

Requirement of CCL17 for CCR7- and CXCR4-dependent migration of cutaneous dendritic cells

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Chemokines are known to regulate the steady-state and inflammatory migration of cutaneous dendritic cells (DCs). The β -chemokine CCL17, a ligand of CCR4, is inducibly expressed in a subset of DCs and is strongly up-regulated in atopic diseases. Using an atopic dermatitis model, we show that CCL17-deficient mice develop acanthosis as WT mice, whereas dermal inflammation, T helper 2-type cytokine production, and the allergen-specific humoral immune response are significantly decreased. Notably, CCL17-deficient mice retained Langerhans cells (LCs) in the lesional skin after chronic allergen exposure, whereas most LCs emigrated from the epidermis of allergen-treated WT controls into draining lymph nodes (LNs). Moreover, CCL17-deficient LCs showed impaired emigration from the skin after exposure to a contact sensitizer. In contrast, the absence of CCR4 had no effect on cutaneous DC migration and development of atopic dermatitis symptoms. As an explanation for the major migratory defect of CCL17-deficient DCs *in vivo*, we demonstrate impaired mobility of CCL17-deficient DCs to CCL19/21 in 3D *in vitro* migration assays and a blockade of intracellular calcium release in response to CCR7 ligands. In addition, responsiveness of CCL17-deficient DCs to CXCL12 was impaired as well. We demonstrate that the inducible chemokine CCL17 sensitizes DCs for CCR7- and CXCR4-dependent migration to LN-associated homeostatic chemokines under inflammatory conditions and thus plays an important role in cutaneous DC migration.

atopic dermatitis | CCL22 | CCR4 | GPCR | Langerhans cells

Chemokines represent a family of low molecular weight chemotactic cytokines and are classified as either constitutive or inducible (1). Constitutive chemokines are generally implicated in the homeostasis of the immune system, whereas inducible chemokines are expressed mainly during inflammatory processes. The migration of dendritic cells (DCs) into and out of the skin is coordinated by multiple chemokine–chemokine receptor pairs. Thus, immigration of immature DCs into the skin appears to be mediated through many different chemokine receptors, including CCR1, CCR2, CCR5, CCR6, CXCR1, CXCR2, and CXCR4 (2–4). In contrast, the homeostatic as well as inducible emigration of DCs from the skin to the skin-draining lymph nodes (LNs) has been shown to depend mainly on CCR7 and CXCR4 (4–6).

The inducible chemokine CCL17, previously termed thymus and activation-regulated chemokine (TARC) (7, 8), is strongly up-regulated through Toll-like receptor stimulation (9). CCL17 and the closely related chemokine CCL22 (MDC) (10, 11) bind to the receptor CCR4, which is known to be expressed on activated/memory T cells with a Th2 preference (12), regulatory T cells (13), Langerhans cells (LCs) (11), basophils, and macrophages (14). CCL17 and its receptor have been reported to play a role in endotoxin shock (14), systemic lupus erythematosus, Hodgkin's lymphoma, and pulmonary fibrosis. Using a reporter mouse model in which the *ccl17* promoter drives expression of EGFP, we previously showed that CCL17 is specifically expressed by CD8 α ⁺CD11b⁺DEC205⁺ DCs. CCL17⁺ DCs are located in peripheral LNs, mucosa, and various non-lymphoid organs, but normally are not found in the spleen. LCs and

dermal DCs (dDCs) in healthy normal skin are CCL17-negative, whereas CCL17 expression is induced in these cells after activation and migration to the skin-draining LNs (9). With the help of CCL17-deficient mice, we previously showed that contact hypersensitivity (CHS) responses are diminished in the absence of CCL17 (9).

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by eczematous skin lesions, flexural lichenification, and intense pruritus (15). Both dysregulation of the immune system and disturbances of the epidermal barrier have been implicated in the etiology of AD (16). The prevalence of AD is particularly high in young children compared with adults (10–20% vs. 1–3%) (17). Recently reported data show enhanced expression of various proallergic and proinflammatory cytokines and chemokines in the lesional skin and serum of AD patients (18). This also applies to the chemokine CCL17, the serum levels of which directly correlate with the severity of the disease (19, 20). The strong correlation of CCL17 expression with disease severity in AD patients prompted us to directly investigate the role of CCL17 in cutaneous immune responses. Using a mouse model of AD, we demonstrate that both local skin inflammation and the systemic humoral immune response are strongly reduced in CCL17-deficient mice compared with WT and CCR4-deficient mice. We provide an unanticipated explanation for the immunostimulatory function of CCL17, demonstrating that this chemokine plays an essential role in the emigration of DCs from the skin to the skin-draining LNs.

Results

Presence of Acanthosis but Reduced Dermal Inflammation in CCL17-Deficient Mice. To determine the influence of CCL17 on the development of AD, we treated heterozygous CCL17/EGFP (CCL17E/+) reporter mice and homozygous CCL17-deficient (CCL17E/E) mice with three cycles of tape-stripping and application of an ovalbumine (OVA)-containing patch on the skin of the back (21, 22). Mice treated with tape-stripping but receiving a patch soaked with NaCl alone were used as negative controls. In general, CCL17E/+ mice responded like normal BALB/c mice and were used as controls (Fig. S1). Mechanical stress induced by tape-stripping and application of a NaCl-soaked patch led to only minor thickening of the epidermis. When the model allergen OVA was applied to the patch on the mechanically stressed skin, acanthosis was induced in both CCL17E/+ and CCL17E/E mice to a similar extent (Fig. S2A and B), indicating that CCL17 has no influence on keratinocyte proliferation. In contrast, thickening of the dermis as a measure of leukocytic inflammation was strikingly diminished in

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OVA-treated CCL17E/+ and CCL17E/E mice (Fig. S7C) or in the number of LCs in OVA-treated WT and CCR4KO mice (Fig. 1C).

Emigration of LCs After Contact Sensitization Requires CCL17. The observation that CCL17 affects DC migration in the AD model prompted us to also examine emigration of skin-resident DCs in a model of acute contact sensitization. For this purpose, we applied the contact sensitizer DNFB on the ears for 4 h, and then cultured them for 48 h to permit emigration of LCs from the epidermis. Remarkably, LC emigration was significantly attenuated in CCL17E/E mice (Fig. 2A). Under steady-state conditions, $\approx 1,000$ LCs/mm² were found in the epidermis of CCL17E/+ and CCL17E/E mice, and treatment with solvent only did not significantly change this number (Fig. 2A and B). In contrast, DNFB application induced the emigration of more than 50% of all LCs in CCL17E/+ mice (Fig. 2A and B) and in WT and CCR4KO mice (Fig. 2C), but not in CCL17E/E mice. In the latter, the number of LCs was not significantly reduced compared with the solvent control and only moderately reduced compared with the steady state (Fig. 2A and B). These findings are in line with the higher frequency of EGFP⁺ LC in epidermal sheets from DNFB-treated CCL17E/E mice compared with CCL17E/+ mice (Fig. S3C). To further analyze the migration kinetics of skin-derived DCs after allergen exposure, we treated mice with DNFB on the dorsal skin and analyzed the numbers of both LCs and dDCs in the draining LNs after 0, 2 and 4 days. In CCL17E/E mice, the number of LCs was significantly reduced by 4 days after treatment; for dDCs, a significant difference could already be detected on day 2 as well as in the steady state (Fig. 2D

and E). These results are in line with our previous finding of impaired CHS responses in CCL17E/E mice (9).

CCL17 Enhances Directional Migration to CCR7 and CXCR4 Ligands. CCR7 and CXCR4 are known to be key regulators of DC migration from the skin to the skin-draining LNs (5, 6, 24–26). Because migration of mature DCs through peripheral tissues involves direct interactions with the extracellular matrix (3, 27), we first analyzed the migration of bone marrow (BM)-derived DCs in 3D collagen gels using live cell imaging microscopy. In this assay, BM-DCs from CCL17E/E mice showed less random motility and migratory speed (velocity) in the absence of a chemokine gradient compared with CCL17E/+ BM-DCs (Fig. 3A and B and Movie S1 and Movie S2). When a CCL19 gradient was applied, BM-DCs of CCL17E/+ mice responded with vigorous migration and good directionality (0.52 ± 0.15) (Fig. 3A, Upper Right and Movie S3). In contrast, the directionality of BM-DCs from CCL17E/E mice toward the CCR7 ligand CCL19 was strongly reduced to 0.28 ± 0.18 (Fig. 3A, Lower Right and Movie S4). Likewise, the y-forward migration index, which indicates the directed migration toward the chemokine gradient along the y axis, was reduced by almost 50% (Fig. 3C). Furthermore, the average velocity of CCL17E/E cells was significantly lower than that of CCL17E/+ cells in the presence and absence of a CCL19 gradient (Fig. 3B).

We next analyzed the directional migratory capacity of BM-DCs from either CCL17E/+ or CCL17E/E mice to the CCR7 ligands CCL19 and CCL21 and to the CXCR4 ligand CXCL12 in transwell migration assays. Again, we found that BM-DCs generated from CCL17E/E mice had a strongly reduced ability to transmigrate toward all three chemokines compared with DCs from CCL17E/+ mice (Fig. 3D). Nevertheless, the migratory ability of CCL17E/E DCs was not entirely abrogated. Interestingly, CCL17E/E DCs also demonstrated reduced random migration when incubated without any chemokine and moderately reduced chemokinesis (Fig. S8A and B). We then asked whether migration of CCL17E/E BM-DCs to CCL19 and CXCL12 could be restored by preincubation with recombinant murine (rec) CCL17. As shown in Fig. 3E–G, rec-CCL17 had to be present for more than 2 h and at concentrations of >100 ng/mL to achieve full migratory capacity. In addition, emigration of LCs from the epidermis also could be induced in vivo in CCL17E/E mice by injection of recCCL17 into the ear pinna before challenge with DNFB (Fig. S8E and F). In contrast, treatment with anti-CCL17 antibodies strongly inhibited CCL17E/+ DC migration (Fig. S8D). Thus, we can conclude that CCL17 is essential for the ability of DCs to respond to CCR7 or CXCR4 ligands with efficient directed chemotaxis in an inflammatory micro-milieu. In contrast, the absence of CCR4 had no influence on the migration of BM-DCs toward CCL19 (Fig. S8G). Although CCL22, which is thought to have similar biological activity as CCL17, is produced at normal amounts in CCL17E/E mice (Fig. S8H), it apparently does not compensate for CCL17-deficiency. But the addition of recCCL22 to CCL17-deficient DCs also restores migration to CCL19 (Fig. S8I), and antibody-mediated blockade of CCL22 slightly inhibits DC migration (Fig. S8J). Furthermore, knockdown of CCL22 by siRNA leads to reduced migration of CCL17-expressing, but not CCL17-deficient, BM-DCs to CCL19 and CXCL12 (Fig. S8K and L). These findings indicate that CCL22 might have a similar function as CCL17, but nonetheless does not compensate for CCL17 in terms of cutaneous DC migration, perhaps because of local cell type-specific differences in production of CCL17 versus CCL22.

