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Visualizing CD4 T-cell migration into inflamed skin and its inhibition by CCR4/CCR10 blockades using *in vivo* imaging model

X. Wang*, M. Fujita[†], R. Prado[†], A. Tousson[‡], H-C. Hsu*, A. Schottelius[§], D.R. Kelly[¶], P.A. Yang*, Q. Wu*, J. Chen*, H. Xu**, C.A. Elmetts**, J.D. Mountz^{*,††}, and C.K. Edwards III[†]

*Department of Medicine, Division of Clinical Immunology and Rheumatology, University of Alabama at Birmingham, Birmingham, AL 35294, USA

†High Resolution Imaging Facility and Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL 35294, USA

¶Department of Pathology, Children's Hospital, University of Alabama at Birmingham, Birmingham, AL 35294, USA

**Department of Dermatology, University of Alabama at Birmingham, Birmingham, AL 35294, USA

†Department of Dermatology, University of Colorado Denver, Aurora, CO 80045, USA

§Department of Development, Genentech, South San Francisco, CA 94080-4990, USA (currently at MorphoSys AG, Martinsried, Germany)

††Veterans Affairs Medical Center, Birmingham, AL 35233, USA

Summary

Background—Chemokines are critical mediators of T-cell homing into inflamed skin. The complex nature of this multicellular response makes it difficult to analyse mechanisms mediating the early responses *in vivo*.

Objectives—To directly visualize T-cell homing into inflamed skin and its inhibition by blockades using a unique noninvasive confocal microscopy.

Materials and methods—A mouse model of allergic contact dermatitis was used. T cells from oxazolone-sensitized and -challenged Balb/c mice were first analysed phenotypically *in vitro*. CD4 T cells were then labelled with a tracker dye and transferred into Balb/c-SCID mice. The recipient mice were challenged with oxazolone and CD4 T-cell homing into inflamed skin was visualized.

Results—T cells with the skin homing receptors CCR4 and CCR10 were increased in the affected skin and draining lymph nodes, and effectively attracted by their specific chemokines CCL17, CCL22 and CCL27 *in vitro*. Using *in vivo* imaging, T-cell migration into the inflamed

Correspondence Mayumi Fujita, mayumi.fujita@ucdenver.edu.

Conflicts of interest

None declared.

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skin was observed at 2 h after application, peaking at 12 h and continuing for 48 h. Simultaneous systemic administration of neutralizing antibodies against CCR4 ligands (CCL17 and CCL 22) and CCR10 ligand (CCL27) led to a significant suppression of T-cell migration and skin inflammation.

Conclusions—Our data indicate that these tissue-selective adhesion molecules and chemokine/receptor pathways act in concert to attract specialized T-cell populations to mediate cutaneous inflammation. The *in vivo* imaging technique can be applicable to other models of cutaneous diseases to help with better understanding of the pathogenesis and monitoring the therapeutic effects.

Keywords

cell trafficking; chemokines; skin homing; T cells

Expression of specific chemokine receptors and adhesion molecules on T-cell subsets accounts for selective recruitment of effector T cells to sites of inflammation.¹ Identification of chemokine ligand–receptor interactions that mediate the earliest events in the recruitment of T cells to the allergen-impregnated skin could potentially have broad implications for the development of therapeutic strategies.

Two chemokine receptors, CCR4 and CCR10, have been proposed to be important for homing of skin-specific T lymphocytes in humans.^{2–6} CCR4 has two known ligands: CCL17 (thymus and activation-regulated chemokine) and CCL22 (macrophage-derived chemokine). Both are expressed abundantly by dendritic cells and facilitate the recruitment of activated T cells into inflamed skin.^{7,8} CCR10 ligand, CCL27 (cutaneous T cell-attracting chemokine), is expressed by skin keratinocytes.^{2,9,10}

