CCR7 Governs Skin Dendritic Cell Migration under Inflammatory and Steady-State Conditions

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Summary

The CC chemokine receptor CCR7 has been identified as a key regulator of homeostatic B and T cell trafficking to secondary lymphoid organs. Data presented here demonstrate that CCR7 is also an essential mediator for entry of both dermal and epidermal dendritic cells (DC) into the lymphatic vessels within the dermis while this receptor is dispensable for the mobilization of Langerhans cells from the epidermis to the dermis. Moreover, a distinct population of CD11c+MHCIIhigh DC showing low expression of the costimulatory molecules CD40, CD80, and CD86 in wild-type animals was virtually absent in skin-draining lymph nodes of CCR7-deficient mice under steady-state conditions. We provide evidence that these cells represent a semimature DC population of DC that is capable of initiating T cell proliferation under conditions known to induce tolerance. Thus, our data identify CCR7 as a key regulator that governs trafficking of skin DC under both inflammatory and steady-state conditions.

Introduction

Chemokines are small chemotactic cytokines that regulate the homeostatic migration of leukocytes through lymphoid organs as well as the inflammation-induced recruitment of immune cells to sites of infection and inflammation (Baggiolini, 1998; Cyster, 1999). Data obtained from gene-targeted mice revealed that the complex interactions between DC, T cells, and B cells within specialized compartments of lymphoid tissues are guided by a network of chemokines and chemokine receptors (Cavanagh and Von Andrian, 2002). While native T and B cells enter lymphoid organs following the interaction of CCR7 with its ligands CCL19 and CCL21, which are expressed at the luminal site of high endothelial venules, antigen-bearing DC migrate from the periphery into lymphoid organs via afferent lymphatics (Forster et al., 1999; Steinman, 1991). Once within the lymphoid organs, DC express CCL19, which is known to attract naive T cells as well as other DC through the interaction with CCR7 (Luther et al., 2000; Sallusto et al., 1998). In contrast to the widely accepted model for lymphocyte migration, molecular mechanisms regulating DC trafficking are still largely unknown.

Based on the chemokine receptor expression profile of in vitro differentiated DC, as well as on chemokines found to be expressed in the inflamed skin, it has been proposed that the production of chemokines at sites of inflammation recruits bone marrow-derived immature DC to these places (Saeki et al., 1999; Sallusto et al., 1998; Sozzani et al., 1998). According to this hypothesis, transendothelial migration and recruitment of immature DC and monocytes to skin is mediated by the expression of several inflammatory chemokines. These include the CCR4 ligands CCL22 and CCL17 (Campbell et al., 1999; Katou et al., 2001); CCL20, the ligand of CCR6 (Dieudosjean et al., 2000); CCL2, which is a ligand of CCR2 (Barker et al., 1991); as well as the CXCR3 ligands CXCL9, CXCL10, and CXCL11 (Filer et al., 2001). Interestingly, most of the chemokine receptors mentioned were found on immature in vitro differentiated DC, while mature DC have been shown to express primarily the chemokine receptors CCR7 and CXCR4, both known to regulate homeostatic trafficking of lymphocytes (Dieu et al., 1998; Sallusto et al., 1998). Thus, it has been postulated that a switch in chemokine receptor expression enables activated DC to leave the site of inflammation and to migrate to the T cell zones of the draining LN via afferent lymphatics. Indeed, we have demonstrated earlier that CCR7 deficiency, in addition to defective T and B cell homing, causes severely impaired migration of activated Langerhans cells (LC) into draining LN (Forster et al., 1999). Since impaired migration of LC has also been observed in antigenically challenged plt/plt mice, which lack expression of the CCR7 ligands CCL19 and CCL21-Ser (Gunn et al., 1999), we were interested in further clarifying the role of this chemokine receptor in DC migration.

We now provide strong evidence that, while CCR7 seems to be dispensable for the initial mobilization of LC within the epidermis, it is indispensable for DC migration into afferent dermal lymphatics. Moreover, the absence of the semimature DC population (CD11c−MHCII−, CD40−, CD80low, CD86low) in LN of unchallenged CCR7-deficient mice demonstrates that CCR7 is essentially required for the steady-state migration of skin-derived DC in the absence of inflammatory signals. We show that these cells induce antigen-specific T cell activation under conditions known to induce tolerance. Since adoptive transfers demonstrate that this function is severely impaired in CCR7-deficient animals, our data point to a role of CCR7 in the maintenance of peripheral tolerance.

