated in the CCR4^{-/-} mice (Figure 3b). According to flow cytometric analysis, similar percentages of T cells in the skin stained positive for IFN- γ and IL-13 in the WT and CCR4^{-/-} mice (Figure 3c); hence, the increased IL-13 mRNA levels were probably a result of increased numbers of CD3⁺ + IL-13⁺ cells.

The increased number of inflammatory cells in the skin may be a result of more intensive recruitment. IL-1 β and tumor necrosis factor- α are known to induce the expression of E-selectin and P-selectin (Harari *et al.*, 1999), adhesion molecules important for the migration of inflammatory cells into the skin. In our experiment, the mRNA expression of both these selectins was significantly increased in OXA-sensitized CCR4^{-/-} mice (Figure 4a). In addition, increased mRNA expression levels of the chemokines CCL3, CCL4, CCL5, and CCL8 (Figure 4b) and corresponding receptors, CCR5 and CCR3, were detected in the skin of CCR4^{-/-} mice

(Figure 4c). Of the CCR4 ligands, CCL17 was significantly elevated in both OXA-treated groups, whereas CCL22 expression was at similar levels in all groups (Figure 4b). Immunofluorescence staining revealed CCR4 expression in the skin of OXA-treated WT mice (Supplementary Figure S1 online), although the relative expression of CCR4 mRNA was lower in OXA-treated WT mice than in vehicle-treated mice (Figure 4c).

The CCL27-CCR10 pathway has been shown to compensate for the lack of the CCL17/CCL22-CCR4 pathway in the recruitment of inflammatory cells to the skin (Reiss *et al.*, 2001; Mirshahpanah *et al.*, 2008). WT mice and CCR4^{-/-} mice both expressed mRNA for CCL27 and CCR10 (Figure 5a). In addition, flow cytometric analysis revealed similar percentages of CCR10⁺ cells in both in the WT and the CCR4^{-/-} mice (Figure 5b). T cells from CCR4^{-/-} mice had greatly impaired chemotaxis toward CCR4 ligands CCL17 and CCL22, but showed similar chemotactic response toward CCL27 compared with WT T cells (Figure 5c).

Increased numbers of CD3⁺ + CD4⁺ cells in the draining LNs of CCR4^{-/-} mice

To further understand the events that lead to increased inflammation in CCR4^{-/-} mice at 24 hours post-challenge,