The role of CCR4 and CCR10 for T-cell trafficking into inflamed skin is a topic of extensive research. However, experimental data for the requirement and redundancy of individual receptors in T-cell homing and the resulting skin inflammation is controversial. Homey *et al.* demonstrated marked reduction of dinitrofluorobenzene (DNFB)-induced allergic contact dermatitis (ACD) with neutralization of CCL27–CCR10 interactions in Balb/c mice.⁵ Alferink *et al.* reported the reduction of DNFB- and fluorescein–isothiocyanate (FITC)-induced ACD in CCL17-deficient mice.¹¹ On the other hand, Reiss *et al.* concluded that simultaneous inhibition of CCR4 and CCR10 is required to block lymphocyte recruitment by showing the necessity of anti-CCL27 to reduce DNFB-induced ACD in CCR4 knockout B57Bl/6 mice.² Similarly, Mirshahpajah *et al.* demonstrated that only combination therapy of neutralizing antibodies to CCL17, CCL22 and CCL27 resulted in oedema inhibition using three different ACD models.⁶

Different models and experiment designs in these studies may in part explain the controversial results. For example, the effect of blocking chemokine ligands or receptors may not be well represented in studies using knockout mice, because immune system and compensatory mechanisms may be developmentally altered in these animals. Given that tissue microenvironment strongly influences chemokine expression and T-cell migration, a functional assay using intact tissue where the physiological context is preserved may be

important to properly assess the dynamic migration of specific T-cell subpopulations and address the role of chemokine ligand–receptor interactions *in vivo* and *ex vivo*.¹²

Here we developed a unique *in vivo* imaging technique that allowed direct and real-time visualization of T-cell migration and its response to chemokines in the inflamed skin. We demonstrate that CCL17, CCL22 and CCL27 play important roles in attracting skin-homing memory T cells into the inflamed skin of oxazolone-induced ACD, and that simultaneous blockade of these interactions is required to efficiently attenuate T-cell migration and inflammation.

Materials and methods

Animals

Female Balb/c and Balb/c-SCID mice (6–8-weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME, U.S.A.) and were kept under specific pathogen-free conditions. Experiment protocols were approved by the Institutional Animal Care and Use Committee of University of Alabama at Birmingham. Animal studies were performed in accordance with National Institutes of Health Animal Care and Use guidelines.

Mouse model of allergic contact dermatitis and antichemokine treatment

The protocol for inducing ACD is illustrated in Figure 1. Balb/c mice were sensitized on day 0 by applying 150 μ L of 3% oxazolone (4-ethoxymethylene-2-oxazolin-5-one; Sigma-Aldrich, St Louis, MO, U.S.A.) in acetone : olive oil (4 : 1) on the shaved abdomen. Mice were challenged on day 6 by epicutaneous application of 1% oxazolone (20 μ L) on the right ear. Left ears were treated with vehicle alone. Mice were sacrificed 24 h later and lymphocytes from skin-draining lymph nodes (LNs) (cervical, axillary and brachial) were prepared using a 70- μ m nylon cell strainer (BD Falcon, San Jose, CA, U.S.A.). Skin-infiltrating T cells were released from the dermis after separating the epidermal sheets as described.¹³ Lymphocytes from bilateral LNs and ears were collected separately and used for phenotypic and functional characterization.

Lymphocytes from right-side draining LNs were purified with CD4 selection columns (R&D Systems, Inc., Minneapolis, MN, U.S.A.). The column-purified CD4⁺ T cells were labelled *in vitro* with 10 μ mol L⁻¹ carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, U.S.A.) according to the manufacturer's instructions. CFSE-labelled T cells (5×10^7) suspended in 200 μ L of phosphate-buffered saline (PBS) were injected via the tail vein into unprimed congenic Balb/c-SCID recipients. Immediately after the adoptive transfer, 1% oxazolone (20 μ L) was applied to the right ears of the recipients while left ears were treated with vehicle only. Both ears were imaged in the confocal microscope.