Results

CCR7 Is Required for the Entry of DC into Dermal Lymphatics

In an earlier study, we reported that CCR7-deficient mice show a severe decrease in the accumulation of cutane-
ous DC in skin-draining LN following skin painting with FITC (Forster et al., 1999). However, it remained unclear at which step CCR7 deficiency interferes with DC migration from the skin to the draining LN. Therefore, we studied the migration process of these cells in greater detail. Using a mAb specific for Langerin, there was no obvious difference regarding shape and distribution of Langerhans cells (LC) within freshly isolated epidermis between wild-type and CCR7-deficient mice (Figures 1A and 1B). In order to test the mobilization capacity of peripheral DC in vitro, skin explants were floated on culture medium. After 24 hr, the epidermis was separated from the dermis and analyzed by immunohistochemistry. Within this period of time, LC changed their morphologic appearance and decreased in numbers in both wild-type and CCR7-deficient skin (Figures 1C and 1D). At the same time, DC in the dermis of wild-type mice gave rise to formations known as “dermal cords” (Figure 1E) representing the accumulation of MHCII+ DC including Langerin-positive cells along dermal lymphatics after the in vitro culture of skin explants (Larsen et al., 1990; Weinlich et al., 1998). Interestingly, formations resembling dermal cords were never observed in the dermis of CCR7-deficient mice, although MHCII+ DC and Langerin+ cells were present (Figure 1F). In contrast, analysis of the culture medium collected 24 and 48 hr after the onset of skin explant culture revealed a 3- to 4-fold increase in the number of MHCII+ CD86+ cells emigrated into the culture medium of skin explants isolated from CCR7-deficient mice when compared to wild-type mice (Figure 1G). These data demonstrate that CCR7 is essentially involved in the migration of DC towards or into lymphatics within the dermis, an observation that is in agreement with the expression of the CCR7-ligand CCL21 in lymphatic endothelium (Saeki et al., 1999).

In Vivo Activation of Langerhans Cells
Analyzing the number and distribution of resident epidermal DC in untreated wild-type and CCR7−/− mice using anti-langerin mAb staining on epidermal sheets isolated from the ear, we could not observe any significant difference with regard to distribution or density of MHC class II+ cells between wild-type and CCR7 mutants. Wild-type epidermis harbored 454 ± 35 MHC class II+ cells/mm² while that of CCR7-deficient animals contained 447 ± 33/mm² (mean ± SD, p < 0.01, Figure 1H, open columns). To further investigate the emigration of LC from the epidermis, we applied an in vivo migration model. LC migration was induced by sensitizing the ear skin with FITC, and 18 hr later the frequency of Langerin+ cells in the epidermis was analyzed. As shown in Figure 1H, LC were mobilized from the epidermis of wild-type and CCR7-deficient mice to the same extent. These data, together with the unimpaired in vitro emigration of CCR7-deficient LC from the epidermis (see Figures 1D and 1G), demonstrate that CCR7 is not required for the initial step of LC mobilization, i.e., the migration from the epidermis toward the dermis. Instead, our observations point to a decisive role for CCR7 in the entry of activated LC into draining lymphatics once these cells reach the dermis.

Figure 1. Effects of CCR7 Deficiency on Distribution and Mobilization of Langerhans Cells and Skin Dendritic Cells
(A–F) To analyze LC distribution in the untreated skin, epidermal sheets were prepared from the ear of wild-type (A) or CCR7−/− (B) mice and stained with rat anti-Langerin mAb (clone 929F3) and mouse anti-rat-Cy3. In the explant model, skin flaps were isolated from wild-type (C and E) and CCR7−/− (D and F) ear and floated on tissue culture medium for 48 hr before epidermis (C and D) was separated from dermis (E and F) followed by staining with a rat anti-Langerin (C–F) or FITC-conjugated anti-MHCII mAb (E and F).

(G) The number of CD86+ MHCII+ cells emigrated into the tissue culture medium 24 hr and 48 hr after the onset of culture.

(H) Ears of C57BL6 and CCR7−/− mice were treated with 0.1% FITC in acetone/dibutylphthalat or carrier alone. Mice were sacrificed 18 hr later and epidermal sheets were stained with rat anti-Langerin mAb (clone 929F3) and mouse anti-rat-Cy3, and the number of Langerin-positive cells was determined by epifluorescent microscopy. Data are derived from three images per epidermal sheet (10× original magnification, 0.8 mm²/image) of at least six ears of each genotype and treatment (**p < 0.01, unpaired t test).
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Figure 2. CCR7-Deficient DC Fail to Migrate to Skin-Draining Lymph Nodes

(A) In vitro differentiated, bone marrow-derived DC from wild-type and CCR7-deficient mice were labeled with CFSE and TAMRA, respectively, mixed at equal numbers, and injected i.c. into the right footpad. After 36 hr, the popliteal LN was removed and analyzed for the presence of injected DC.