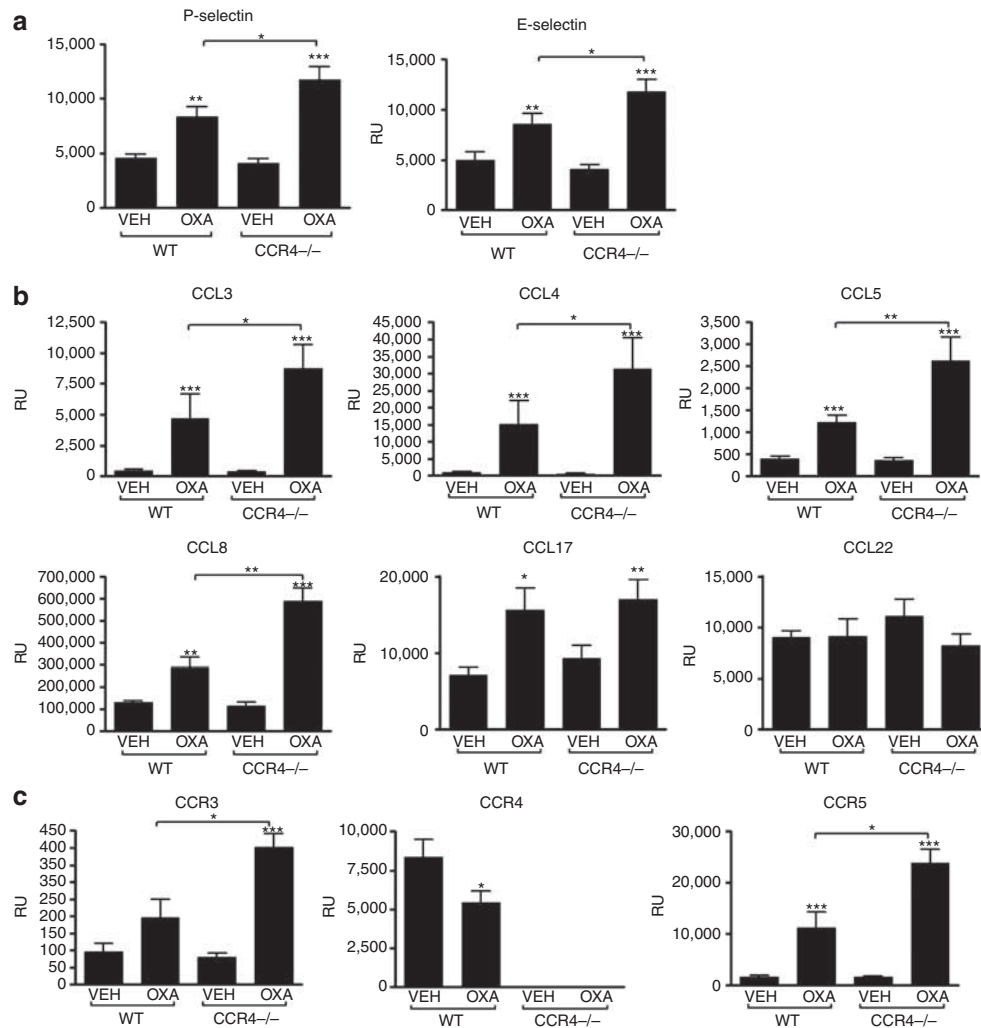


Figure 4. The mRNA expression analysis of ear tissue. (a) P- and E-selectins, (b) multiple chemokines (CCL3, CCL4, CCL5, CCL8, CCL17, and CCL22), and (c) corresponding chemokine receptors (CCR3, CCR4, and CCR5) 24 hours after oxazolone (OXA) elicitation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; bars represent mean $\pm$ SEM, $n = 8$. RU, relative units; VEH, vehicle; WT, wild type.

mice were sensitized as before and the numbers of CD3⁺CD4⁺ and CD3⁺CD8⁺ cells were determined 0, 2, 4, and 7 days after sensitization as well as 0, 4, 12, and 24 hours post-elicitation. The number of the cells was calculated by counting the number of total cells in the draining LN, analyzing with flow cytometry, and multiplying the cell number by the percentage of CD3⁺CD4⁺ or CD3⁺CD8⁺ cells. In the sensitization phase, there were no differences in the numbers of CD3⁺CD4⁺ or CD3⁺CD8⁺ cells, except at 7 days post-sensitization, at which point the number of CD3⁺CD4⁺ cells was down-regulated in the WT but not in the CCR4^{-/-} mice compared with 4 days after sensitization (Figure 6a). In the elicitation phase, the number of CD3⁺CD4⁺ cells was significantly elevated at 4 and 12 hours post-elicitation in CCR4^{-/-} mice compared with WT mice (Figure 6b). There was also a slight increase in the number of CD3⁺CD8⁺ cells in CCR4^{-/-} mice during the elicitation phase, but this difference was not statistically significant (Figure 6b). In addition, we analyzed

the ear swelling responses at 2, 4, 6, 12, and 24 hours after elicitation and observed an increased ear swelling response as early as 12 hours post-elicitation in the CCR4^{-/-} mice, although the increase was statistically significant only at 24 hours post-challenge (Figure 6c).

Increased number of CD3⁺IL-13⁺ cells in the draining LNs of CCR4^{-/-} mice after elicitation

LN cell suspensions from different time points after elicitation were prepared, stimulated with phorbol myristate acetate and ionomycin, and analyzed with flow cytometry. The number of CD3⁺IL-13⁺ cells was slightly increased in CCR4^{-/-} mice throughout the elicitation phase, whereas the number of CD3⁺IFN- γ ⁺ cells was comparable at all time points studied (Figure 7a). The mRNA analysis of stimulated cells at the 24 hours time point was consistent with these data, showing a slight increase in IL-13 mRNA expression in CCR4^{-/-} mice and no difference in IFN- γ expression (Figure 7b).