CCL17 Regulates CCR7 and CXCR4 Function Without Altering Their Expression Levels. To test whether the impaired responsiveness of CCL17E/E DCs to CCR7 and CXCR4 ligands was caused by reduced expression of CCR7 or CXCR4, we stained BM-DCs for these receptors. Under normal conditions, CCR7 and CXCR4 are expressed by stimulated BM-DCs, and despite the impaired responsiveness of CCL17E/E DC to CCL19, CCL21, and CXCL12,

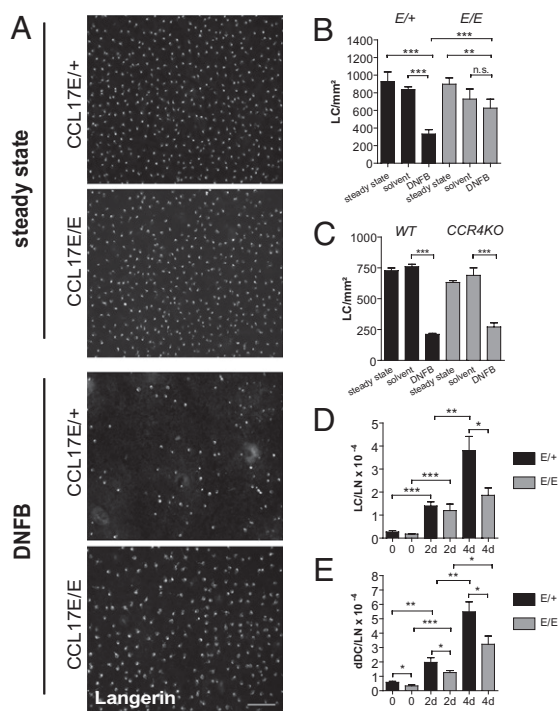


Fig. 2. Emigration of DCs after contact sensitization requires CCL17. (A) CCL17E/+ or CCL17E/E mice were treated with 0.5% DNFB on the ears for 4 h. After culture for 2 days, the skin layers were separated, and the epidermis was stained for Langerin. (Scale bar: 100 μ m.) (B and C) Quantification of the number of LCs per mm² of epidermis of CCL17E/+ and CCL17E/E mice (B), and WT and CCR4KO mice (C) for the different treatment groups as indicated. (D and E) 0.5% DNFB was applied on the shaved back of CCL17E/+ and CCL17E/E mice. After 2 and 4 days, the numbers of immigrated dDCs (E) and LCs (D) in skin-draining LNs were compared with steady-state levels in untreated mice (0). CCL17E/+ and WT mice are denoted by black bars; CCL17E/E and CCR4KO mice, by gray bars. Mean values $\pm$ SEM of two or three independent experiments are shown.

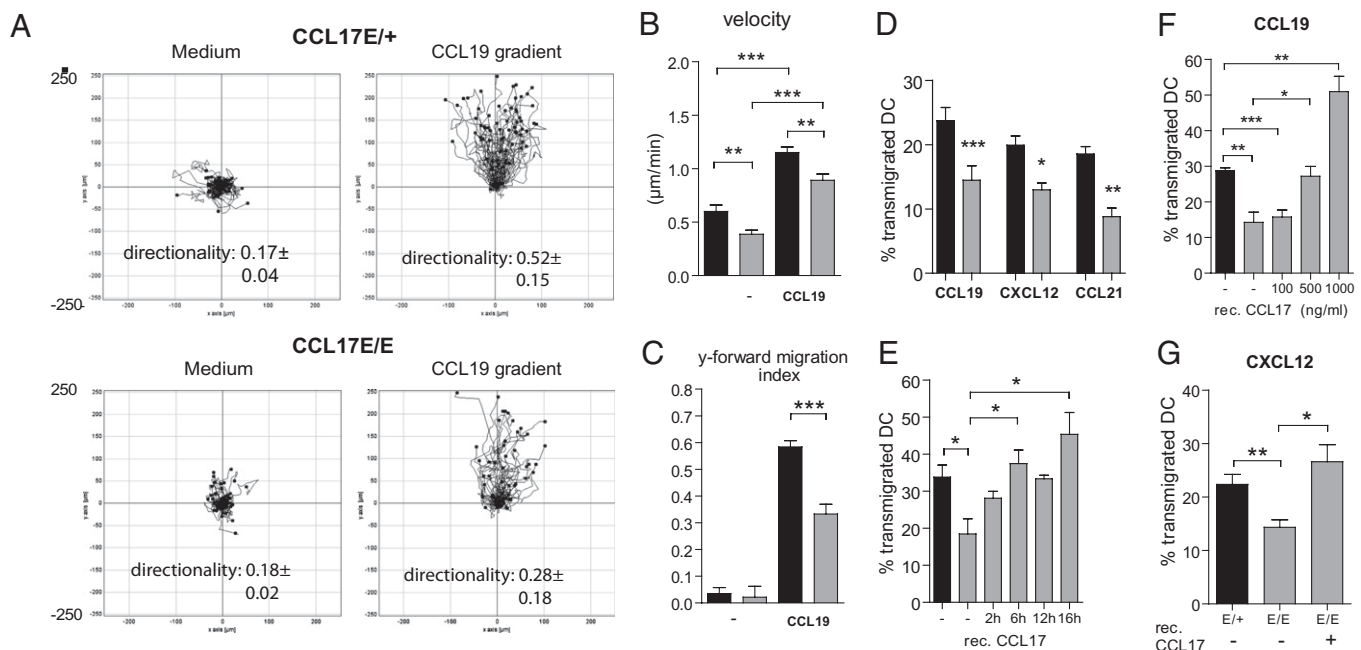


Fig. 3. Migration toward CCR7 and CXCR4 ligands is impaired in the absence of CCL17. (A–C) Chemotactic migration in 3D collagen matrices of BM-DCs from CCL17E/+ and CCL17E/E mice in response to CCL19 or medium only. Plots of 60 cells/sample tracked by live cell imaging from one representative experiment out of three are depicted. Quantifications of directionality (A), velocity (B), and y-forward migration index (C) represent mean values ± SEM from all three independent experiments. (D) BM-DCs were stimulated on day 6 with LPS overnight and transferred into transwell inserts. Cells were incubated with CCL19, CXCL12, or CCL21 as indicated, and migrated DCs were counted. Results of three independent experiments were pooled, and mean values ± SEM were calculated. (E) BM-DCs were stimulated on day 6 with 100 ng/mL of LPS overnight and with 1,000 ng/mL of CCL17 at different time points. Cells were incubated with CCL19, and migrated DCs were counted. (F) BM-DCs were stimulated on day 6 with 100 ng/mL of LPS and graded doses of recCCL17 overnight as indicated. Cells were incubated with CCL19. (G) BM-DCs were stimulated on day 6 with 100 ng/mL of LPS and 1,000 ng/mL of recCCL17 overnight. Cells were stimulated with CXCL12. CCL17E/+ mice are denoted by black bars; CCL17E/E mice, by gray bars.

we found no reduction in CCR7 or CXCR4 expression (Fig. 4A). We also stained for CCR4 and found that equivalent amounts of CCR4 were expressed on unstimulated DCs of CCL17E/E and CCL17E/+ mice (Fig. 4B, Left). After LPS stimulation, the expression levels of CCR4 on DCs from CCL17E/E mice were slightly higher than those on DCs from CCL17E/+ mice (Fig. 4B, Right). We also analyzed CCR7 expression on ex vivo isolated LCs of WT, CCL17E/+, CCL17E/E, and CCR4KO mice. Whereas CCR7 expression was not detectable directly after isolation of the cells (Fig. 4C and D, Left), it was strongly up-regulated after LPS stimulation, with no difference in LCs from WT, CCL17E/+, CCL17E/E, and CCR4KO mice (Fig. 4C and D, Right). This indicates that CCL17 deficiency does not affect surface expression of CCR7 and CXCR4, but rather alters their functionality. As a consequence of signal transduction through chemokine receptors, cells normally react with the release of intracellular calcium stores. Thus, we compared the ability of CCR7 to induce calcium flux in CCL17E/+ versus CCL17E/E DCs after stimulation with CCL19 or CXCL12 (Fig. 4E–N). Remarkably, both CCL19- and CXCL12-induced Ca^{2+} flux was strongly reduced in CCL17E/E DCs, whereas Ca^{2+} flux could be induced in all genotypes by treatment with ionomycin (Fig. 4E–N).

Discussion

Chemokines are known to exacerbate inflammatory reactions in allergic skin diseases. In AD, serum levels of several chemokines were found to correlate with disease activity (18). Here we studied the pathogenic role of CCL17 in a murine AD model using CCL17-deficient mice. Although CCL17 and CCL22 are structurally homologous, and both bind to CCR4, the absence of CCL17 alone, and not the absence of CCR4, leads to major amelioration of disease symptoms in the AD model. Our results demonstrate an as-

sociation of CCL17 with cutaneous DC migration. We show that emigration of DCs, in particular LCs, from the skin to skin-draining LNs is inhibited in models of AD and CHS in CCL17-deficient mice. This finding of a major migratory defect of CCL17-deficient DCs in vivo is further substantiated by direct live cell imaging in vitro, demonstrating that CCL17 is required to sensitize activated BM-DCs for CCR7- and CXCR4-dependent migration and allow Ca^{2+} flux. Thus, chemokine-dependent emigration of DCs from the skin under inflammatory conditions is strongly enhanced by the inducible chemokine CCL17 through sensitization of at least two different receptors for homeostatic chemokines.