(B) Same as (A) with the exception that wild-type DC were labeled with TAMRA and CCR7-deficient DC with CFSE and cells were injected into the left footpad of the same mice used in (A).

(C) Mean ± SEM of the data derived from 8 popliteal LN of the experiments shown in (A) and (B).

In Vitro Differentiated CCR7-Deficient DC Fail to Migrate into the Draining LN

To further substantiate the hypothesis that CCR7 is required for the entry of DC into lymphatics, we used bone marrow-derived in vitro differentiated DC. TNFα/PGE2-matured DC from wild-type and CCR7-deficient mice were labeled with the fluorescent dyes CSFE (green) and TAMRA (red), respectively, mixed at equal numbers and injected i.c. into the right footpad of wild-type mice. In order to rule out that the dye-labeling procedure adversely influences the migration of the DC, TAMRA-labeled wild-type and CSFE-labeled CCR7−/− DC were injected into the left footpad of the same animals. As demonstrated in Figures 2A and 2B, only DC derived from wild-type mice could be identified in significant numbers in the popliteal LN 36 hr after transfer, while the CCR7-deficient DC failed to immigrate into the LN (Figure 2C). These data are consistent with previous findings (Martin-Fontecha et al., 2003) and further support the hypothesis that CCR7 is essentially required for DC to migrate from the skin toward the LN via the CCL21 expressing lymphatics.

Lack of CD11c+MHCIiHigh Cells under Steady-State Conditions in Skin-Draining LN of CCR7-Deficient Mice

Since several distinct DC populations have been identified in lymphoid organs in the mouse, presumably serving different biological functions (Shortman and Liu, 2002), we further analyzed the subpopulations of DC present in peripheral LN. While the total number of CD11c− DC was reduced to 60% in CCR7-deficient mice (Figure 3), there was no significant difference in numbers regarding CD4+ CD8− DC. In contrast, CD4+ CD8− DC were reduced to 42% and 34%, respectively. Of interest, DC expressing both CD4 as well as CD8 were particularly scarce in the LN of CCR7−/− mice (only 17% of the cell count of wild-type mice; Figure 3). These data indicate that CCR7 deficiency causes an imbalance among defined subpopulations of DC present in peripheral LN in the steady state. Further analysis revealed a population of CD11c+MHCIiHigh DC present in all skin-draining but not organ-draining LN of wild-type mice that was almost completely absent in skin-draining LN of CCR7 mutants (Figures 4A–4C). Since we noticed impaired migration of skin DC after skin sensitization in CCR7-deficient mice (data not shown and Forster et al., 1999), and based on the observation that 24 hr after FITC-skin sensitization all FITC− cells in the draining LN of wild-type mice display this particular CD11c+ MHCIiHigh phenotype (data not shown), we further characterized this cell population. In wild-type animals within the population of CD11c+ MHCIiHigh, a subpopulation exclusively expressed CD207/Langerin, a marker thought to be specific for LC (Figure 4D). These data indicate that these skin-derived LC show a CD11c+ MHCIiHigh phenotype once they reached the draining LN. Neither the very few remaining cells in the gate containing CD11c+ MHCIiHigh cells nor CD11c− cells with lower expression levels of MHCIi in the skin-draining LN of CCR7−/− mice expressed CD207 (Figure 4E), indicating that CCR7 is indispensable for the steady-state migration of LC to lymphoid organs.