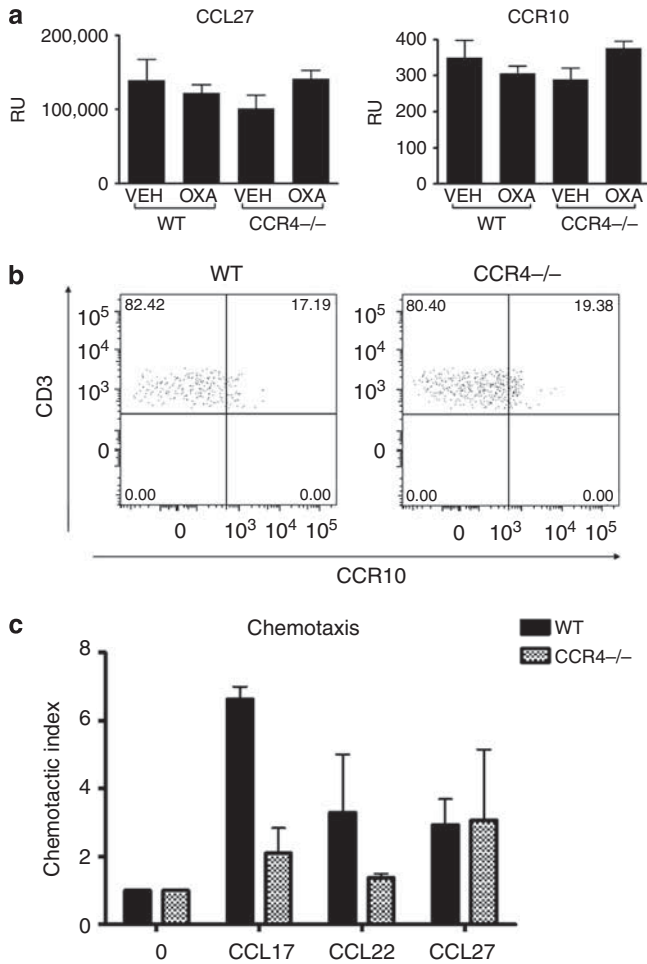


Figure 5. CCL27 and CCR10 expression in the skin and chemotaxis of draining LN cells. (a) The mRNA expression analysis of skin samples shows no differences in the expression levels of CCL27 or CCR10, $n=8$. (b) Flow cytometric analysis of CD3⁺ cells in the skin show that the same proportion of T cells is positive for CCR10 expression in the oxazolone (OXA)-sensitized and challenged wild-type (WT) and CCR4^{-/-} mice. Gated on CD3⁺ cells. (c) CCR4^{-/-} mice show severely impaired migration toward CCL17 and CCL22, whereas the chemotactic response toward CCL27 is similar to that of the WT. Bars represent mean + SEM. RU, relative units; VEH, vehicle.

Tregs are able to infiltrate the skin of CCR4^{-/-} mice

CCR4 has proven to be important for the function of Tregs and the recruitment of Tregs to the skin and draining LN (Denning *et al.*, 2005; Baatar *et al.*, 2007; Sather *et al.*, 2007; Yuan *et al.*, 2007). In our experiment, at 24 hours post-elicitation, Foxp3 mRNA was significantly increased in the skin of CCR4^{-/-} OXA mice (Figure 8a). This finding was further confirmed with flow cytometry and immunohistochemical stainings (Figure 8b). In the draining LN, the number of Foxp3⁺ cells followed the kinetics of CD3⁺CD4⁺ cells in both the WT and the CCR4^{-/-} mice in the sensitization phase as well as in the elicitation phase (Figure 8c). In the skin, OXA-sensitized CCR4^{-/-} mice expressed at 24 hours significantly more mRNA for the suppressive cytokine TGF- β , but the expression level of another important suppressive cytokine, IL-10, was only slightly elevated in CCR4^{-/-} mice (Figure 8a).

