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CUTTING EDGE

Cutting Edge: Chemokine Receptor CCR4 Is Necessary for Antigen-Driven Cutaneous Accumulation of CD4 T Cells under Physiological Conditions[1]

James J. Campbell,[2]† Daniel J. O'Connell,‡ and Marc-André Wurbel*†*

Dual expression of chemokine receptor CCR4 and E-selectin ligand is characteristic of skin-tropic CD4 T cells from blood, lymphoid organs, and the skin itself. A strong and specific correlation exists among CCR4, its ligand CCL17/TARC, and the cutaneous lymphocyte-homing process. Nevertheless, whether CCR4 function is required for skin-specific trafficking remains an open question, which we address in this study. We developed an Ag-specific, TCR-transgenic, murine CD4 T cell adoptive transfer model that induces a mixed Th1 and Th17 cutaneous response. Within the hosts, both CCR4$^{+/+}$ and CCR4$^{-/-}$ donor CD4 T cells contribute equally well to the circulating E-selectin ligand^{+} pool in response to Ag. However, only CCR4$^{+/+}$ donor cells accumulate efficiently within the skin. CCR4$^{-/-}$ cells home normally to the peritoneum, showing that they do not have a general defect in lymphocyte trafficking. We conclude that under physiological conditions, CCR4 is a nonredundant, necessary component of skin-specific lymphocyte trafficking. The Journal of Immunology, 2007, 178: 3358–3362.

Since we first described the correlation between cutaneous T cell homing and CCR4-CCL17/TARC interaction (1, 2), three intriguing discoveries have engendered a reassessment of these molecules and their roles in skin-specific lymphocyte trafficking. First is the discovery that a second chemokine receptor, CCR10, is associated with cutaneous T cells (3, 4). This discovery raised the possibility that CCR10 might be redundant with, or more important than CCR4 in skin-specific trafficking. Second is confirmation of the notion proposed by Cyster et al. (5), that competition between individual cell populations, each expressing disparate homing receptor repertoires, controls entry into limited microenvironments (6–8). This finding implies that skin-homing experiments performed directly on chemokine receptor-deficient mice may not be physiological, because they lack a competitive component (9, 10). Third is the recent demonstration that classical murine models of hapten-mediated skin delayed-type hypersensitivity, i.e., 2,4-dinitro-1-fluorobenzene (DNFB[3])- or oxazalone-induced skin swelling, are not T cell-dependent events (11, 12). This discovery raises the possibility that conclusions drawn from studies using these techniques may not have direct relevance to T cell-mediated skin inflammation (11).

In this study, we assessed the role of CCR4 in a truly Ag-dependent model of cutaneous T cell homing. We established a system in which skin inflammation was mediated by adoptively transferred TCR-transgenic CD4 T cells. This approach allowed us to monitor a naive TCR-transgenic CD4 population as it acquired tissue-specific homing properties within the host, in response to exogenously applied Ag. This model recapitulates the physiological conditions under which a small number of Ag-specific lymphocytes must compete with a vastly larger population of nonspecific lymphocytes to accumulate within a given tissue.

Materials and Methods

Mice

C57BL/6N (Charles River Laboratories), B6.SJL-ptprc(a)Pep3(b)BoyJ (CD45.1^{+}), and OT-2 Tg (both obtained from The Jackson Laboratory), and CCR4$^{-/-}$ (6, 13) and CCR9$^{-/-}$ (8) were maintained in our facility, backcrossed >12× on the C57BL/6N. All animals were housed at the Children's Hospital Boston (CHB)-Karp2 animal facility, and all procedures were approved by the CHB Institutional Animal Care and Use Committee.

Inflammation

Skin. Each side of each ear was treated as described in Ref. 14 with minor modifications. Briefly, both sides of each ear were gently stripped 10 times with Scotch Tape (3M), then 25 μl of acetone was applied and evaporated. Then, 25 μl of cholera toxin (CT) (0.5 mg/ml, type Inaba 569B; Calbiochem) or CT plus $OVA_{323-339}$ (2.5 mg/ml; Peptides International) was spread evenly with a small paintbrush.

Peritoneum. One hundred microliters of CT at 0.05 mg/ml (10-fold less than used on skin) or CT plus OVA (2.5 mg/ml) were injected i.p. for lymphocyte harvest by lavage on day 5. DNFB skin inflammation was performed exactly as described in Ref. 10.