In order to further substantiate the hypothesis that CD11c+ MHCIiHigh cells represent DCs that migrate to LN under steady-state conditions, Balb/c mice that were born and maintained in a germ-free environment were analyzed. In skin-draining LN of these animals, CD11c+ MHCIiHigh cells were identified in numbers comparable to those obtained from SPF animals (data not shown). It has recently been suggested that epidermal DCs are CD40HighCD11cHigh while dermal DCs are CD40LowCD11cLow in LN (Ruedl et al., 2000). Expression of these markers was assessed on DCs isolated from wild-type and CCR7-deficient LN. We observed that CD40HighCD11cHigh LC are virtually absent in CCR7-deficient mice (popula-
Figure 4. Lack of Skin-Derived DC and Langerhans Cells in Skin-Draining LN of CCR7-Deficient Mice
(A and B) LN cells derived from 10 skin-draining LN (inguinal, axillary, brachial, popliteal, and facial) of C57BL6 (A) and CCR7-deficient C57BL6 (B) mice were pooled and analyzed for the expression of CD11c and MHCII.
(C) Number of cells falling within the upper right gate shown in (A) and (B) (data derived from three wild-type and three mutant mice of one experiment; similar data were derived from at least six additional mice of each genotype).
(D) Langerin+ cells were found only in the CD11c+ MHCIIhigh DC population (solid line, upper right gate shown in [A] and [B]) present in wild-type mice, whereas MHCIIint DC (dotted line, middle right gate shown in [A] and [B]) do not express Langerin.
(E) In CCR7-deficient mice, neither the residual MHCIIhigh nor the MHCIIint DC population contained any Langerin+ cells.
(F and G) Skin-draining lymph nodes of CCR7-deficient mice lack CD40int DC populations containing skin-derived DC. Cells from skin-draining LN of four wild-type mice (F) and four CCR7−/− mice were enriched for DC by density gradient centrifugation. DC were stained with anti-CD11c and anti-CD40.

Figure 5. Expression of CCR7 on LN Dendritic Cells and Freshly Isolated Epidermal Dendritic Cells
Lymp node cells (upper panel) or cells isolated from ear epidermis (lower panel) of C57BL6 mice were stained with anti-MHCII, anti-CD11c, and rat anti-mCCR7 (solid line) or isotype control (shaded area) and mouse anti-rat-Cy5. Expression of CCR7 on CD11c+ cells expressing intermediate levels of MHCII (left column) and high levels of MHCII (right column) is shown.

Expression of CCR7 on Lymph Node and Epidermal DC
Since CCR7-deficient mice missed several DC populations in peripheral LN, it was of interest whether CCR7 is differently expressed on DC. Applying a recently generated anti-CCR7 mAb, the CD11c+ MHCIIhigh population, which is missing in CCR7-deficient mice, was found to uniformly express CCR7, while this chemokine receptor was only expressed on a subpopulation of CD11c+ MHCIIint DC (Figure 5, upper panel). The expression of CCR7 on LC residing in the epidermis was then investigated. Analyzing freshly isolated epidermal LC, it was possible to separate these cells based on the amount of surface MHCII molecules into two groups: MHCIIint and MHCIIhigh (data not shown). However, no CCR7 expression was found on MHCIIint LC, while CCR7 expression was evident on MHCIIhigh LC to some degree (Figure 5, lower panel). These data indicate that some DC in the skin start to spontaneously upregulate CCR7, thereby allowing steady-state trafficking to the draining LN.

CD11c+ MHCIIhigh Cells Are Semimature
DC at Steady State
It has been reported that skin DC migrating under noninflammatory conditions into the draining LN display a semimature phenotype, since they express high levels of MHCII molecules typical for mature DC, but lack high expression levels of costimulatory molecules such as CD80 and CD86 (Lutz and Schuler, 2002; Stoitzner et al., 2003). Therefore, we further characterized CD11c+ MHCIIhigh cells from skin-draining LN for the expression of costimulatory molecules with and without prior FITC skin painting. The epicutaneous application of FITC induced a strong mobilization of CD11c+ MHCIIhigh cells, increasing the proportion of this particular cell population within all LN cells from 3.4% to 8.6% (mean of 6
The phenotype of the CD11c+ FITC-positive DC isolated from draining LN (Figure fold when comparing draining and nondraining LN in skin-derived DC essentially contribute to the population nondraining LN, while this number was increased 165-

Intracutaneous/subcutaneous (i.c./s.c.) application of various amounts of antigen (0.1 μg to 10 μg) together with CpG-DNA as an adjuvant induced a strong and dose-dependent induction of antigen-specific T cell proliferation in wild-type mice (Figure 7A, upper panel). This proliferative response was markedly reduced when OT-II cells (CCR7-positive) were transferred to CCR7-deficient mice. Under these experimental conditions—CCR7 deficiency affects DC but not T cell migration—a moderate proliferative response was identified at the highest amount of antigen applied (10 μg), while proliferation was barely detectable at lower doses (Figure 7A, lower panel).