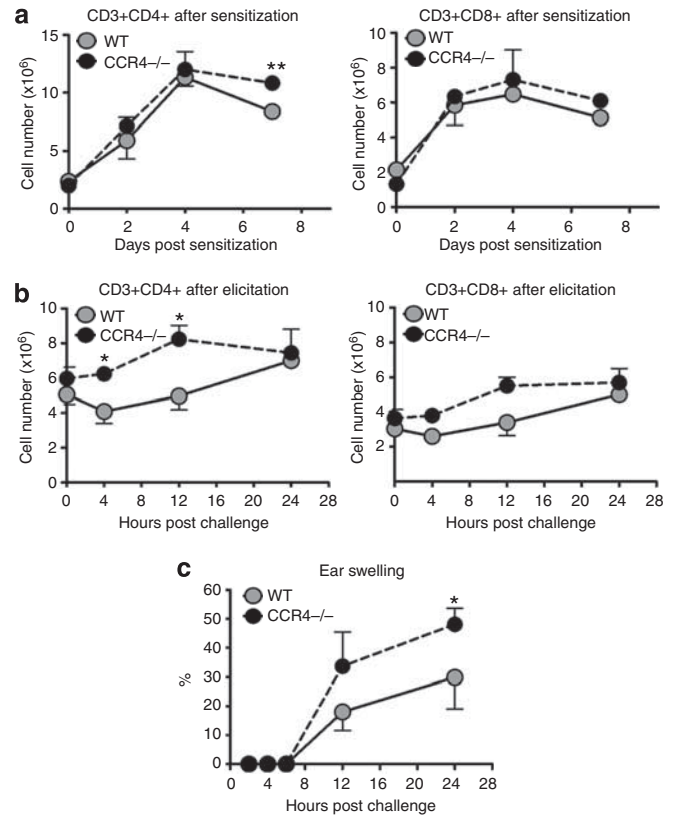


Figure 6. The number of CD3⁺CD4⁺ and CD3⁺CD8⁺ cells in the draining LNs of wild-type (WT) and CCR4^{-/-} mice. (a) 0, 2, 4, and 7 days after sensitization ($n=2-3$) and (b) 0, 4, 12, and 24 hours after elicitation ($n=2-3$). (c) Ear swelling response 2, 4, 6, 12 ($n=2-3$), and 24 hours after elicitation ($n=8$). * $P<0.05$, ** $P<0.01$; bars represent mean + SEM.

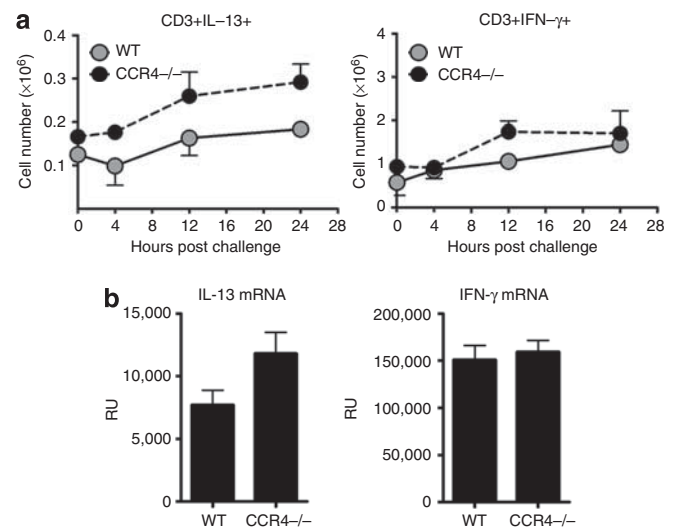


Figure 7. IL-13 and IFN- γ expression in the stimulated draining LN cells. (a) The number of CD3⁺IL-13⁺ and CD3⁺IFN- γ ⁺ cells and (b) mRNA expression of IL-13 and IFN- γ in the stimulated lymph node cells of oxazolone-sensitized and challenged wild-type (WT) and CCR4^{-/-} mice at 0, 4, 12, and 24 hours post-elicitation, $n=2-3$. RU, relative units.