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[3] Abbreviations used in this paper: DNFB, 2,4-dinitro-1-fluorobenzene; CT, cholera toxin; LN, lymph node; DLN, draining LN; E-lig, E-selectin ligand; WT, wild type; BM, bone marrow.

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Adoptive transfers

Splenic $CD4^+$ OT-2 Tg cells were prepared from donor mice (OT-2 Tg, $CD45.1^+$ OT-2 Tg, $CCR4^{-/-}$ OT-2 Tg, or $CCR9^{-/-}$ OT-2 Tg). $CD4^+$ OT-2 Tg donor cells (5×10^6) were adoptively transferred i.v. into recipient mice ($CD45.1^+$ or C57BL/6N). For CFSE experiments, donor cells were labeled with CFSE (Fluka; catalog no. 21888) as previously described (15) before transfer.

Isolation of lymphocytes

Inflamed skin. Ears from five identically treated mice were pooled for each experiment. Lymphocytes were isolated by the EDTA technique described in Ref. 10. Isolated cells were stained with CD45 isoform marker, B220, CD4, and CD3.

Lymph node (LN) and PBL. Cells were isolated from LN and blood exactly as described in Ref. 6.

Intracellular cytokine staining

Draining LN (DLN) cells from day 6 chimera were cultured briefly with PMA, ionomycin, and brefeldin A to enhance assessment of intracellular cytokines, as described in Ref. 6. Cells were stained with CD45.1, CD4, and E-selectin ligand (E-lig), then permeabilized and stained for intracellular cytokines (stimulation and permeabilization did not affect the staining profile of CD45.1, CD4, or E-lig; data not shown).

Results and Discussion

A model for homing of Ag-specific T cells to skin

Splenocytes from OT2 TCR-transgenic mice (16) were transferred i.v. into normal hosts. Donors and hosts differed in CD45 allotype (i.e., CD45.1 vs CD45.2).

Topical immunization was chosen to bias the response toward cutaneous T cell memory. The Ag for the transgenic OT2 TCR (OVA peptide 323–339) was applied to the ears as described in Ref. 14 with modifications (see *Materials and Methods*), along with CT as an adjuvant. The treated chimeric animals were sacrificed at various time points to isolate the cervical and axillary LNs. Donor-derived OT2 cells from these DLN were assessed for E-lig expression by flow cytometry. LN CD4 cells isolated directly from OT2 mice did not contain significant numbers of $E\text{-lig}^+$ cells, nor did OT2 populations from hosts treated only with CT (Fig. 1*a*). In contrast, OT2 DLN populations from Ag-treated hosts contained high numbers of $E\text{-lig}^+$ cells (~15%).

Donor OT2 populations isolated directly from OVA-inflamed skin (Fig. 1*b*) contained dramatically more $E\text{-lig}^+$ cells (~92%) than those from draining nodes of the same mice (~12%). The staining for E-lig was Ca^{2+} dependent, and therefore specific (Fig. 1*b*).

The $CD4^+/E\text{-lig}^{high}$ population from OVA-treated chimeric mice contained dramatically more donor-derived OT-2 cells (15.6%) than $CD4^+/E\text{-lig}^-$ cells (3.3%) from the same mice (Fig. 1*c*). The size of the OT2 population within the $CD4^+/E\text{-lig}^+$ gate reached its peak at day 6 after topical OVA immunization (Fig. 1*d*). This time point was used for all subsequent assays. Transgenic TCR was required for this response, because transferred normal wild-type (WT) donor splenocytes did not contribute significantly to the $CD4^+/E\text{-lig}^{high}$ DLN population (Fig. 1*d*). Topical OVA immunization (but not CT alone) induced a dramatic proliferative response that reached its peak earlier than E-lig expression (Fig. 1*e*).