We next tested the potential of i.c./s.c. applied LPS-free antigen to promote antigen-specific proliferation of T cells in the draining LN in the absence of adjuvant and danger signals. In accordance with previous results, proliferation of adoptively transferred MHCI-restricted OT-II cells was strongly induced in the draining LN of wild-type mice after i.c./s.c. application of small amounts of soluble antigen (Figure 7C), while no proliferating antigen-specific T cells were identified after PBS injection to the contralateral side (Figure 7D). As described above for inflammatory conditions, a dose-dependent proliferation of OT-II cells was observed only if cells were transferred into wild-type animals (Figure 7C, upper panel) but not after transfer into CCR7-deficient recipients (Figure 7C, lower panel). In the latter case, no proliferation was observed at 0.1 μg and 1 μg OVA while a very weak response was found at 10 μg OVA.

Conjugation of antigen to the anti-CD205 mAb NLDC145 has been shown to allow efficient presentation of the conjugated antigen in MHCI molecules and alsopermits effective crosspresentation in MHCI molecules (Bonifaz et al., 2002; Hawiger et al., 2001). Application of the conjugate antigen to the anti-CD205 mAb NLDC145 has been shown to allow efficient presentation of the conjugated antigen in MHCI molecules (Bonifaz et al., 2002; Hawiger et al., 2001). Application of this antigen:mAb conjugate allows antigen targeting to DC without maturation, thus resembling DC migration and antigen presentation in the steady state. Again, following the application of OVA coupled to anti-CD205, no substantial proliferation of OT-II cells occurred in the draining LN of CCR7-deficient mice. Draining LN contained only approximately 4 times the number of proliferating T cells compared to those observed in the nondondraining LN, while this number was increased 165-fold when comparing draining and nondondraining LN in wild-type animals (Figure 7E, data not shown). In order to rule out that priming did occur in CCR7-deficient mice but was not sufficient to induce proliferation, the expression of the early activation marker CD69 was analyzed 36 hr after immunization with 1 μg OVA without adjuvant. As shown in Figure 7F, a profound upregulation of CD69 on OT-II cells was observed upon adoptive transfer into wild-type recipients but not when trans-
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intravenously into wild-type and CCR7-deficient mice. A comparable proliferative response of the OT-II cells was observed irrespective of transfer to wild-type or CCR7-deficient recipients (Figure 7G).

Discussion

Several lines of evidence suggest that DC reprogram their chemokine receptor expression profile during defined maturation stages. One of the two chemokine receptors expressed on mature but not on immature bone marrow-derived DC is CCR7 (Sallusto et al., 1998; Yanagihara et al., 1998), a receptor also known to regulate entry of lymphocytes into LN (Forster et al., 1999) and the antigen-induced migration of B cells from the B cell follicle to the outer T cell area during the initiation of an B cell immune response (Reif et al., 2002). In addition, it has been shown previously that the migration of skin DC toward the draining LN is impaired in CCR7-deficient mice under inflammatory conditions (Forster et al., 1999).

Within the heterogeneous group of DC, LC represent the population of skin-resident DC that have been most intensely studied with regard to their migratory properties. LC reside within basal and suprabasal laminae of the epidermis and are equipped with the machinery to capture and process potentially harmful pathogens at the time that they invade the body, thereby rapidly initiating an immune response (Schuler and Steinman, 1985; Stingl et al., 1980). It has been known for a long time that skin DC, including LC, are mobilized to migrate to the draining LN once they have been activated, but recent work demonstrated that these cells are also found in the draining LN in the absence of an inflammatory event, suggesting a continuous turnover of DC, a process described as steady-state migration (Hemmi et al., 2001; Merad et al., 2002; Yoshino et al., 2003).

Data presented here strongly suggest that CCR7 is involved in both activation-induced as well as steady-state turnover of skin DC. FITC-bearing CD11c<sup>+</sup>MHCII<sup>high</sup> cells are absent in the draining LN after skin sensitization of CCR7-deficient mice (inflammatory condition). More importantly, CD11c<sup>+</sup>MHCII<sup>high</sup> cells are also missing in the skin-draining LN under steady-state conditions. This phenotype is corroborated by the observation that a considerable proportion of CD11c<sup>+</sup>MHCII<sup>high</sup> cells express CCR7 at a moderate level already in the epidermis, indicating their imminent emigration. In skin-draining LN of wild-type mice, either after skin sensitization or in the steady state, a subpopulation of these CD11c<sup>+</sup>MHCII<sup>high</sup> cells expresses the Langerhans cell marker Langerin, CD207 (see Figure 4), suggesting that CCR7 is involved in recruiting LC as well as other skin-derived DC into skin-draining LN under inflammatory and steady-state conditions.