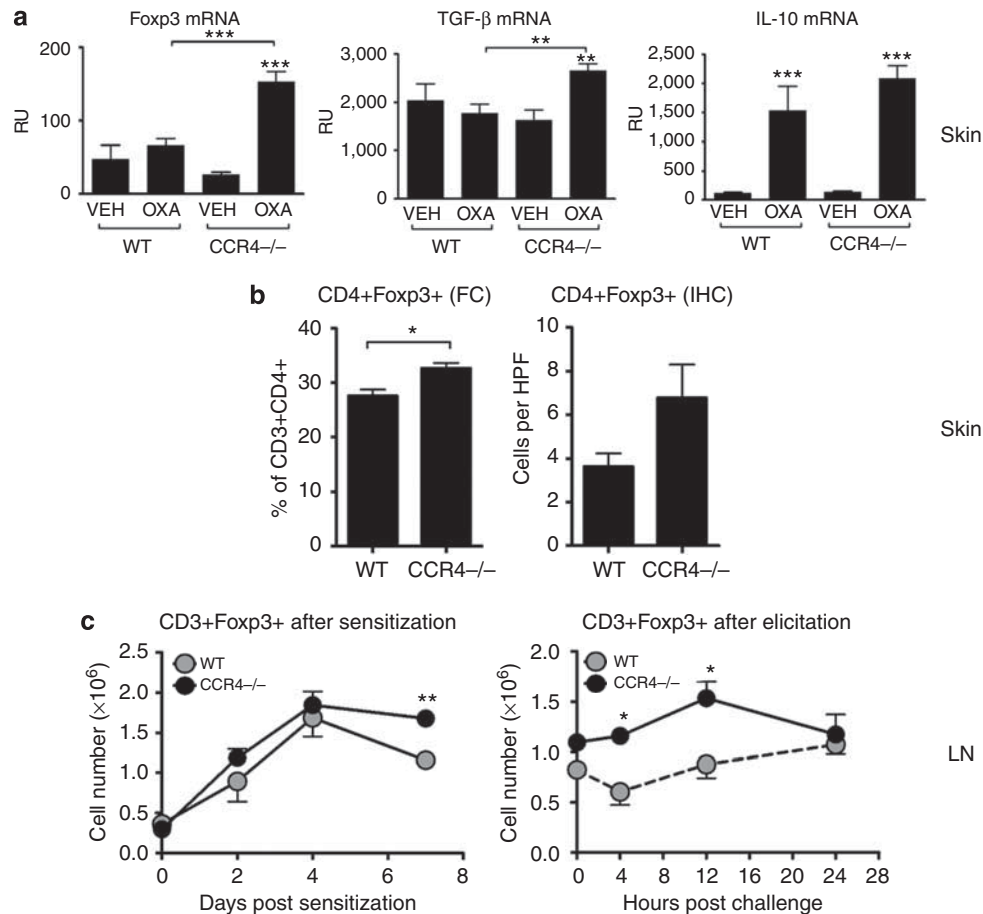


Figure 8. Fcγ expression in the skin and draining LN cells. (a) According to mRNA analysis from the skin at 24 hours post-challenge, Fcγ and TGF-β are significantly upregulated in the CCR4^{-/-} mice, whereas there are no differences in the IL-10 expression, $n=8$. (b) Flow cytometric (FC) and immunohistochemical (IHC) analysis reveal an increased number of Fcγ⁺ cells in the skin of oxazolone-sensitized and challenged CCR4^{-/-} mice compared with wild-type (WT), $n=3$ (FC), $n=4$ (IHC). (c) The number of CD3 + Fcγ⁺ cells in the draining lymph nodes (LNs) follows the kinetics of CD3 + CD4⁺ cells, in both the WT and the CCR4^{-/-} mice, $n=2-3$. (c) * $P<0.05$, ** $P<0.01$, *** $P<0.001$; bars represent mean + SEM. RU, relative units.