Assessment of intracellular cytokine induction by Ag-induced $E\text{-lig}^{high}$ OT2 cells demonstrated that cells expressing Th2-associated cytokines (i.e., IL-4, IL-5, and IL-10) were rare (Fig. 1*f*). However, appreciable populations expressed IFN-γ

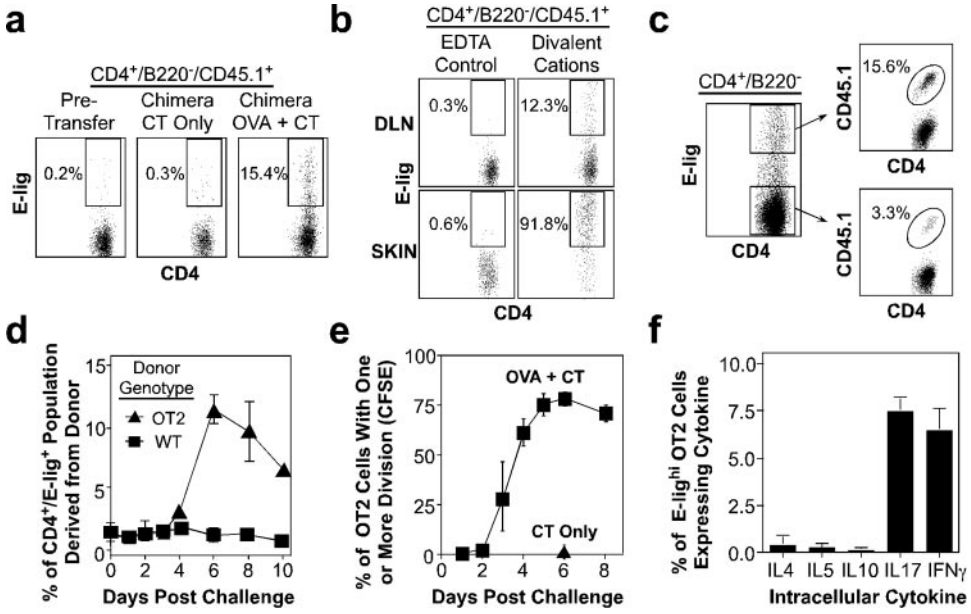

FIGURE 1. Establishment of adoptive transfer model to generate Ag-specific cutaneous CD4 T cells. *a*, E-lig expression by OT2 cells obtained directly from LN of an unmanipulated donor mouse (*left panel*), donor-derived OT2 cells from DLN of a CT-treated WT host mouse (*middle panel*), or donor-derived OT2 cells from DLN of an OVA + CT-treated WT host mouse (*right panel*). Representative of 3–16 repeats at day 6. *b*, Expression of E-lig by donor OT2 cells from DLN (*top panels*) vs OVA-inflamed skin (*bottom panels*). Specificity demonstrated by sensitivity to EDTA (*left panels*). A single experiment is shown with pooled cells from 10 mice at day 6. *c*, Proportion of donor-derived OT-2 cells within $E\text{-lig}^{high}$ and $E\text{-lig}^-$ components of the total CD4 T population in day 6 chimeric DLN. Representative of 16 repeats. *d*, Time course of donor cell representation within the $CD4^+/CLA^{high}$ DLN population. Donor cells were OT2 or WT on the CD45.1 background. *e*, Time course of OT2 cell division (CFSE loss) from day 6 chimera DLN. OVA + CT (■) vs CT only (▲) shown. The mean of two experiments with at least two data points/time point/experiment. *f*, Intracellular cytokine expression by donor-derived $E\text{-lig}^{high}$ OT2 cells from DLN of chimeras at day 6 as assessed by flow cytometry. The mean and range of two experiments with DLN pooled from 5 to 10 mice per experiment. Cells that stained positively with isotype control in parallel wells were subtracted from the number shown.

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or IL-17 at robust levels. The populations expressing IFN-γ or IL-17 did not overlap (data not shown). Thus, our topical inflammation protocol appears to yield a mixture of cutaneous Th1 and Th17 cells.

Using the new model to assess requirement for CCR4 in skin-specific homing

OT2 cells were dramatically enriched within the skin vs blood and DLN after topical OVA immunization (Fig. 2, *a* and *b*). This accumulation was Ag-dependent, because CT alone did not induce an influx of OT2 cells (Fig. 2*b*). The response also depended upon the Ag-specific TCR, because WT donor cells did not accumulate in OVA-treated skin. Although hapten-mediated inflammation (DNFB) caused more severe swelling and redness than our OVA protocol (data not shown), it did not induce accumulation of OT2 cells (Fig. 2*b*). Together, these latter findings exclude the possibility that OT2 accumulation is a nonspecific effect of general skin inflammation.