Although less pronounced than observed in CCR7<sup>−−</sup> mice, impaired mobilization of activated skin DC has also been reported in a naturally occurring mutant, the plt<sup>−−</sup> mouse (Gunn et al., 1999). This strain carries a deletion leading to loss of CCL19 expression as well as expression of one of the two CCL21 genes. The CCL21 gene missing in plt<sup>−−</sup> mice codes for a CCL21-ser gene product that differs...
in a single position from a second, CCL21-leu gene that is still present in plt/plt mice. While the CCL21-ser gene is specifically expressed in lymphoid organs, CCL21-leu is in particular found in nonlymphoid organs including lymphatic vessels of the skin (Luther et al., 2000; Nakano and Gunn, 2001; Vassileva et al., 1999). This residual expression of CCL21-leu in plt/plt mice might explain why activated skin DC are still able to form "dermal cords" in a skin explant model and to migrate to draining LN in plt/plt mice (Gunn et al., 1999) but not in CCR7−/− mice. Residual expression of CCL21 in plt/plt mice might also be responsible for unaffected steady-state migration that has been recently reported in these mice (Yoshino et al., 2003).

There is now accumulating evidence for a considerable proportion of DC of LN and spleen constitutively presenting self-antigens in the steady state, a mechanism believed to induce tolerance (Hugues et al., 2002; Wilson et al., 2003). Application of endotoxin-free antigens without adjuvant, or antigens specifically coupled to an anti-CD205 mAb, mimics confrontation of an organism with a harmless or self-antigen, and recent studies demonstrated that DCs presenting antigen under such conditions, i.e., DCs under steady state, will not induce an inflammatory immune response. Although they are able to vigorously stimulate T cell proliferation, these proliferated T cells are unresponsive to further stimulation and are eliminated a few days later, most likely due to lack of costimulatory signals (Bonifaz et al., 2002; Hawiger et al., 2001). The characteristics of these tolerance-inducing cells, which have also been called "semimature DC," have been reviewed recently (Lutz and Schuler, 2002). Stoizner et al. (2003) characterized steady-state migration of LC. Using a mAb specific for Langerin, they demonstrate that in steady state, LC present in skin-draining LN express low levels of maturation markers such as 2A1, CD86, and CD40 and high levels of MHCII as well as CD11c. These observations are in accordance with the data presented here. Langerin+ cells are identified as part of the CD11c+ MHCII+ population, which is entirely missing in CCR7-deficient mice under steady-state conditions. Peripheral LN of germ-free mice invariably possess this DC population, excluding the possibility that our observation of a continuously mobilized DC population was caused by ongoing but clinically not apparent infectious processes in the skin.

Our data derived from skin explant cultures confirm the hypothesis that CCR7 is selectively required to allow skin DC with a mature or semimature phenotype to enter skin lymphatics. In vitro cultures of skin explants induce the accumulation of DC along dermal lymphatics in wild-type mice, the so-called "dermal cords," an artificial structure occurring in experimental situations when the flow of lymph fluid is abrogated (Larsen et al., 1990). The formation of dermal cords is also observed in explant culture of skin derived from plt/plt mice, which express CCL21-Leu in dermal lymphatics (Gunn et al., 1999). In contrast, cultured skin derived from CCR7-deficient mice is completely devoid of these structures, pointing to a critical role of CCR7 in the migration of skin DC toward and into dermal lymphatics. The decisive and selective role for CCR7 in guiding cutaneous DC into skin lymphatics was corroborated by in vivo mobilization experiments, indicating that CCR7 is not required for the initial mobilization of LC within the epidermis, since no differences regarding the emigration of LC from the epidermis were observed between wild-type and CCR7-deficient mice following activation with FITC (Figure 1H).

The impaired migration of cutaneous DC observed in CCR7-deficient mice is reflected by the reduced capacity of CCR7-deficient mice to induce antigen-specific T cell proliferation after s.c. or i.d. application of antigen. Small amounts of antigen without additional adjuvant, reflecting tolerance-inducing conditions, failed to induce proliferation of antigen-specific lymphocytes in the draining LN of CCR7-deficient hosts. However, this small dose of antigen is capable of inducing a vigorous proliferation of the adoptively transferred OT-II T cells in wild-type hosts (Figure 7). The correlation of impaired DC migration and lack of antigen-specific proliferation in CCR7-deficient mice suggests that efficient T cell stimulation after low-dose antigen application can only be triggered by specialized antigen-presenting cells, most likely by CD11c+ MHCIIhigh DC entering the draining LN. In contrast, a higher dose of antigen together with adjuvant was sufficient to induce a weak proliferation of antigen-specific T cells in CCR7-deficient hosts. Passive transport of soluble antigen with the lymph fluid or by cells other than the described CD11c+ MHCIIhigh cells might contribute to presentation to and stimulation of T cells in the LN, although at lower efficiency. The idea that impaired migration of DC from the skin to the draining LN represents the limiting factor for the delivery of antigen to the LN of CCR7-deficient mice is supported by our finding that intravenous application of antigen induced a profound proliferative response of OT-II cells in both wild-type and CCR7-deficient animals. Nevertheless, since LN are morphologically altered in CCR7-deficient mice, we cannot entirely exclude the possibility that disturbed LN architecture might partially contribute to the reduced proliferative response of adoptively transferred T cells following antigen application.