For antichemokine experiments, 6 h and 2 h prior to the adoptive transfer, Balb/c-SCID mice received intravenously either 100 μ g neutralizing antichemokine antibodies (rat antimouse CCL17, goat antimouse CCL22, rat antimouse CCL27; R&D Systems) in PBS (200 μ L), or the same volume of IgG isotype control antibodies (rat IgG2A, goat IgG and rat IgG2B, respectively). Ear thickness was measured in a blinded fashion using Mitutoyo Dial Thickness Gage (APIS Inc., Middleburg Heights, OH, U.S.A.) at 0, 2, 12 and 48 h.

Flow cytometry analysis

Single-cell suspensions from bilateral LNs and skin were stained with goat FITC-conjugated anti-CD4, Cy5-conjugated anti-CD8, phycoerythrin (PE)-conjugated anti-CD62L, APC-conjugated anti-CD44 and APC-conjugated anti-CD3 (BD-Pharmingen, San Diego, CA, U.S.A.). They were also stained with goat anti-CCR4 (Abcam, Cambridge, MA, U.S.A.) and anti-CCR10 (Capralogics Inc., Hardwick, MA, U.S.A.), followed by cychrome-conjugated or biotinylated polyclonal antigoat IgG (Abcam). Cutaneous lymphocyte-associated antigen (CLA)-expressing cells were identified by flow cytometry using a recombinant mouse E-Selectin/Fc chimera (R&D Systems, Inc.)^{14,15} followed by PE-conjugated goat antihuman IgG (Jackson ImmunoResearch, Inc., West Grove, PA, U.S.A.).

Immunohistochemistry

Cryosections (6- μ m) of mouse ear skin and LNs were fixed in Fix-Frozen cocktail solutions (University of Alabama at Birmingham Center for Immunohistochemistry, Birmingham, AL, U.S.A.) for 20 min, and treated with avidin-biotin block solutions (Biogenex, San Ramon, CA, U.S.A.) and FcR block (Innovex Biosciences, Richmond, CA, U.S.A.) consecutively for 10 min each at room temperature. Sections were incubated overnight at 4 °C with primary antibodies (rabbit antimouse CCR4 or goat antimouse CCR10; Alexis Biochemicals, San Diego, CA, U.S.A.) or isotype control antibodies. To block endogenous peroxidase activity, 3% aqueous hydrogen peroxide (Sigma-Aldrich) was used for 15 min at room temperature. Biotinylated goat antirabbit IgG or mouse antigoat IgG (Biocare Medical, Walnut Creek, CA, U.S.A.) were incubated for 40 min at room temperature, followed by horseradish peroxidase-conjugated streptavidin. Slides were developed using diaminobenzidine tetrachloride substrate, counterstained with haematoxylin and analysed using light microscopy.

Chemotaxis assay

Single-cell suspensions were prepared from draining LNs as above. Standard transmigration assays using 3- μ m pore Millipore Multiscreen MIC (Millipore, Billerica, MA, U.S.A.) were performed. CCL17, CCL22 or CCL27 (100 nmol L⁻¹; R&D Systems, Inc.) or the combination of three chemokines was added to the 96-well feeder tray (lower chamber). Recombinant murine CCL21 (secondary lymphoid tissue chemoattractant, SLC; PeproTech, Rocky Hill, NJ, U.S.A.) was used as a positive control. Donor mouse CD3⁺ T cells (1 $\times$ 10⁶) or purified CD4⁺ T cells (> 95% pure) were suspended in 100 μ L chemotaxis buffer (1 $\times$ Hank's balanced saline solution with 20 mmol L⁻¹ HEPES, pH 7.4 and 0.5% bovine serum albumin) and placed into the upper chamber. After 3-h incubation at 37 °C and 5% CO₂, 16 μ L of 6.25 μ g mL⁻¹ Calcein AM (Molecular Probes) was added into each well and incubated for 45 min at 37 °C in the dark. Migration of T cells to chemoattractants was measured using a Fusion™ Universal microplate analyser (Packard Bioscience, Boston, MA, U.S.A.) at excitation : emission wavelengths of 585 : 530 nm. The percentage migration was calculated by dividing the number of migrated T cells by the number of input cells multiplied by 100.