The molecular mechanisms that regulate DC migration from the skin to the draining LNs are only partially understood. Triggering of CCR7 was shown to be required for DC migration under both steady-state and inflammatory conditions (5). In addition, signaling via CXCR4 was found to contribute to the migration of cutaneous DCs after contact sensitization (6, 26). In steady state, the number of LCs within the epidermis is similar in CCL17E/E and CCL17E/+ mice (Fig. 2B). In contrast to CCR7KO mice, which lack skin-derived DCs in peripheral LNs (5), peripheral LNs of CCL17E/E mice have only slightly reduced numbers of skin-derived DCs (Fig. S6L and M). This slightly reduced skin-derived DC migration might be the result of CCL17 production induced by unintentional environmental stimuli under steady-state conditions. On the other hand, there is an obvious deficiency in the mobilization of LCs from the epidermis after allergic sensitization in CCL17-deficient mice, but not in CCR7KO mice (5). This difference is likely explained by the fact that CXCR4-dependent DC migration is impaired in CCL17E/E mice as well.

In vivo, the CCL17-dependent migratory defect was observed primarily in LCs. We cannot exclude the possibility that migration of dDCs is affected as well, because the number of CD11c⁺ DCs in the

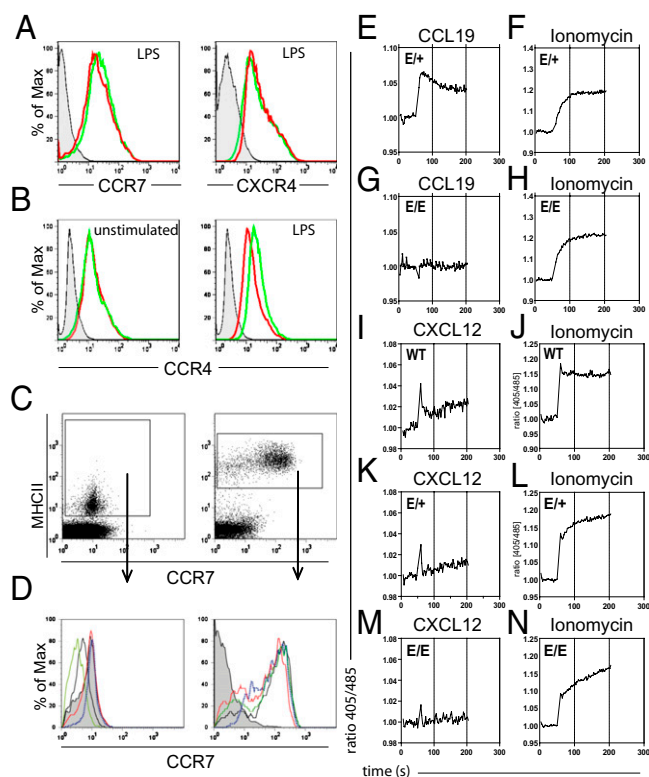


Fig. 4. Normal expression of CCR7 and CXCR4, but impaired Ca^{2+} flux, in CCL17E/E DCs. Unstimulated and LPS-stimulated BM-DCs of CCL17E/E (green line) and CCL17E/+ (red line) mice were stained with anti-CCR7 (A, Left), anti-CXCR4 (A, Right), or anti-CCR4 (B). As a negative control, splenic cells of CCR4KO mice were stained with the CCR4 antibody. The negative control for CCR7 and CXCR4 was unstained cells. The data are representative of three independent experiments. (C and D) LCs were isolated from the dorsal skin of C57BL/6 mice (black line), CCR4KO mice (blue dotted line), CCL17E/+ mice (green line), and CCL17E/E mice (red line) and stained for MHCII and CCR7 either immediately (Left) or after stimulation with LPS overnight (Right). The histograms in D depict CCR7 expression of gated MHCII⁺ cells as indicated in C. (E–H) LPS-stimulated BM-DCs from CCL17E/+ and CCL17E/E mice were loaded with Indo-1 AM, and CCL19-induced Ca^{2+} -flux was analyzed by calculating the ratio of calcium-bound to free Indo-1 over time. Shown is the baseline ratio (0–50 s), followed by stimulation with CCL19 or ionomycin (50–200 s). (I–N) Before staining with Indo-1 AM, CD11c⁺ BM-DCs from CCL17E/E, CCL17E/+, and WT mice were enriched by MACS on day 6 and stimulated with LPS overnight. CXCL12-induced Ca^{2+} flux was calculated as described above (I, K, and M). As a control, DCs were treated with ionomycin (J, L, and N).

dermis might be kept high in the inflamed skin through a continuous influx of DCs from the circulation. As for the absolute numbers of LC and dDC descendants in the draining LNs, both subsets were similarly reduced in OVA-treated CCL17E/E mice compared with CCL17E/+ controls. The concept that CCR7 is not constitutively able to signal cell migration in the presence of its ligands CCL19/CCL21 has been reported for CD47-deficient DCs (28) and WT DCs that have been differentiated in vitro in the absence of prostaglandin E2 (PGE2) (29, 30). Like CCL17-deficient DCs, these cells express normal levels of CCR7, but fail to undergo efficient directional migration to CCR7 ligands. Thus, the expression of CCR7 on the cell surface alone is not sufficient to guarantee its full functionality (31). The molecular mechanisms by which PGE2 or CCL17 act on CCR7 and potentially other chemokine receptors remain enigmatic, however. In line with previous findings regarding the influence of PGE2 on the responsiveness of human DCs to CCR7 ligands (29), we have shown that the release of intracellular Ca^{2+} stores in response to CCL19 and CXCL12 is markedly impaired in the absence of CCL17. Therefore, CCL17 may be required to engage important signal

transduction components downstream of CCR7 and CXCR4. At present, we also cannot exclude the possibility that CCL17 deficiency leads to a general impairment of DC mobility, because chemokinesis was reduced in CCL17E/E DCs as well (Fig. S8A and B). A similar phenomenon of sensitization of chemokine receptor function has been described in vitro for CXCR4 and CXCR3, which act synergistically to induce migration of plasmacytoid DCs (pDCs) (32, 33). In the present study, we now provide an in vivo proof of the relevance of cooperation of inducible and homeostatic chemokines during allergic inflammation.

CCL17 has long been implicated in the preferential attraction of Th2 cells (8, 12), which upon recognition of allergens, release IL-4, IL-5, IL-10, and IL-13. We have demonstrated that cytokine expression in the skin is strongly attenuated in the absence of CCL17. In addition, the numbers of mast cells and eosinophils are reduced in OVA-treated CCL17E/E mice, correlating with decreased production of allergen-specific IgE and IgG antibodies. We anticipate that the amelioration of CD4⁺ T cell infiltration in the skin of CCL17E/E mice is mainly a consequence of reduced T-cell priming in the skin-draining LNs due to deficient DC migration. Although the possibility of a direct skin-homing deficiency of activated T cells in CCL17E/E mice cannot be excluded, this is quite unlikely, because CCR4 and CCR10 are known to play redundant roles in the attraction of memory T cells to the skin (34), in line with the present observation that CCR4KO mice develop allergic skin inflammation similar to WT mice.

Our finding that deficiency of CCL17 alone leads to a blockade of cutaneous DC migration also sheds some light on the relationship and possible redundancy of CCL17 and CCL22, which both bind and activate the receptor CCR4. As shown in this study, recCCL22 can be used like recCCL17 to reconstitute DC migration in vitro, and inhibition of CCL22 leads to reduced DC mobility. CCL22 protein is normally expressed in our CCL17E/E knock-in mice (Fig. S8H), excluding the possibility that the genetic manipulation of *ccl17* in these mice also affected the closely linked *ccl22* gene. However, under physiological conditions, CCL17 and CCL22 appear to be locally produced by different cell types and in different amounts (35, 36). Furthermore, CCL22 has a greater affinity to the D6 decoy receptor compared with CCL17, and it is susceptible to cleavage by the dipeptidyl-peptidase IV (CD26) (37). These differences may explain why CCL22 does not fully compensate for CCL17 deficiency in vivo. The finding that CCR4KO mice show no defect in cutaneous DC migration and no amelioration of AD pathology suggests that CCL17 and CCL22 must bind to an additional receptor besides CCR4. The only other chemokine receptor that has been postulated to bind CCL17 is CCR8 (38), although this was not seen by another group (39). Therefore, it will be important to identify the as-yet unknown additional interaction partners of CCL17 and CCL22 in DCs.

Consistent with our findings in the mouse model, the number of epidermal LCs is reduced in lesional skin of AD patients compared with normal skin, and LC migration from the epidermis to the dermis correlates with enhanced expression of thymic stromal lymphopoietin (TSLP) in the epidermis (40, 41). In this context, it is interesting to note that TSLP induces maturation and CCL17 expression in DCs. In addition, transgenic expression of CCL17 in keratinocytes was shown to enhance Th2-dependent CHS and AD-like symptoms (42).

In conclusion, we have demonstrated that the chemokine CCL17 plays a unique and essential role in cutaneous DC migration and the pathogenesis of allergic skin inflammation. Regarding the design of novel therapeutic drugs for treatment of AD, it has been suggested that blockade of CCR4 represents a promising target (43). Based on our present findings that CCL17-deficient mice, but not CCR4KO mice, display a defect in cutaneous DC migration and are protected from AD, we propose that local inhibition of CCL17 might be more suitable for prevention of allergic skin inflammation than CCR4 blockade.

Materials and Methods

AD Mouse Model. Atopic skin inflammation was induced as described previously (21, 22). In brief, mice were tape-stripped four times using Tegaderm 1624W (3M Medica). First, 100 μ g of OVA (grade V; Sigma-Aldrich) in NaCl or NaCl alone was placed on a patch of sterile gauze and attached to the skin. The patches were applied on days 1 and 4 and maintained for a total of 1 week. After 2 weeks of rest, mice were tape-stripped again, and an identical patch was reapplied to the same site. Mice received a total of three 1-week patch exposures.

Contact Sensitization. Mice were treated on both sides of the ear with 10 μ L of 0.5% DNFB in acetone/olive oil (5:1). After 4 h, the ears were removed and cultured for 2 days at 37 °C. Epidermal sheets were stained with mouse anti-Langerin (clone 929F3.01; Acris Antibodies). To investigate the *in vivo* migration of DCs, the dorsal skin was shaved, and 80 μ L of 0.5% DNFB was applied. After 2 and 4 days, skin-draining LNs were analyzed by flow cytometry.

In Vitro Migration Assay. BM-DCs were generated as described previously (9). On day 6, nonadherent cells were harvested and activated with LPS overnight. DC migration was measured in 24-well Costar transwell chambers

(Corning). After a 3-h incubation at 37 °C, the migrated cells were counted and stained for FACS analysis.