The present study also reveals that in addition to recently immigrated skin DC, other subsets of DC are also diminished in LN of CCR7 mutants. The function and kinetics of the different DC subpopulations identified in lymphoid organs of man and mouse are currently under intense investigation. In mouse LN and spleen, various DC subsets expressing different surface markers were recently identified (reviewed in Shortman and Liu, 2002). One of these subsets, classified by its expression of CD8 (formerly designated as the lymphoid DC subset), is in particular missing in CCR7-deficient mice. Since it has been suggested that CD8+ DC are involved in the induction of TH1 responses (Maldonado-Lopez et al., 1999; Pulendran et al., 1999) as well as in the induction of tolerance (Belz et al., 2002) to peripheral self-antigen, it will be worth studying these mechanisms in mice carrying a DC-specific deletion of CCR7.

Our data provide ample evidence that CCR7 is essentially required for skin DC and LC migration into dermal lymphatics under inflammatory conditions, thus guiding them on their way to the draining LN. A CCR7-based mechanism is also effective in directing semimature skin DC and a subpopulation of LC under steady-state conditions to the skin-draining LN, cells that are thought to play a pivotal role in the maintenance of peripheral tolerance. Since CCR7−/− mice are not prone to spontane-
ously develop autoimmune diseases, it seems likely that other mechanisms are able to compensate the impaired steady-state DC migration in the induction of peripheral tolerance. Alternatively it seems plausible that potentially autoreactive T cells present in CCR7-deficient mouse are not efficiently activated, since naive T cells are also severely hampered from gaining entry into LN of CCR7−/− mice, leading to impaired T cell–DC interaction.

In summary, our data identify CCR7 as a conductor within an orchestra of molecules directing the egress of DC from the skin under inflammatory and steady-state conditions.

Experimental Procedures

Mice

CCR7−/− mice (mixed Balb/c × 129Sv/Ev genetic background) have been described earlier (Forster et al., 1999) and were used for the experiments shown in Figure 1G. For all other experiments, CCR7-deficient mice were used that had been backcrossed for seven generations to the C57BL/6 genetic background. Mice were maintained under SPF conditions and used at 8–12 weeks of age. All animals including germ-free Balb/c mice were maintained at the central animal facility of Hannover Medical School. The MHCII-restricted T cell receptor transgenic OT-II mice have been described earlier (Barnden et al., 1999).

Preparation of Epidermal Sheets and Fluorescent Epikut Staining of Skin DC

Epidermal sheets were obtained from ears of wild-type and CCR7−/− mice as described earlier (Price et al., 1997). Briefly, ears were split into dorsal and ventral halves and split open with a sharp razorshaped blade (for the edges) over a large petri dish containing PBS supplemented with 10% FCS and 50 μM β-mercaptoethanol; one volume of fresh medium was added at day 3, and on day 5, one volume of fresh medium was added. GM-CSF was produced by a NIH-3T3 cell line infected with Psi2-pM5DG#6 (Qin et al., 1997). On days 7 to 9, the culture medium was supplemented with 30 ng TNF/ml (R&D Systems) and 1 μg/ml prostaglandin-E2 (PGE2, Sigma) to induce maturation. Analysis of cells obtained with this procedure revealed that 90% expressed CD11c with 50%–60% showing high levels of MHCII and in the case of wild-type donors these cells also expressed high levels of CCR7.

For labeling cells with fluorescent dyes, 105 cells/ml DC in HBSS/2%FCS were incubated with either 3 μM CFSE or 10 μM TAMRA (Molecular Probes) for 10 min at 37°C. A mixture of each 105 CFSE- and TAMRA-labeled DC was injected in a volume of 30 μl into the foot pad of wild-type recipients.