***In vivo* imaging**

The hair on the ear of Balb/c-SCID mice was removed using 'Nair® lotion' 48 h before imaging. Ears of recipient mice were positioned on a glass transparent microscope cover slip and imaged after the adoptive transfer. During imaging, mice were maintained under anaesthesia with 200 mg kg⁻¹ pentobarbital sodium by intraperitoneal injection with constant heart rate and body temperature monitoring. Imaging was performed using a Leica DMIRBE inverted epifluorescence/Nomarski microscope outfitted with Leica TCS NT Laser Confocal optics (Leica, Inc., Exton, PA, U.S.A.). Excitation of CFSE was provided with an Argon neon laser using the 488 nm line and 553 nm for blood vessels. Precise control of fluorochrome excitation and emission was afforded by an acoustooptical tunable filter and TCS SP prism spectrophotometer (Leica, Inc.), respectively. Streaming video wide-field fluorescence microscopy was performed using a digital Canon camera attached on the ocular head of the Leica DM IRBE microscope. In this case, fluorescence excitation of the CFSE label was provided by a Mercury vapour lamp.

Statistical analysis

Data were analysed using standard statistical analysis (analysis of variance and Student's *t*-test). Data are presented as mean ± SEM and differences were considered significant if *P* < 0.05.

Results

Allergic contact dermatitis to oxazolone induces increased number of CCR4 and CCR10 positive cells in the skin and draining lymph nodes

Induction of ACD was confirmed by oedema and marked infiltration of neutrophils, lymphocytes and plasma cells histologically (Fig. S1a,b). Immunohistochemical staining revealed CCR4⁺ cells and CCR10⁺ cells in oxazolone-treated but not vehicle-treated skin and LNs (Fig. S1c–j).

As expression of CCR4 and CCR10 has been well characterized in CD4⁺ T cells,^{10,16} their expression in skin and draining LNs was further analysed for the presence of CLA on the CD4⁺CD62L^{lo}CD44^{hi} memory subpopulation (Fig. 2a).¹⁷ CLA⁺ memory CD4⁺ T cells were increased in draining LNs of oxazolone-treated skin compared with controls (Fig. 2b). CD4⁺CD62L^{lo}CD44^{hi}CLA⁺ T cells expressing CCR4 and CCR10 were significantly increased in oxazolone-challenged ear and LNs, compared with those of controls (Fig. 2c). These findings confirm that oxazolone-induced ACD results in upregulation of CCR4 and CCR10 on CD4⁺CD62L^{lo}CD44^{hi}CLA⁺ T cells in the LNs, leading to the selective recruitment and enrichment of T cells expressing these chemokine receptors in the oxazolone-treated ear skin.

CCL17, CCL22 and CCL27 chemoattract skin-homing CD4⁺ T cells

We next determined if the expression of CCR4 or CCR10 induced by oxazolone sensitization correlates with their functional response to the ligands CCL17, CCL22 and CCL27 in a chemotaxis assay.¹⁸ After 3-h incubation with either CCL17, CCL22 or CCL27 alone, or all three in combination, CD4⁺ T cells from draining LNs migrated efficiently

towards the individual or the combined chemokines (Fig. 3). Interestingly, migration to the combined chemokines was not greater than migration to the individual chemokines. Unfractionated CD3⁺ T cells exhibited lower migration responses to these ligands, but migrated equally well to a positive control chemokine, SLC.2,14. These results indicate that CD4⁺ T cells from oxazolone-treated draining LNs respond effectively to CCR4 ligands (CCL17, CCL22) or CCR10 ligand (CCL27). There appear to be little additive or synergistic effect of these chemokines for effective T-cell recruitment to skin *in vitro*.