We bred an OT2/CCR4$^{-/-}$ strain to assess the requirement for CCR4 in cutaneous OT2 accumulation. The ability of OT2/CCR4$^{-/-}$ cells to accumulate in OVA-inflamed skin was significantly and dramatically decreased with respect to the original OT2 cells (Fig. 2*b*). Similar experiments with OT2 cells deficient in CCR9 (a receptor not associated with skin) showed no significant difference from the original OT2 cells. This response suggests that the effect is truly CCR4 dependent, and not a general artifact of any chemokine receptor gene deletion. Because our model appears to be Th1 and Th17 biased (Fig. 1*f*), it should be noted that the observed requirement for CCR4 is not consistent with a role for this receptor as a Th2 marker (discussed in Refs. 1, 2, 6).

Generation of circulating E-lig$^+$ "cutaneous" memory T cells

We next assessed the ability of OT2/CCR4$^{-/-}$ cells to generate a population of E-lig$^+$ memory cells in the host circulation. In experiments where OT2/CCR4$^{+/+}$ donor cells were transferred into WT hosts, ~10% of the total CD4$^+$/E-lig$^+$ cells were donor-derived (Fig. 3*a*). This number was not significantly different for OT2/CCR4$^{-/-}$ donors.

This result suggests that the presence or absence of CCR4 on the donor cells does not affect the Ag-induced generation of E-lig$^+$ CD4 memory T cells and their subsequent release into the circulation. Therefore, it is likely that the poor accumulation of OT2/CCR4$^{-/-}$ cells in the skin results from the inefficient entry of E-lig$^+$/CCR4$^{-/-}$ cells into the skin from the blood.

In contrast to peripheral blood, the representation of OT2/CCR4$^{-/-}$ was reduced in the CD4/E-lig$^+$ population in the DLN (Fig. 3*a*). For each type of transfer, representation within the E-lig$^+$ DLN population closely paralleled accumulation in skin (compare with Fig. 2). This finding is consistent with our hypothesis (6) that many (or most) E-lig$^+$ CD4 cells in DLN of

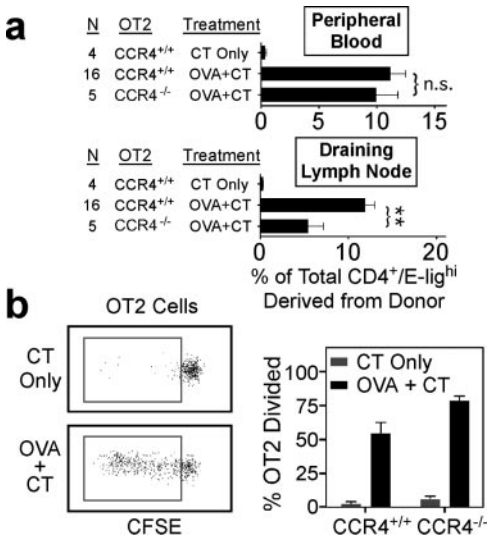

FIGURE 3. E-lig induction and replication of donor-derived CCR4$^{+/+}$ and CCR4$^{-/-}$ OT-2 cells. *a*, Representation of donor cells within total chimeric CD4$^+$/E-lig^{high} populations from blood or DLN at day 6 after immunization. E-lig^{high} populations were gated as in Fig. 1*c* (*upper gate*), and percentage derived from donor in blood (*upper panel*) and DLN (*lower panel*) are shown in the bar graphs. N indicates the number of experiments performed, each experiment consisting of pooled cells from five mice. **, Significant difference ($p < 0.05$) from positive control (i.e., OT2 donor cells in WT recipient treated with OVA + CT) as determined by the Mann-Whitney rank order test. *b*, *left panels*, FACS gates of CFSE data used to identify replicated cells at day 6 shown in the *right panel*. *Right panel*, Replication of CCR4$^{+/+}$ vs CCR4$^{-/-}$ donor-derived OT-2 cells at day 6. For CCR4$^{+/+}$ donors $n = 5$, and for CCR4$^{-/-}$ donors $n = 6$.