DISCUSSION

Human allergic contact dermatitis shares many features with murine CHS, and valuable information regarding allergic contact dermatitis has been collected by studying CHS in mouse models induced by various chemicals. CCR4 is a chemokine receptor expressed especially by Th2 cells (Imai *et al.*, 1999) and is also expressed by CLA⁺ cells, having a function in cutaneous inflammation (Andrew *et al.*, 2001; Iellem *et al.*, 2003). Previous studies have revealed an important function for CCR4 and corresponding ligands, CCL17 and CCL22, in CHS (Kusumoto *et al.*, 2007; Mirshahpanah *et al.*, 2008). To further clarify the role of CCR4 in CHS, we studied the effects of OXA-induced CHS in CCR4^{-/-} mice.

The absence of CCR4 had a clear effect on the inflammatory response. CCR4^{-/-} mice demonstrated significantly elevated ear swelling responses, increased infiltration of inflammatory cells, and enhanced expression of inflammatory cytokines and chemokines in the skin compared with WT. In addition, the number of CD3 + CD4⁺ cells was increased in OXA-treated CCR4^{-/-} mice. These

results suggest that CCR4 is involved in the regulation of the immune response during OXA-induced CHS.

The CCL27-CCR10 pathway has been shown to have overlapping roles with the CCL17/CCL22-CCR4 pathway in the cellular recruitment to the skin (Reiss *et al.*, 2001; Mirshahpanah *et al.*, 2008). In line with these reports, in our experiment CCR4^{-/-} mice expressed mRNA for the skin-homing receptor CCR10 and had percentages of CD3 + CCR10⁺ cells that were similar to those for WT mice (Figure 5a and b), indicating that CCR10-mediated recruitment of the inflammatory cells is available in CCR4^{-/-} mice. Chemotaxis assay, however, suggests that CCR4^{-/-} mice do not possess any enhanced migration capacity toward CCL27 compared with WT mice (Figure 5c), which is also in accordance with previous studies (Reiss *et al.*, 2001).

In the sensitization phase, the numbers of CD3 + CD4⁺ and CD3 + CD8⁺ cells in the draining LN followed the same kinetics in the WT and CCR4^{-/-} mice, except at day 7 post-sensitization, when the number of CD3 + CD4⁺ cells was downregulated in the WT but was sustained at a similar level in CCR4^{-/-} mice compared with day 4 after sensitization.

In the elicitation phase, the numbers of CD3 + CD4 + cells at 4 and 12 hours post-elicitation were significantly elevated in CCR4^{-/-} mice (Figure 6a and b). These results suggest that the sustained numbers of CD3 + CD4 + cells in the sensitization phase led to a more rapid increase in the number of these cells in the secondary phase. In addition, this increase was followed by elevated amounts of CD3 + CD4 + cells at the site of inflammation at 24 hours post-elicitation, inducing a shift in the CD4/CD8 balance in the skin of CCR4^{-/-} mice (Figure 2). Moreover, the number of IL-13-producing CD3 + cells had increased in CCR4^{-/-} mice after elicitation (Figure 7a), and a slightly elevated ear swelling response was observed as early as 12 hours post-elicitation in the CCR4^{-/-} mice compared with WT mice (Figure 6c). In conclusion, these results indicate that the secondary response to OXA starts earlier and is more dominated by CD4 + cells in CCR4^{-/-} mice, which most likely is the reason for augmented inflammation seen at the 24 hour time point in CCR4^{-/-} mice compared with WT mice.