Flow Cytometry

To obtain single cell suspensions of LN and spleen, organs were minced through a nylon mesh and washed with PBS supplemented with 3% FCS and 5 mM EDTA. Erythrocytes of the spleen were removed by hypotonic lysis with NH4Cl. Cells were stained with antibodies against MHCII, CD4, CD11c, CD40, CD80, CD86, V5–2, and Vj5.1-5.2 (Becton Dickinson) and CD86, CD69 (Caltag, Germany) as described earlier (Forster et al., 1994). CCR7 expression on LN or epidermal DC was detected by staining with rat-anti-mCCRF7 (clone 4B12) that will be described elsewhere (U. Ritter et al., submitted). The specificity of this antibody has been confirmed in the present study by lack of staining on DC and T cells isolated from CCR7−/− mice (data not shown). For the detection of Langerin (Cd207) expression, cells were fixed with 1% paraformaldehyde for 10 min on ice, washed, and resuspended in 0.1% saponin (Sigma, Germany) in PBS containing 3% FCS for 15 min at RT. Cells were stained with anti-Langerin (clone 929F3) or an isotype control antibody. The anti-Langerin mAb was detected using mouse anti-rat-Cy5.

Isolation of Langerhans Cells from the Epidermis

Epidermal sheets of untreated mice were prepared as described above and cut into small pieces, which were digested with 0.1 mg/ml collagenase D (Sigma, Germany). After blocking with 5% mouse serum, sheets were stained with rat anti-mouse Langerin mAb (clone 929F3, kindly provided by Sam Saeland, Schering-Plough, France) followed by mouse anti-rat-Cy3 (Dianova, Germany). All staining and washing steps were performed in PBS supplemented with 0.1% saponin. Sheets were mounted with 17.5% Moviol (Calbiochem) in 25% glycerol and 100 mM Tris and images were made using an Axiosvert 200M microscope and Axiovision 3.0 software (Carl Zeiss, Germany).

Skin Explant Culture and Staining of Emigrated Cells

As described elsewhere (Price et al., 1997), ears were split into dorsal and ventral halves and split open with a sharp razorshaped blade (for the edges) over a large petri dish containing RPMI, 10% FCS, and 50 μM β-mercaptoethanol for 24 HEPES (pH 7.4), and 5 μM EDTA. Erythrocytes of the spleen were removed with NH4Cl. Cells were stained with antibodies against MHCII, CD4, CD11c, CD40, CD80, CD86, V5–2, and Vj5.1-5.2 (Becton Dickinson). Total LN cells isolated from OT-II mice were labeled with CFSE as described previously (Bonifaz et al., 2002). Cells were transferred into recipient mice by i.v. injection. After 24 hr, mice were immunized s.c./i.d. in the ear and hind footpad with variable amounts of ovalbumin (0.1–10 μg OVA, Grade VI, Sigma) and 10 μg CpG1668 (TIB MoleBiol, Germany) and contralaterally with 10 μg CpG alone. Mice were sacrificed 72 hr later. Antigen-specific V5–2 Vj5.1-5.2 CD4+ cells of the OVA draining and contralateral nondraining LN were analyzed for proliferation, which is indicated by the dilution of CFSE-fluorescence intensity.

To test the stimulatory capacity of skin DC under steady-state conditions, experiments were performed according to those described by Hawiger et al. (2001) and Bonifaz et al. (2002). OT-II cells were CFSE labeled and adoptively transferred as described above. Various amounts of LPS-free OVA (5–50 EU/mg, prepared by filtration on Dextran columns [Pierce]) and test DNA with LAL QCL-1000 [BioWhittaker-Cambrex], according to the manufacturer’s instructions) were injected s.c./i.d. into the ear and hind footpad. In another set of experiments, ovalbumin was conjugated to an anti–CD205 mAb (clone NLDC145) as described previously (Bonifaz et al., 2002). Following adoptive transfer of OT-II cells, mice received 1 μg anti–CD205/ova conjugate in PBS s.c.
Acknowledgments

We thank Martin Lipp, MDC Berlin, for providing CCR7-deficient mice on a mixed 129Sv x Balb/c background. We are grateful to Sam Sæland, Schering-Plough, for providing the anti-Langerin antibody, to Eva Stüwe for excellent technical assistance, and to Oliver Pabst and Günther Bernhardt for valuable suggestions on the manuscript. We are also grateful to Sheila Fryk for critically reading the manuscript. Requests regarding the GM-CSF producing cell line should be sent to Thomas Blankenstein (tblanken@mdc-berlin.de). This work has been supported by a DFG grant Fo 334/1-1 to R.F.

Received: February 6, 2004
Revised: June 15, 2004
Accepted: June 23, 2004
Published: August 17, 2004

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