Three-Dimensional Collagen Gel Chemotaxis Assay. The 3D chemotaxis assays were performed as described previously (27) with some modifications. Collagen-BM-DC mixtures were placed in custom-made chemotaxis chambers and incubated at 37 °C in 5% CO₂ for 90 min. After collagen polymerization, 600 ng/mL of CCL19 (R&D Systems) was added on top of the gel. Time-lapse series were recorded using a fully automated inverted TE Eclipse microscope (Nikon). BM-DC were tracked over 3 h (5 min/frame) using the ImageJ manual tracking plug-in.

Other Methods. Detailed descriptions of all methods used are provided in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Mice. CCL17E/+ and CCL17E/E mice, described previously (1), were backcrossed to BALB/c or C57BL/6 background for eight generations. In brief, an EGFP cDNA was inserted into the murine *ccl17* locus such that EGFP was expressed instead of CCL17. Whereas heterozygous CCL17E/+ mice produce about half the normal amounts of CCL17 (Fig. S8C), homozygous CCL17E/E mice represent CCL17-KO mice. The WT mice on a C57BL/6 background were originally purchased from Harlan-Winkelmann. The CCR4KO mice (2) on a C57BL/6 background were obtained from Y. Chvatchko (Serono Pharmaceutical Research Institute). All mice were bred under specific pathogen-free conditions in the animal facility of the Institut fuer Umweltmedizinische Forschung and were used for experiments at age 6–10 weeks. All animal experiments were performed with permission of the North Rhine-Westphalia State Office for Nature, Environment, and Consumer Protection (50.05–230-79/05 and 50.05–230-3/06).

AD Mouse Model. Atopic skin inflammation was induced according to the protocol of Spergel et al. (3) with the modification of additional tape-stripping to introduce a standardized disturbance of the epithelial barrier (see also ref. 4). In brief, female CCL17E/+ and CCL17E/E mice (BALB/c background) and WT and CCR4KO mice (C57BL/6 background) were anesthetized with isoflurane (CP Pharma), shaved on the back, and tape-stripped four times using Tegaderm 1624W (3M Medica). Then 100 µg of OVA (grade V; Sigma-Aldrich) in 100 µL of 0.9% NaCl or NaCl alone was placed on a patch of sterile gauze (1 × 1 cm) and attached to the skin with Tegaderm 1624W. The patches were applied on days 1 and 4 and maintained for a total of 1 week. After 2 weeks of rest, the mice were tape-stripped again, and an identical patch was reapplied to the same site. The mice received a total of three 1-week patch exposures, totaling 7 weeks.

Contact Sensitization. CCL17E/+ and CCL17E/E mice (on a C57BL/6 background) were anesthetized, and the dorsal and ventral sides of the ear were treated with 10 µL of 0.5% DNFB in acetone/olive oil (5:1). After 4 h, the mice were killed, and the ears were harvested, divided at the cartilage site, and placed in RPMI 1640 medium supplemented with 10% FCS (PAN Biotech), L-glutamine, penicillin-streptomycin, and 2-ME (all from Gibco). After 2 days of culture at 37 °C, the epidermis and dermis were separated by EDTA incubation (20 mM in PBS for 2 h). Epidermal sheets were fixed with acetone, and unspecific binding was blocked with PBS/5% mouse serum. The first antibody, mouse anti-Langerin (clone 929F3.01; Acris Antibodies), was applied for overnight staining. After washing, the sheets were incubated with the second antibody. Sheets were washed again and embedded into Mowiol-DABCO (Roth) and analyzed by microscopy using Zeiss AxioVision 4.6 software.

To investigate the in vivo migration of DCs from the skin to skin-draining LNs in a short-term model, the dorsal skin was shaved, and 80 µL of 0.5% DNFB in acetone/olive oil (5:1) was applied to the skin. After 2 and 4 days, skin-draining LNs (brachial, axillary, and inguinal) were harvested and analyzed by flow cytometry.

For in vivo rescue of CCL17 deficiency, 25 µL of recCCL17 (1,000 ng/mL) in PBS or PBS alone as a solvent control was injected into the ear pinna before application of 10 µL of 0.5% DNFB in acetone/olive oil (5:1). Epidermal sheets were prepared as described above.

To analyze the expression pattern of green fluorescent LCs in epidermal sheets, mice were treated with 0.5% DNFB for 4 h. The

ears were divided at the cartilage site and placed in RPMI 1640 medium with all supplements. After 2 days of culture at 37 °C, the epidermis and dermis were separated by EDTA incubation, and the epidermis was fixed in 4% PFA for 3 h and a sucrose gradient, and then embedded in Mowiol (Roth) before analysis.

Isolation of LCs. The hair of the dorsal skin was removed, the skin was washed with 70% alcohol, and subcutaneous fat was removed. Skin pieces were placed on 0.25% trypsin/PBS for 2 h at 37 °C. The epidermal layer was peeled from the underlying dermis, and cells were released by breaking up epidermal sheets with forceps, followed by vigorous pipetting. Cells were immediately stained for FACS analysis or taken into culture with 1 µg/mL LPS (O11: B4; Sigma-Aldrich) overnight at 37 °C before staining.

ELISA. Serum was collected 1 day after the end of the AD treatment period. Total IgE level was determined by ELISA using the standard BD Biosciences protocol. In brief, 96-well microtiter plates (Nunc) were coated with 2 µg/mL of purified rat anti-mouse IgE mAb (clone R35-72; BD Biosciences). Bound IgE was detected with 2 µg/mL of biotin-conjugated rat anti-mouse IgE (clone R35-118; BD Biosciences). Purified mouse IgE (clone C38-2; BD Biosciences) was used as a standard. OVA-specific IgG1 and IgG2a were measured on OVA-coated microtiter plates [100 µg/mL OVA in coating buffer (eBioscience)] at room temperature overnight. Plates were washed with PBS-Tween 20 (0.05%) and blocked with PBS/3% BSA for 2 h at room temperature. Serial dilutions of the serum in PBS/1% BSA were incubated overnight at 4 °C. Subsequently, 2 µg/mL of biotin-conjugated rat anti-mouse IgG1 (clone A85-1) or IgG2a (clone R19-15; both from BD Biosciences) were added for 1 h at room temperature. Streptavidin-HRP (1/2,000; Amersham Biosciences) in PBS/1% BSA was incubated for 30 min at room temperature. Peroxidase substrate (TMB Plus ready-to-use substrate; Kem-En-Tec Diagnostics) was added, and absorbance was read with an automated ELISA reader (Gen5; BioTek). Antibody concentrations were calculated compared with an internal standard and are depicted in units.

Because IgE antibodies represent only a minor proportion of the total OVA-specific immunoglobulins in serum, a different method was used to determine OVA-specific IgE. Therefore, plates were coated with rat anti-mouse IgE (clone R35-72; BD Biosciences) in 0.1 M NaHCO₃ overnight at 4 °C (see also ref. 2). The plates were blocked, and serial dilutions of serum were incubated overnight at 4 °C. After washing, biotin-labeled OVA was added, and the mixture was incubated for 2 h. After streptavidin-HRP and TMB substrate were added, the absorption was read and reported as OD values.

RT-PCR. Skin biopsy specimens were frozen on dry ice and kept at –70 °C until being homogenized in EUROzol (Euroclone) using an Ultra-Turrax T8 (IKA). Total RNA was isolated according to the manufacturer's instructions and reverse-transcribed using an Applied Biosystems High-Capacity cDNA Reverse-Transcription Kit. mRNA expression levels of cytokines were determined by real-time quantitative PCR (AbiPrism 7500 Fast Real-Time PCR System; Applied Biosystems). PCR primers and probes for IL-4, IL-13, IL-10, IL-1β, IFN-γ, and 18S rRNA were purchased from Applied Biosystems as predeveloped reagents. Results were calculated by the comparative CT method according to the Applied Biosystems protocol, using ribosomal 18S rRNA as an endogenous control.

Flow Cytometry. Skin-draining LNs were incubated for 15 min at 37 °C with 1 mg/mL of collagenase D and 300 U/mL of DNaseI (both from Roche), and single-cell suspensions were generated and stained

with anti-mouse CD16/CD32 Fc block (clone 93; eBioscience). To analyze skin-derived DCs, cells were stained with anti-mouse CD11c (clone N418; eBioscience), anti-mouse MHCII (clone M5/114.15.2; eBioscience), and anti-mouse CD24a (clone M1/69; eBioscience). For receptor staining, BM-DCs or skin-derived DCs were incubated with anti-mouse CD16/CD32 Fc block (clone 93; eBioscience), anti-CD11c (clone N418; eBioscience), anti-CXCR4 (clone 247506; R&D Systems), and anti-MHCII and polyclonal goat anti-mouse CCR4 (Abcam) for 30 min on ice, or with anti-CCR7 (clone 4B12; eBioscience) for 4 h on ice. After washing, cells were stained with secondary antibodies for an additional 1 h when necessary. Cell suspensions were measured by FACS and analyzed with CellQuest 3.3 (BD Biosciences) and Flow-Jo (Tree Star).

Histology. Skin samples were obtained from patched areas 24 h after the third sensitization week, fixed in 4% PFA, and embedded in paraffin. Sections were stained with H&E or Giemsa. Mast cells were counted in six to eight randomly chosen areas of skin, and mean values $\pm$ SEM per mm of skin covering the entire dermis were calculated. Eosinophils per high-power field at 400 $\times$ magnification were counted. Pictures were taken with a Nikon Eclipse E100 microscope and a Hamamatsu C4742-80 digital camera, and analyzed with Zeiss AxioVision AutoMeasure software.

Immunofluorescence. Skin samples from affected areas were embedded in tissue-freezing medium (Jung) on dry ice. Then 7- μ m frozen sections were fixed with cold acetone, and unspecific binding was blocked by incubation with 10% FCS, 1% goat serum, and 1% rat serum, followed by an avidin D and biotin block (Vector Laboratories). After washing in PBS, sections were stained with the following antibodies: rat anti-mouse CD4 (clone RM4-5; BD Biosciences), rat anti-mouse MHCII (clone M5/114.15.2; BD Biosciences), and biotin rat anti-mouse Fc ϵ RI α (clone MAR-1; eBioscience) in 1% FCS. After washing, sections were incubated with goat anti-rat IgG Texas red (Invitrogen) or streptavidin FITC. Skin-resident DCs were detected with hamster anti-mouse CD11c (clone HL3; BD Biosciences). After washing, skin sections were incubated with biotinylated mouse anti-hamster mixture (clones G70-204 and G94-56; BD Biosciences), followed by streptavidin-FITC. All sections were embedded with VECTASHIELD HardSet (Vector Laboratories).