Sather *et al.* (2007) have shown that CCR4 deficiency in Tregs results in severe leukocyte infiltration and inflammation in the skin. They observed impaired homing of CCR4-deficient Tregs to the skin and concluded that this would be the reason for spontaneous inflammation seen in the skin of mice that lack CCR4 in their Tregs. In our experiment, the mRNA levels of Foxp3 were significantly elevated in the skin of CCR4^{-/-} mice compared with the WT, indicating that CCR4^{-/-} Tregs were able to migrate into the skin. This finding was confirmed with flow cytometry and immunohistochemistry stainings (Figure 8a and b). These results suggest that during homeostasis CCR4 is important for homing of Tregs to the skin, but that during an inflammatory response Tregs are able to accumulate in the skin even in the absence of CCR4. Flow cytometric analysis revealed that approximately 10% of the Tregs from LNs of OXA-sensitized mice expressed CCR10 (Supplementary Figure S2 online), which most likely means that CCR10 can compensate for the lack of CCR4 also in Tregs. We also studied the number of Tregs in the draining LNs at different time points after sensitization and elicitation and noticed that the numbers of Foxp + CD3 + cells followed the kinetics of CD3 + CD4 + T-cell expansion in both the WT and the CCR4^{-/-} mice. These results indicate that Tregs are able to infiltrate the skin and draining LN in spite of CCR4 deficiency and that they also proliferate at similar rates in the WT and CCR4^{-/-} mice. However, we cannot rule out the possibility that the function of Tregs is impaired in CCR4^{-/-} mice. Tregs use multiple mechanisms to suppress inflammation. In our experiment, the mRNA expression level of TGF- β , which has been shown to be important in Treg-mediated suppression in several *in vivo* models (Vignali *et al.*, 2008), was also significantly increased in the skin of CCR4^{-/-} mice. In contrast, IL-10, an important suppressive cytokine produced by Tregs during CHS (Ring *et al.*, 2006; Cavani, 2008), was elevated only slightly in CCR4^{-/-} mice compared with WT. However, both these cytokines are produced by many cell types, and no conclusions about the functionality of Tregs can be made on the basis of these mRNA data.

In conclusion, CCR4 has a function in the control of inflammation in the skin, as the absence of CCR4 exacerbates the inflammation. In the future, more studies are needed to fully understand the effects of CCR4 during inflammation at both the systemic and the tissue-specific levels. In addition, manipulation of chemokine responses—for example, with biological drugs such as recently discovered evasins (Deruaz *et al.*, 2008)—at both the sensitization and the elicitation phases may help us to find new ways to induce tolerance to selected haptens.

MATERIALS AND METHODS

Mice and sensitization protocol

Heterozygous B6;129P-Ccr4^{tm1Pwr} mice were purchased from The Jackson Laboratory (Bar Harbor, ME) kept in pathogen-free conditions in the animal facilities of the Finnish Institute of Occupational Health, bred, and genotyped. All the procedures concerning animals were accepted by Social and Health Services of the State Provincial Office of Southern Finland.

Seven-week-old littermates homozygous for the WT allele or deleted CCR4 allele were selected for the sensitization protocol, which was carried out as previously described, with a few modifications (Lauerma *et al.*, 1995). Briefly, 50 μ l of OXA (10 mg ml⁻¹) or vehicle (acetone/olive oil, 4:1) was pipetted onto the shaved and tape-stripped skin. One week later, mice were anesthetized, ear swelling was measured, and 25 μ l of OXA (3 mg ml⁻¹) was pipetted onto the dorsal side of both ears. After 24 hours the mice were killed, ear thickness was measured, and samples were collected. For kinetic experiments, mice were sensitized with OXA as described above. The mice were killed and samples collected 0, 2, 4, or 7 days after sensitization and 0, 4, 12, or 24 hours after elicitation. Ear swelling was measured at 0, 2, 4, 6, 12, and 24 hours after elicitation.

Real-time PCR

Ears were homogenized in Trizol (Invitrogen, Camarillo, CA), and RNA was extracted according to standard protocols and subjected to complementary DNA synthesis. Real-time quantitative PCR was performed using commercial or self-designed primers and probes with an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Commercial primers and probes for ribosomal 18S (Applied Biosystems) were used as an endogenous control to ensure an equal amount of RNA in each sample. Self-designed primer sequences and the formula for calculating relative units are given in the Supplementary Materials and Methods online.