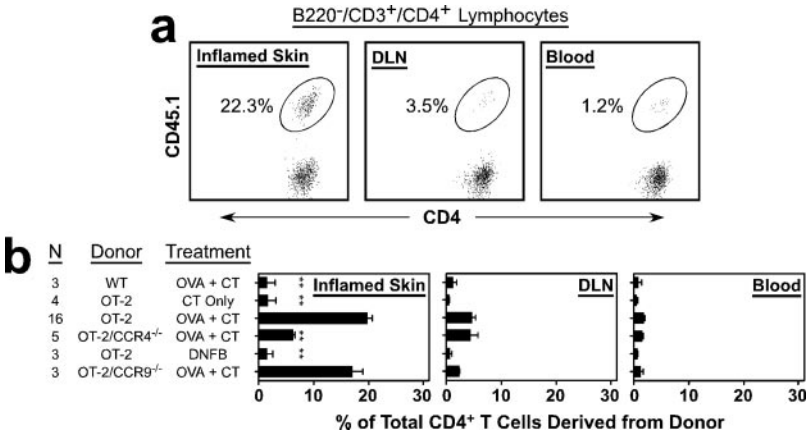

FIGURE 2. Donor-derived OT-2 cells lacking CCR4 are dramatically impaired at reaching OVA-inflamed skin. Representation of donor cells within total chimeric CD4 population of various organs at day 6. *a*, Gating criteria used for bar graphs in *b* for total CD4 cells isolated from blood, DLN, and skin from the same positive control (pooled from five mice). *b*, Bar graphs showing mean and SEM for blood (*right panel*), DLN (*middle panel*), and skin (*left panel*) of chimeric mice treated as indicated. N indicates the number of experiments performed, each experiment with cells pooled from five mice. **, Significant difference ($p < 0.05$) from positive control (i.e., OT2 donor cells in WT recipient treated with OVA + CT) as determined by the Mann-Whitney rank order test.

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inflamed skin are not "newly minted" cutaneous CD4 cells, but are instead cells that have returned to the DLN though the lymph after homing to the skin from the blood.

Proliferative response of OT2/CCR4$^{-/-}$ cells in vivo

Donor OT2 cells were stained with CFSE before transfer, and the chimeric hosts were treated exactly as in the previous experiments. This method allowed us to assess OT2 proliferation in DLN by CFSE loss. OT2 cells showed little or no proliferation in hosts treated with only CT, but the vast majority of OT2 cells proliferated in hosts treated with OVA + CT (Fig. 3*b*, *left panels*).

We found that OT2/CCR4$^{-/-}$ cells proliferated slightly better than OT2/CCR4$^{+/+}$ cells, but this difference was not significant (Fig. 3*b*, *right panel*). This result demonstrates that CCR4$^{-/-}$ cells are not impaired in their ability to locate and respond to Ag in the DLN. Again, these data are consistent with the notion that defective accumulation of OT2/CCR4$^{-/-}$ cells within inflamed skin is a direct result of their inability to enter the skin from the blood, rather than ineffective exposure to Ag in the DLN.

Tissue specificity of the OT2/CCR4$^{-/-}$ homing defect

We established an OT2 peritonitis model for comparison to the topical cutaneous model. Naive OT-2 or OT2/CCR4$^{-/-}$ cells were transferred i.v. exactly as in the cutaneous model. However, instead of treating the ear skin, CT alone or OVA + CT was injected i.p. into the host on day 0. The peritoneum of each sacrificed mouse was lavaged on day 5 (the peak time point; data not shown), and the total CD4 population was assessed for its content of OT2 cells. Only a negligible percentage of OT2 cells was found among the peritoneal CD4 population of CT-treated mice (Fig. 4). In contrast, nearly half of the peritoneal CD4 cells from OVA + CT i.p.-treated mice originated from the OT2 donor. Although the phenotype of the original donor population was naive, those isolated from the OVA + CT-treated peritoneum were essentially 100% memory (as assessed by CD44 and CD45RB levels; data not shown). OT2/CCR4$^{-/-}$ cells actually accumulated somewhat better than normal OT2 cells in the inflamed peritoneum, although this increase was not significant. Thus, although OT2/CCR4$^{-/-}$ cells are impaired at homing to inflamed skin, they are not impaired at homing to inflamed peritoneum.