For detection of EGFP-expressing cells, skin samples were fixed in 4% PFA for 3 h and stabilized by a sucrose gradient. Skin sections were fixed with cold acetone and, after blocking of unspecific binding, incubated with anti-GFP, rabbit IgG fraction (Invitrogen). After washing, slides were stained with Alexa-Fluor 488 goat anti-rabbit IgG (Invitrogen). All skin samples were analyzed with AxioVision 4.6 (Zeiss).

In Vitro Migration Assay. BM-derived DCs were generated from WT, CCR4KO, CCL17E/+, and CCL17E/E mice on the C57BL/6 background as described previously (1). On day 6, nonadherent cells were harvested and activated, if not indicated otherwise, by incubation with 1 μ g/mL of *Escherichia coli* LPS overnight. On day 7, cells from the BM culture were harvested and counted. DC migration was measured in 24-well Costar transwell chambers (8- μ m pores) (Corning), as described previously (5). The upper chamber was loaded with 2×10^5 cells in 300 μ L of RPMI-1640/0.5% FCS. The lower chamber was filled with 700 μ L of RPMI 1640/0.5% FCS with or without 200 ng/mL of recombinant CCL19, CCL21, or CXCL12 (R&D Systems). After 4 h of incubation at 37 $^{\circ}$ C, the migrated cells were counted in a blinded fashion. In all experiments, the frequency of DCs among the migrated cells was determined by FACS and used to calculate the absolute number of transmigrated DCs.

For rescue of CCL17 deficiency, BM-DCs were incubated with different concentrations of recCCL17 (R&D Systems) and with LPS (100 ng/mL) overnight. To analyze the influence of CCL22 on the migratory ability of BM-DCs, recCCL22 (100 ng/mL;

R&D Systems) was added to BM-DCs stimulated with LPS (100 ng/mL) overnight. To inhibit CCL17 or CCL22, BM-DCs were incubated with a neutralizing antibody for CCL17 (100 ng/mL and 200 ng/mL) or CCL22 (10 μ g/mL) (both from R&D Systems), according to the manufacturer's instructions, overnight during LPS stimulation, along with 2 μ L/mL of purified anti-mouse CD16/32 antibodies (eBioscience) to block Fc receptors. As an internal control, purified isotype antibodies were used at the same concentration as the neutralizing antibodies.

siRNA. On day 6, 4×10^6 BM-DCs were transferred to a 4-mm cuvette (Bridge) and incubated in 100 μ L of Optimem (Invitrogen) and with oligonucleotides (10 μ g siRNA against the target sequence of CCL22 or 10 μ g of AllStars Negative siRNA (both from Qiagen) for 3 min before electroporation in a Gene Pulser X cell + CE module (Bio-Rad). Pulse conditions were square wave, 1,000 V, 2 pulses, and 0.5-ms pulse length. Three days after electroporation, immature DCs were stimulated with 500 ng/mL of LPS to maturity. RNAi efficiency was evaluated by measuring the CCL22 concentration by ELISA in the supernatant after overnight LPS stimulation. Analysis of the migratory behavior was performed in a transwell system.

Calcium Flux Assay. LPS-stimulated (100 ng/mL) cultures of BM-DCs (1×10^7 cells/mL) were loaded with 3 mM Indo-1 AM (Invitrogen) in RPMI 1640 without phenol red containing 10% FCS (PAN Biotech), L-glutamine, penicillin-streptomycin, and 2-ME (all from Gibco), and incubated for 30 min at 37 $^{\circ}$ C in the dark. Cells were washed once and transferred into Hepes buffer (pH 7.5) supplemented with 2 mM CaCl $_2$ and 2 mM MgCl $_2$ and incubated at 37 $^{\circ}$ C for an additional 15 min before measurements. The emissions of free and calcium-bound Indo-1 AM were analyzed by measuring 1.5×10^6 cells in 300 μ L at wavelengths of 485 and 405 nm, respectively, before and after stimulation with 500 ng/mL of CCL19, or as a positive control with ionomycin (1 μ g/mL). Analyses were performed with a Tecan Infinite M200 fluorescence microplate reader. For analyzing CXCL12 (500 ng/mL)-induced calcium flux, CD11c $^{+}$ DCs were purified from BM cultures to exclude CXCL12-responsive non-DCs from the analysis. For this purpose, BM-DC cultures were enriched on day 6 for CD11c $^{+}$ cells by MACS (Miltenyi Biotech) and stimulated with LPS (100 ng/mL) before staining with Indo-1 AM as described above. The DCs were >95% pure.

Three-Dimensional Collagen Gel Chemotaxis Assay. The 3D chemotaxis assays were performed as described by Friedl et al. (6) with the following modifications. Collagen I (PureCol; Inamed) was mixed with 0.7% sodium bicarbonate (Invitrogen) and 1 $\times$ MEM (Sigma-Aldrich) on ice. BM-DCs in RPMI 1640/0.5% FCS were carefully mixed with the collagen solution at a 2:1 ratio, resulting in gels with a collagen concentration of 1.7 mg/mL and a final cell concentration of 1.5×10^6 cells/mL gel. Collagen-BM-DC mixtures were placed in custom-made chemotaxis chambers and incubated in 5% CO $_2$ for 90 min at 37 $^{\circ}$ C. After collagen polymerization, 600 ng/mL of CCL19 (R&D Systems) in RPMI 1640/0.5% FCS was added on top of the gel. Time-lapse series of motile BM-DCs in 3D collagen gel were recorded using a fully automated inverted Nikon TE Eclipse microscope equipped with a 10 $\times$ phase contrast objective, motorized xyz-stage, a climate chamber (37 $^{\circ}$ C in 5% CO $_2$) and a Vosskühler CCD-1300 camera. Before analysis of chemotaxis, BM-DCs were tracked over 3 h (5 min/frame) using the ImageJ manual tracking plug-in. Subsequently, cell directionality, forward migration index, and velocity were calculated for 60 randomly chosen cells per sample using the Image J chemotaxis and migration tool plug-in (ibidi).

Statistics. All data were analyzed using the Student *t* test, if not indicated otherwise. *P* values are depicted in the figures as follows: ****P* < 0.001; ***P* < 0.01; **P* < 0.05.

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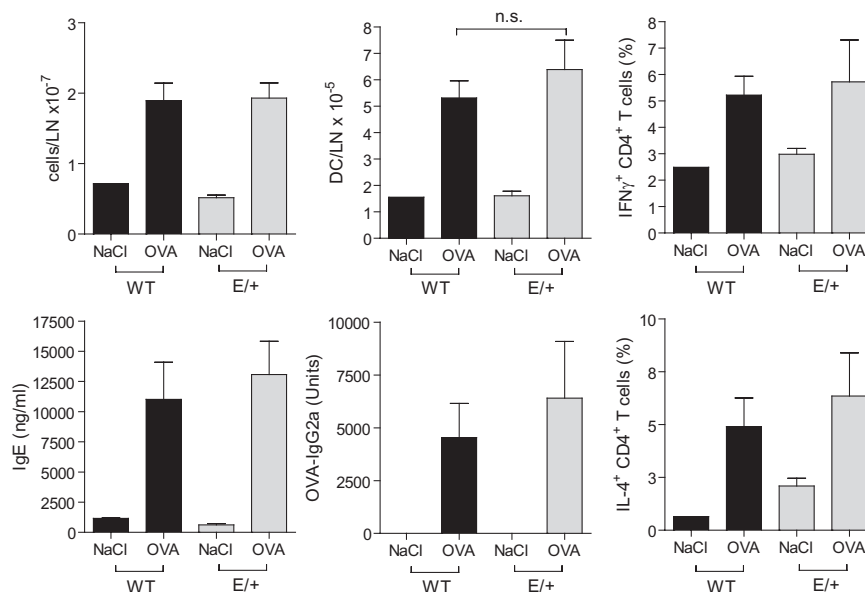


Fig. S1. Development of AD pathology in WT and CCL17E/+ mice. Comparison of AD symptoms of WT and CCL17E/+ mice (both BALB/c background) after treatment with three cycles of tape-stripping and application of a patch containing 1% OVA in NaCl (OVA) or solvent alone (NaCl). Shown are absolute numbers of total LN cells and DCs (*Upper Left and Center*), total serum IgE and OVA-specific IgG2a levels (*Lower Left and Center*), and frequency of IFN- γ - and IL-4-producing T cells (*Upper and Lower Right*). Black bars represent WT mice, and gray bars represent CCL17E/+ mice.