Histological stainings

Part of the ear was fixed in 10% formalin and embedded in paraffin. Multiple 4- μ m-thick sections were stained with hematoxylin-eosin or toluidine blue.

Immunohistochemical and immunofluorescence stainings

Four- μ m-thick frozen sections were fixed with acetone and stained with monoclonal anti-CD4, anti-CD8 (BD Biosciences, San Jose, CA), or anti-Foxp3 (eBiosciences, San Diego, CA). For immunofluorescent staining of CCR4, frozen sections were stained with goat polyclonal anti-CCR4 (Abcam, Cambridge, UK) and Alexa Fluor 568 rabbit anti-goat IgG (H + L) secondary antibody (Invitrogen).

Single-cell suspension preparation from skin and flow cytometric analysis

A piece of inflamed ear skin was cut into very small pieces, crushed through a 70- μ m cell strainer (BD Biosciences), washed with phosphate-buffered saline, and filtered again through a 40- μ m cell strainer (BD Biosciences). Unstimulated cells were surface stained with Alexa Fluor 488-conjugated anti-CD3, PerCP-Cy5-conjugated anti-CD4, and Alexa Fluor 700-conjugated anti-CD8 (eBiosciences). After surface stainings, cells were fixed and permeabilized for intracellular phycoerythrin-conjugated Foxp3 staining using a commercial Foxp3 staining kit according to the manufacturer's instructions (eBiosciences). CCR10 was stained using anti-CCR10 (Capralogics, Hardwick, MA) and a secondary phycoerythrin-conjugated anti-goat antibody (R&D Systems, Minneapolis, MN). For intracellular cytokine analysis, cells were stimulated with phorbol myristate acetate (20 ng ml^{−1}) and ionomycin (1 μ g ml^{−1}) in the presence of Brefeldin A (10 μ g ml^{−1}) for 5 hours, after which the cells were washed, surface stained as above, fixed, and permeabilized for intracellular staining of phycoerythrin-conjugated anti-IL-13 and allophycocyanin-conjugated IFN- γ (eBiosciences) using a Fix and Perm kit from Caltag (Burlingame, CA) as instructed by the manufacturer. Samples were analyzed with FACS Cantoll (BD Biosciences) using FACS Diva software, and the data were analyzed using FlowJo software.

LN preparations

For kinetic studies, cells from axillar LNs (after sensitization) or cervical LNs (after elicitation) were isolated by crushing the LNs, filtering through a 100- μ m filter, and washing with phosphate-buffered saline. The number of cells was calculated, and a million cells were stained directly for CD3, CD4, CD8, and Foxp3 expression, in a way similar to that used for the skin samples. For intracellular stainings, cells were stimulated and stained as described for the skin samples. At the 24 hour time point, a sample of stimulated cells was also lysed in Trizol (Invitrogen), and RNA was isolated as mentioned above and subjected to complementary DNA synthesis and subsequent real-time-PCR analysis.

Chemotaxis assay

For the chemotaxis assay, cells were isolated from the draining LNs of sensitized mice and enriched for CD4⁺ T cells using a mouse CD4⁺ T-cell enrichment kit according to the manufacturer's instructions (Stem Cell Technologies, Vancouver, BC, Canada). After enrichment, cells were suspended in complete RPMI 1640 with 1% BSA. Each chemokine, CCL17, CCL22, or CCL27 (R&D Systems), was added at a concentration of 100 nM to the feeder well (Costar Transwell, Corning, NY) and 1 million cells were added to the insert with a 5- μ m pore size. Chemotaxis was allowed to proceed for 3.5 hours, after which the number of migrated cells was counted and the chemotactic index was calculated by dividing the number of migrated cells by the number of cells that had migrated into the medium alone.

Statistical analysis

Statistical analyses were carried out with Student's *t*-test or the nonparametric Mann-Whitney *U*-test using GraphPad Prism software (GraphPad Software, La Jolla, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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