Requirement of CCR4 in cutaneous T cell homing

In a previous study, we examined competition between CCR4$^{+/+}$ and CCR4$^{-/-}$ CD4 T cell populations in the development of E-lig^{+} cutaneous memory cells (6). These experiments involved equal numbers of transferred CCR4$^{+/+}$ and CCR4$^{-/-}$ bone marrow (BM) cells developing side-by-side within irradiated, lymphocyte-deficient RAG-2$^{-/-}$ adoptive hosts. Over several weeks, precursors from both BM sources developed equally well into B cells, NK cells, neutrophils, and naive CD4 and CD8 T cells. However, the E-lig^{+} CD4 population contained a disproportionately larger number of cells from the CCR4$^{+/+}$ donor.

We considered two hypotheses to explain the competitive advantage of CCR4$^{+/+}$-derived over CCR4$^{-/-}$-derived naive cells at joining the E-lig^{+} cutaneous memory CD4 population (6). The first theory proposed that CCR4 might have a role in generating E-lig^{+} cells from naive CD4 cells within the DLN. Thus, the competitive advantage would occur at the earliest time point in generating E-lig^{+} memory cells.

The second hypothesis proposed that the competitive advantage occurred when E-lig^{+} cells attempted to enter the skin from the blood. Thus, E-lig^{+} memory cells from CCR4$^{+/+}$ donors would enter the skin more efficiently than E-lig^{+} memory cells from CCR4$^{-/-}$ donors. CCR4$^{-/-}$-derived E-lig^{+} cells would therefore be less likely to encounter Ag within the skin, and their maintenance within the E-lig^{+} memory CD4 T cell pool would be compromised in comparison to CCR4$^{+/+}$-derived cells.

The original BM transfer experiments (6) could not distinguish between these two hypotheses. However, we favored the latter, because the predominance of CCR4$^{+/+}$-derived cells was more dramatic among E-lig^{+}/CD103^{+} cells than E-lig^{+}/CD103^{-} cells. Expression of CD103, the intraepithelial integrin (α_e), is likely to indicate that a given E-lig^{+} cell has spent significant time within the skin (17). Thus, the effects of CCR4 deficiency appeared to be more dramatic on the E-lig^{+} subpopulation that had spent time within the skin (CD103^{+}) as opposed to the newly minted E-lig^{+} population more recently released from the DLN (CD103^{-}). The present study supports the latter hypothesis, because OT2/CCR4$^{-/-}$ cells are not impaired in their ability to develop into E-lig^{+} cells that enter the circulation, but they do not accumulate efficiently within the skin when in competition with host-derived CCR4$^{+/+}$ CD4 T cells.

Ag-specific T cell homing to skin

The present model was developed partly in response to the emerging consensus that classical DNFB and oxazalone models, previously believed to represent delayed-type hypersensitivity, are actually T cell-independent phenomena (11, 12). In fact, NK cells appear to play a larger role in these models than T cells (12). One DNFB-based study concluded that CCR4 and CCR10 played redundant roles in T cell-mediated skin inflammation (10). Another concluded that CCR10 alone was required for T cell-mediated skin inflammation (9). With our present knowledge, these previous studies may suggest a role for CCR10 in NK cell rather than T cell-mediated inflammation. It should be noted that CCR4 has a second ligand, CCL22, which does not appear to be involved in cutaneous trafficking (1).

In this study, we have attempted to accurately model T cell-mediated skin inflammation. We assessed T cell accumulation within inflamed skin in response to a T cell-specific Ag, while including the physiologically important factor of competition

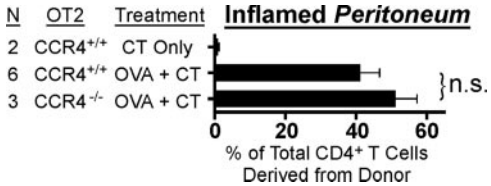

FIGURE 4. Donor-derived OT-2 cells lacking CCR4 traffic efficiently to OVA-inflamed peritoneum. Lymphocytes harvested from peritoneum by lavage on day 5 after indicated i.p. treatment. Percentage of the total CD4 population was calculated for adoptively transferred donor cells. N indicates the number of experiments performed, one mouse per experiment. Blood contamination of peritoneal lavage was negligible (data not shown).

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among cell populations. We cannot exclude the possibility that CCR10 might also play a role in this model system, but we can clearly assert that CCR4 is not redundant with CCR10. We conclude that CCR4 is necessary for homing of CD4 T cells to inflamed skin under physiological conditions.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

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