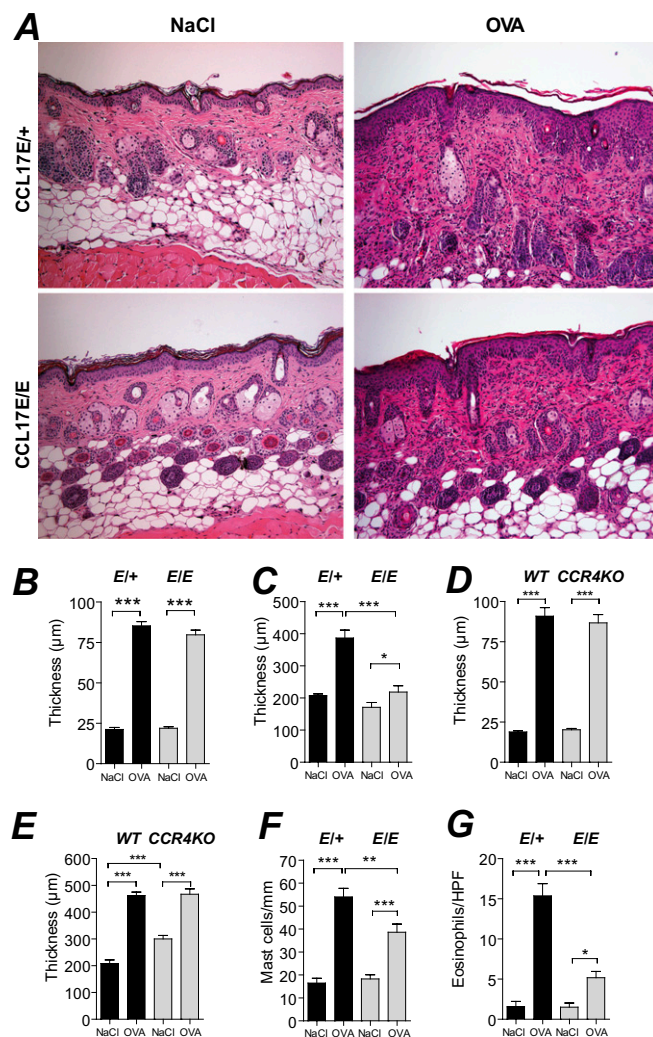


Fig. S2. Acanthosis and dermal inflammation in lesional skin. CCL17E/+ (E/+) and CCL17E/E (E/E) mice were treated with three cycles of tape-stripping and application of a patch containing 1% OVA in NaCl (OVA) or solvent alone (NaCl). At 24 h after the last treatment, the affected skin was fixed in 4% PFA and embedded in paraffin. Skin sections were stained with H&E (A). The size bar indicated in the figure has a length of 200 μ m. The thickness of the epidermis (B) and dermis (C) of CCL17E/+ versus CCL17E/E mice and of the epidermis (D) and dermis (E) of WT versus CCR4KO mice was measured at six randomly chosen sites per section, and mean values were calculated for a total of 12 mice from two independent experiments (6 mice/group). Mast cells (F) and eosinophils (G) were counted in sections from CCL17E/+ and CCL17E/E skin samples stained with Giemsa and H&E, respectively ($n = 6$ mice/group; two independent experiments). Data are presented as mean $\pm$ SEM. CCL17E/+ (B, C, F, and G) and WT (D and E) mice are indicated by black bars; CCL17E/E (B, C, F, and G) and CCR4KO (D and E) mice, by gray bars.

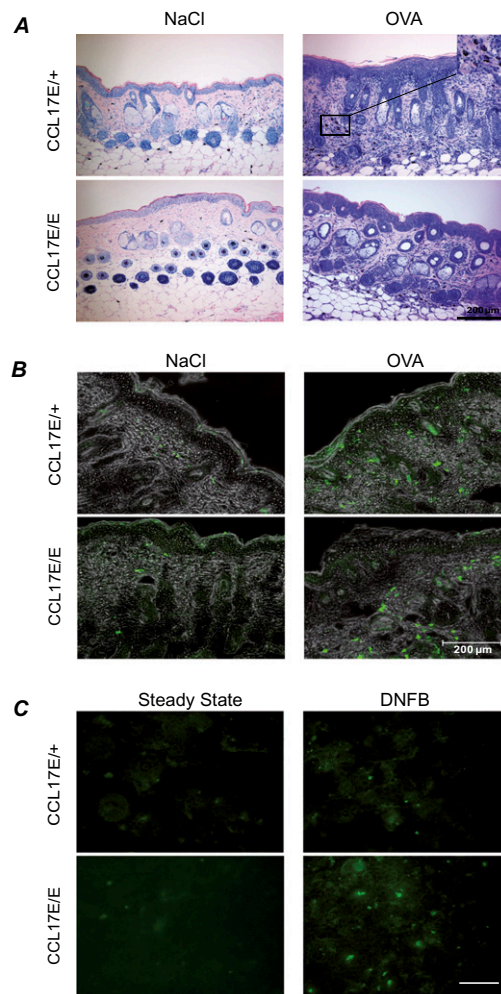


Fig. S3. Mast cell infiltration in AD-treated skin and up-regulation of CCL17/EGFP expression in treated skin. (A) Reduced mast cell inflammation in lesional skin. CCL17E/+ (E/+) and CCL17E/E (E/E) mice were treated with three cycles of tape-stripping and application of a patch containing 1% OVA in NaCl (OVA) or solvent alone (NaCl). At 24 h after the last treatment, the affected skin was fixed in 4% PFA and embedded in paraffin. For detection of mast cells, skin sections were stained with Giemsa. The insert depicts mast cells containing dark-staining vesicles at a higher magnification. (B) CCL17E/+ and CCL17E/E mice were treated with three cycles of tape-stripping and application of a patch containing 1% OVA in NaCl (OVA) or solvent alone (NaCl). PFA-fixed and frozen skin sections were stained with α -GFP ALEXA 488. Green fluorescence was overlaid with dark field. (Original magnification 200 $\times$; scale bar is indicated in the figure). (C) Green fluorescent cells in epidermal sheets of ears from CCL17E/+ and CCL17E/E mice in steady state and after application of 0.5% DNFB. Mice were killed 4 h after DNFB treatment, and ears were divided at the cartilage site and placed in culture for 2 days before separation of the epidermis and dermis by EDTA. The epidermal layer was fixed in 4% PFA for 3 h, followed by a sucrose gradient. (Scale bar: 200 μ m.)

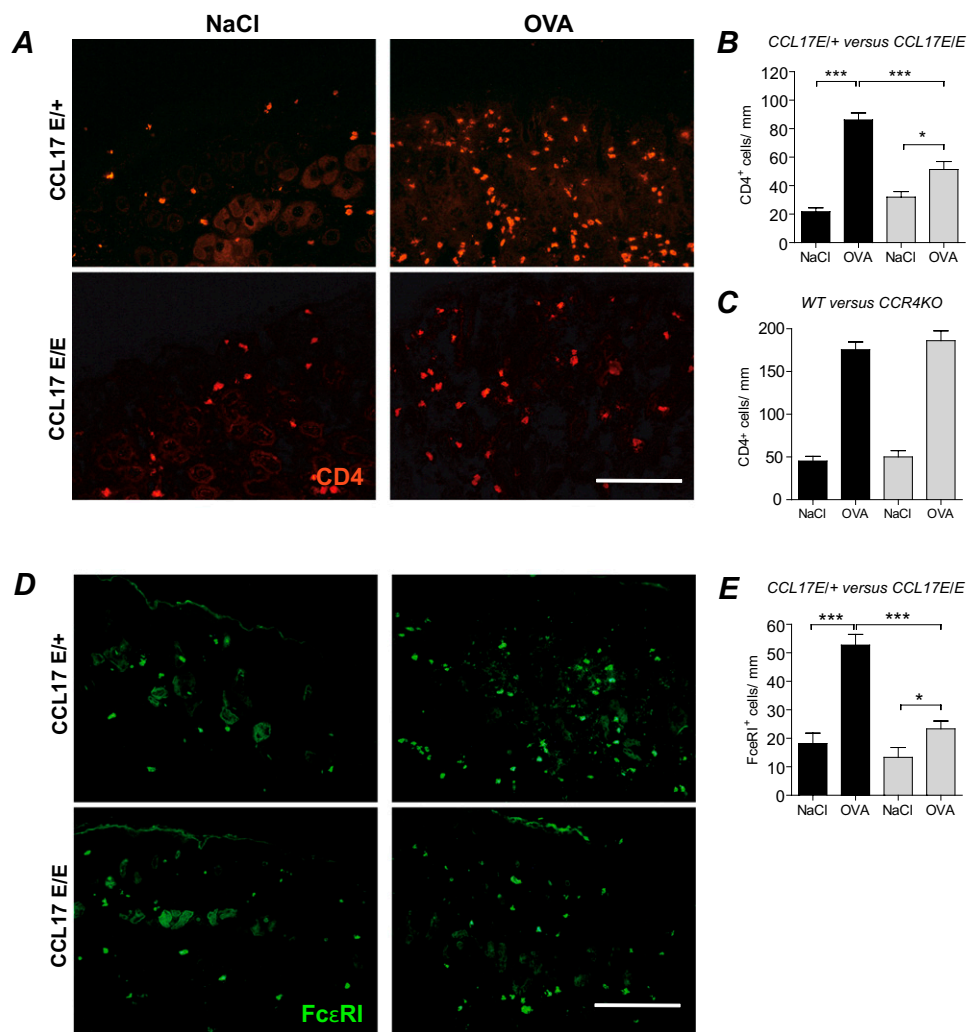


Fig. S4. Immunohistochemical analysis of lesional skin. (A–E) Infiltration of CD4⁺ and FcεRI⁺ cells in treated skin. Frozen skin sections of mice treated with three cycles of tape-stripping and application of a patch containing 1% OVA in NaCl (OVA) or solvent alone (NaCl) were stained with anti-CD4 Texas red (A–C) or anti-FcεRI FITC (D and E). (A, B, D, and E) CCL17E/+ mice were compared with CCL17E/E mice. In C, the number of CD4⁺ T cells in the skin is compared between treated WT mice and CCR4KO mice. Between six and eight independent fields per section were counted, and the mean value ($\pm$ SEM) per mm of skin covering the entire dermis was calculated. Data are representative of two or three independent experiments with between five and seven mice per group. Black bars represent CCL17E/+ mice (B and E) or WT mice (C); gray bars represent CCL17E/E mice (B and E) or CCR4KO mice (C).

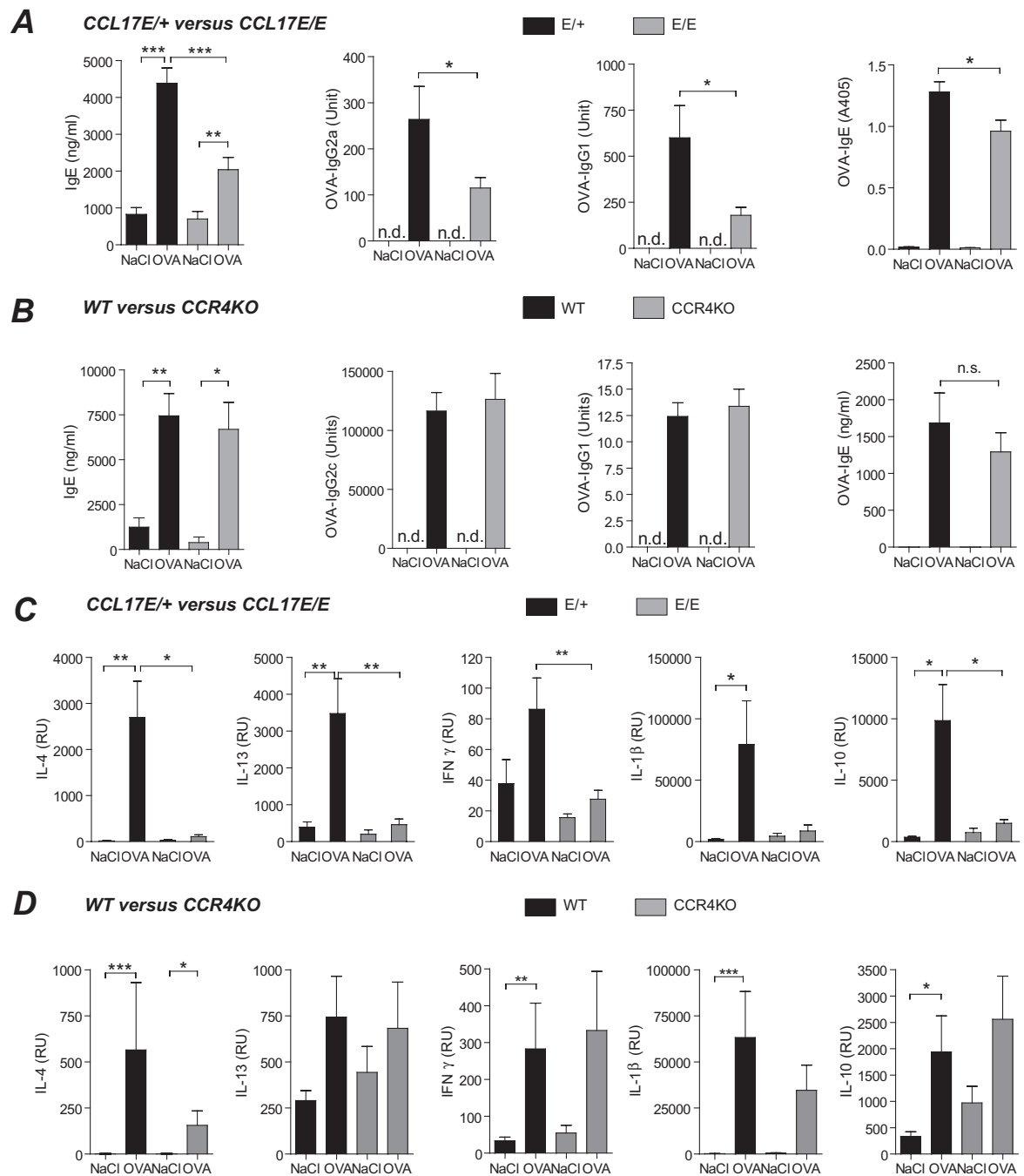


Fig. S5. Reduced allergen-specific humoral immune response and cytokine production in CCL17-deficient mice, but not in CCR4-deficient mice. CCL17E/+ (E/+) and CCL17E/E (E/E) (A) or WT and CCR4KO mice (B) were treated with three cycles of tape-stripping and application of a patch containing 1% OVA in NaCl or solvent alone as indicated. (A and B) At 24 h after the last treatment, the mice were bled, and total serum IgE was measured by ELISA. OVA-specific IgE was measured by an indirect approach using biotin-labeled OVA. The mean values of the ODs of two independent experiments with between five and seven mice per group are indicated as bars $\pm$ SEM. OVA-specific IgG1 (A and B) and IgG2a (A) or IgG2c (B) were measured using OVA-coated ELISA plates. Titers were expressed as units compared with a standard reference serum. Results were pooled from two or three independent experiments with between five and seven mice per group; data are reported as mean $\pm$ SEM. At 24 h after the last treatment, skin samples from CCL17E/+ and CCL17E/E mice (C) and from WT and CCR4KO mice (D) were shock-frozen, and total RNA was isolated and reverse-transcribed into cDNA. RT-PCR was performed with primers specific for IL-4, IL-10, IL-13, IFN- γ , and IL-1 β . Samples of between five and eight mice per group were analyzed individually; data are reported as mean $\pm$ SEM. (C and D) For calculating significance, the Mann-Whitney test was applied.

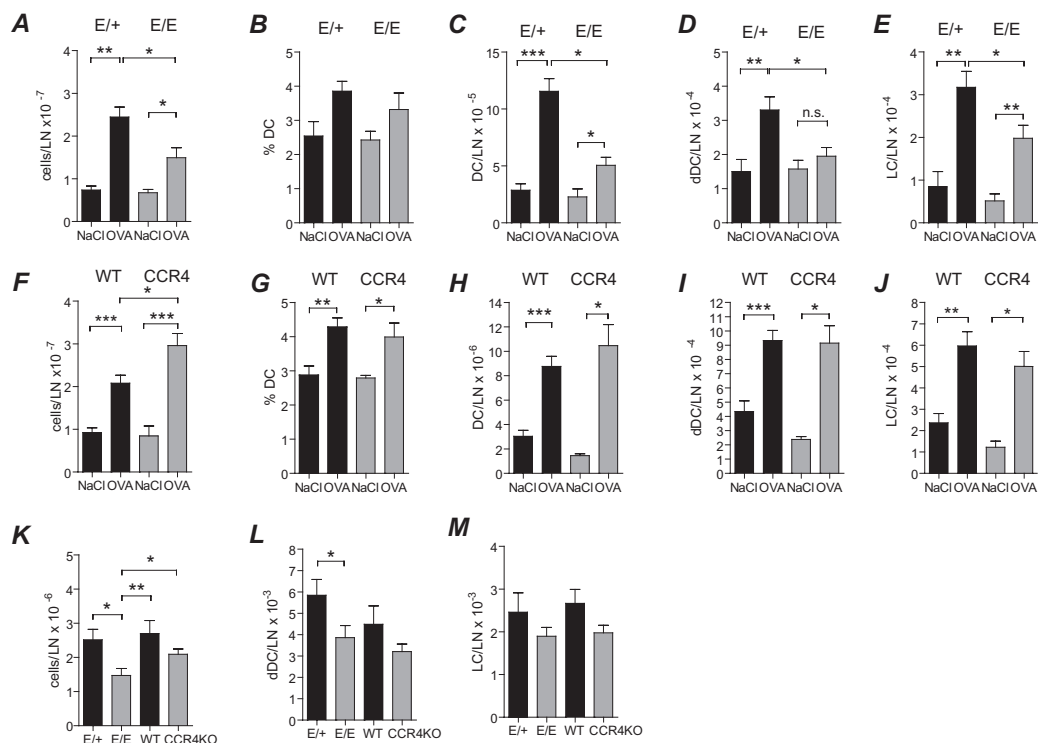


Fig. S6. Reduced numbers of skin-derived DCs in CCL17E/E mice, but not in CCR4KO mice. CCL17E/+ and CCL17E/E (A–E) or WT and CCR4KO mice (F–J) were treated with three cycles of tape-stripping and application of a patch containing 1% OVA in NaCl or solvent alone as indicated. Total numbers of leukocytes (A and F), frequency of CD11c⁺ DCs (B and G) and number of total DCs (C and H), dDCs (D and I) and LCs (E and J) per LN were calculated. For the NaCl-treated controls, LNs from a three mice were pooled for each measurement, whereas LNs from OVA-treated mice were analyzed individually. In A–E, CCL17E/+ mice are depicted by black bars, and CCL17E/E mice are depicted by gray bars. In F–J, WT mice are depicted by black bars, and CCR4KO mice are depicted by gray bars. (K–M) LNs of CCL17E/+, CCL17E/E, WT, and CCR4KO mice on C57BL/6 background were analyzed in steady state for total cellularity and numbers of dDCs and LCs. Data were collected from two or three independent experiments with between four and seven mice per group.

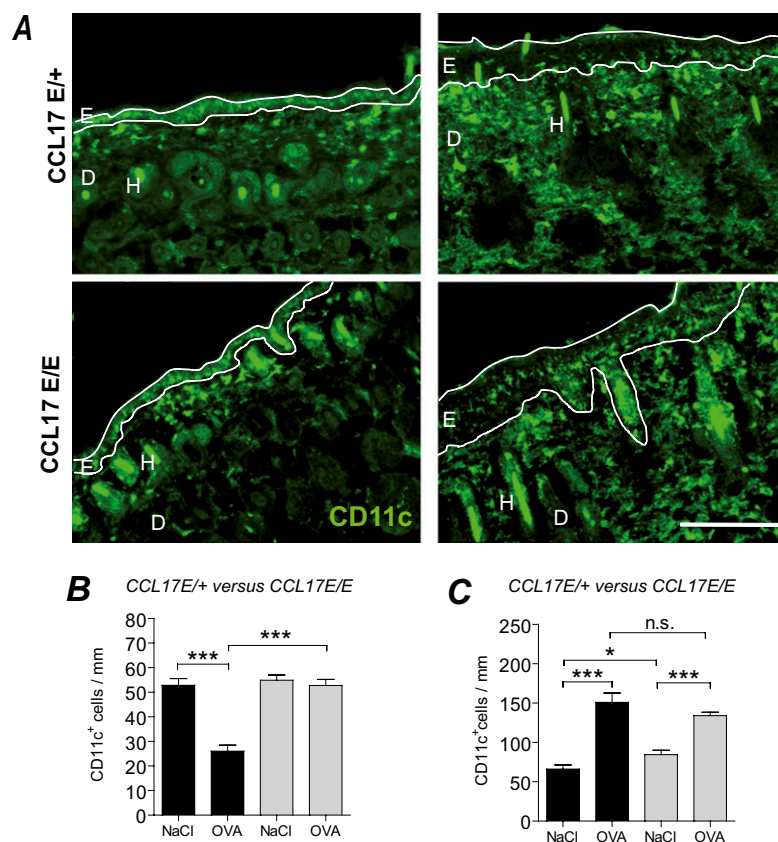


Fig. S7. Impaired emigration of DCs from lesional skin in the absence of CCL17. CCL17E/+ and CCL17E/E mice were treated with three cycles of tape-stripping and application of a patch containing 1% OVA in NaCl (OVA) or solvent alone (NaCl). At 24 h after the last treatment, the lesional skin was isolated and analyzed by immunofluorescence staining. CD11c FITC⁺ cells are localized in the epidermis (marked with a white "E") and dermis (marked with a white "D") (A). The demarcation between the epidermis and dermis is shown as a white line. Hair follicles ("H") exhibit an unspecific green fluorescence. The number of CD11c⁺ epidermal cells (B) and dermal cells (C) was analyzed. Between six and eight independent fields per section were counted, and mean $\pm$ SEM per mm of skin covering the entire dermis was calculated. Data are representative of two or three independent experiments with between five and seven mice per group. The black bars represent CCL17E/+ mice; the gray bars, CCL17E/E mice.

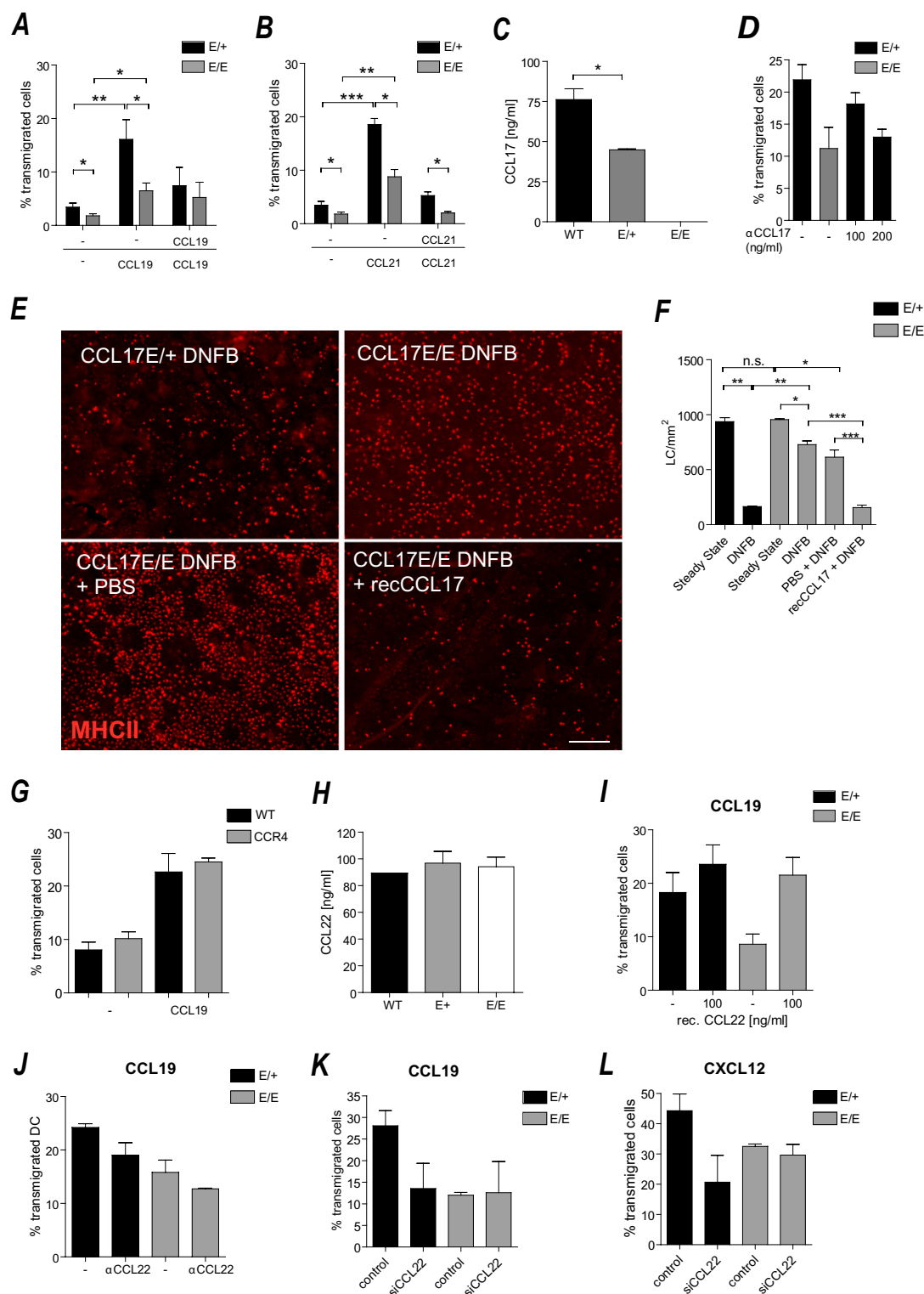
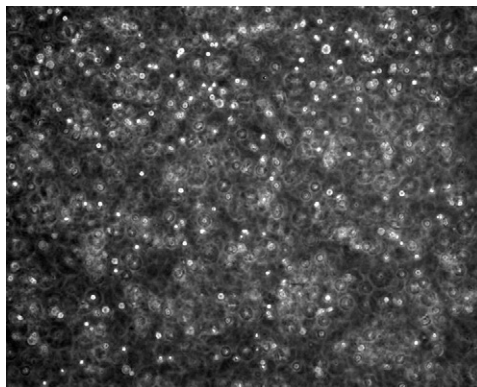


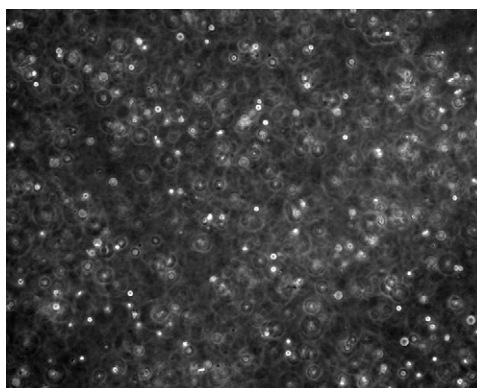
Fig. S8. Dependency of DC mobility and migration on CCL17. (A and B) Impaired chemotaxis and chemokinesis of CCL17-deficient DCs. BM-DCs were stimulated on day 6 with 1 μ g/mL of LPS overnight and analyzed in a transwell system (8 μ m pores) with or without a gradient of CCL19 (200 ng/mL) or CCL21 (200 ng/mL). After 3 h, DCs in the lower well were counted. (C) Amounts of CCL17 produced by BM-DC cultures of WT (black bar), CCL17E/+ (gray bar), and CCL17E/E (white bar) mice stimulated on day 6 with 100 ng/mL of LPS was measured by ELISA (R&D Systems). (D) Inhibition of CCL17 by neutralizing antibodies in vitro. BM-DCs were stimulated on day 6 with 100 ng/mL of LPS and incubated with or without a neutralizing antibody to CCL17 (R&D Systems) overnight. The black bar indicates CCL17E/+ BM-DCs, and the gray bar indicates CCL17E/E BM-DC. (E and F) In vivo rescue of LC migration by recCCL17. (E) Ears of CCL17E/+ and CCL17E/E mice were treated with 0.5% DNFB, and epidermal sheets were stained for MHCII (Upper). In addition, epidermal sheets were prepared after CCL17E/E mice have been injected with 25 μ L of PBS per ear (Lower Left) or with 25 μ L of recCCL17/PBS (1 mg/mL) (Lower Right) before DNFB (0.5%) application. (F) Quantification of the number of LCs per mm² of MHCII-stained epidermal sheets of ears of CCL17E/+ (black bars) and CCL17E/E mice (gray bars) in steady state, and after DNFB application with or without PBS or recCCL17 injection as indicated. (G) BM-DCs of WT mice (black bar) and CCR4KO mice (gray bar) were stimulated on day 6 with 1 μ g/mL of LPS and analyzed in a transwell system for migration toward CCL19 (200 ng/mL). (H) Concentration of CCL22 in BM-DC cultures incubated with LPS (100 ng/mL) Legend continued on following page

overnight. The black bar depicts WT-derived BM-DCs; the gray bar, CCL17E/+ derived BM-DCs; the white bar, CCL17E/E derived BM-DCs. (I) BM-DCs were stimulated with 100 ng/mL of LPS and recCCL22 (R&D Systems) overnight and analyzed in a transwell system for migration toward a gradient of CCL19 (200 ng/mL). After 4 h, DCs in the lower well were counted. Shown are representative results from one of three independent experiments. (J) Inhibition of CCL22 by neutralizing antibodies in vitro. BM-DCs were stimulated on day 6 with 100 ng/mL of LPS and incubated with or without a neutralizing antibody to CCL22 overnight. (K and L) CCL22 inhibition by siRNA reduces DC migration toward CCL19 and CXCL12. BM-DCs were electroporated with control or CCL22 siRNA on day 6 and stimulated on day 10 with 500 ng/mL of LPS overnight. Cell migration toward CCL19 or CXCL12 (200 ng/mL; R&D Systems) was analyzed in transwell assays. The black bars depict CCL17E/+ BM-DCs, and the gray bars depict CCL17E/E BM-DCs.



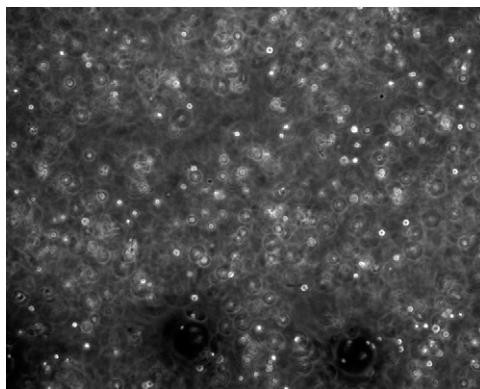
Movie S1. Random motility of LPS-stimulated CCL17E/+ BM-DCs within a 3D collagen I gel (1.6 mg/mL) with no applied chemotactic gradient. The time lapse over 3 h (one frame/5 min) with a 10× phase-contrast objective was monitored.

[Movie S1](#)



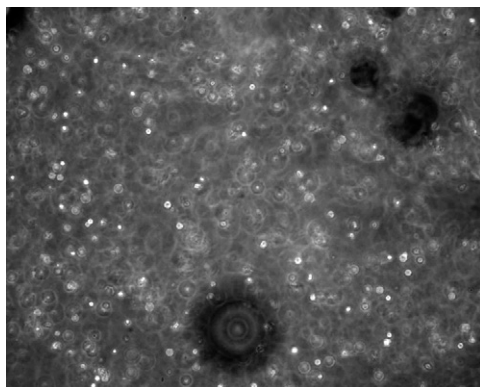
Movie S2. Random motility of LPS-stimulated CCL17E/E BM-DCs within a 3D collagen I gel (1.6 mg/mL) with no applied chemotactic gradient. The time lapse over 3 h (one frame/5 min) with a 10× phase-contrast objective was monitored.

[Movie S2](#)



Movie S3. Directional migration of LPS-stimulated CCL17E/+ BM-DCs within a 3D collagen I gel (1.6 mg/mL) toward a diffusive chemotactic gradient (600 ng/mL CCL19 on top). The time lapse over 3 h (one frame/5 min) with a 10× phase-contrast objective was monitored.

[Movie S3](#)



Movie S4. Directional migration of LPS-stimulated CCL17E/E BM-DCs within a 3D collagen I gel (1.6 mg/mL) toward a diffusive chemotactic gradient (600 ng/mL CCL19 on top). The time lapse over 3 h (one frame/5 min) with a 10× phase-contrast objective was monitored.

[Movie S4](#)