Oxazolone-induced allergic contact dermatitis increases migration and extravasation of sensitized T cells in skin

To enable *in vivo* imaging of T cells, we applied a two-step T-cell migration model as shown in Figure 1. Oxazolone-sensitive donor CD4⁺ T cells were labelled *in vitro* with CFSE (Fig. 4a), and injected intravenously in unprimed recipient Balb/c-SCID mice. Balb/c-SCID mice were used as recipients, due to their lack of lymphocytes, allowing clear evaluation of migration of donor lymphocytes without the effects from the recipient's immune system. Ears of recipient mice were challenged, and migration of the adoptively transferred donor T cells was analysed by time-lapse confocal microscopy (Fig. 4b,c) or streaming video wide-field fluorescence microscopy (Fig. 5) when cell movement was observed. When cell movement was markedly decreased, migration of T cells was analysed by static wide-field fluorescence microscopy (Fig. 4d–g). Together, these microscopy methods provided information in X-Y-Z-T dimensions. Wide-field fluorescence microscopy was also helpful in acquiring images of T cells when fluorescence intensity of the labelled T cells was weak or when a higher background fluorescence of the tissue was needed to trace the path of the T cells through the dark, fluorescent-negative blood vessels.

Immediately after adoptive transfer, circulating CFSE⁺ cells were visible within blood vessels of both oxazolone-treated and vehicle-treated ears. From 0 h up to 2 h post-transfer, no difference in the number of circulating T cells was noted between oxazolone-treated and vehicle-treated ears (Fig. 4b,c), although capillaries were enlarged in the oxazolone-treated ears. Shortly after 2-h post-transfer, transmigration of intravascular CFSE⁺ cells to the tissue was noted in the oxazolone-treated ears but not vehicle-treated ears. This extravasation of T cells peaked at 12 h (Fig. 4d,e), and continued until 48 h after the application of oxazolone (Fig. 4f,g). Detailed evaluation of individual extravasating T cells was performed using streaming-video still frames, which allowed the documentation of the entire process, including the intravascular movement of the T cell (Fig. 5a–d), reduction of intravascular cell velocity and rolling, with subsequent adherence to the endothelium (Fig. 5e,f), crossing of the vessel wall (Fig. 5f,g) and migration into the skin tissue (Fig. 5h,i). It was noted that cells remained adherent to the endothelium for at least 20 s prior to transmigration.

Neutralization of CCL17, CCL22 and CCL27 impairs lymphocyte recruitment to the inflamed ear skin

To determine the effects of CCR4 and CCR10 ligands on the recruitment of T cells into the skin, recipient mice were administered anti-CCL17, anti-CCL22 and anti-CCL27 neutralizing antibodies 6 h and 2 h before adoptive transfer (Fig. 1). Isotype control antibodies were used in control mice. Extravasated T cells were observed in oxazolone-

treated ears but not vehicle-treated ears after 2 h (Fig. 6a,b). Blocking CCR4 (with a combination of anti-CCL17 and anti-CCL22) or CCR10 (with anti-CCL27) did not reduce the recruitment of CD4⁺ cells into the inflammation sites (data not shown). However, simultaneous administration of all three neutralizing antibodies led to a marked decrease in trafficking of CD4⁺ cells at 12 h and 48 h compared with the administration of isotype control antibodies (Fig. 6b,d). As expected, vehicle-treated ears demonstrated only a small number of CD4⁺ cells, which was not changed with administration of neutralizing antibodies (Fig. 6a,c). Videos are available as online supplementary material.

Cryostat sections of ear tissues demonstrated reduction of green fluorescing CFSE⁺ skin-infiltrating T cells after administration of all three neutralizing antibodies compared with those after isotype control treatment (Fig. 6e,f).

Quantitative analysis of the *in vivo* migration was performed by counting the number of extravasated CFSE⁺ T cells in 10 nonconsecutive microscopic images of each ear at each time point, in a blinded fashion. As shown in Figure 6g, administering three neutralizing antibodies led to a significant decrease in trafficking of CD4⁺ cells into oxazolone-treated ear compared with treatment with control antibodies at 12 h and 48 h of inflammation.

Inhibition of CCR4 and CCR10 chemokines *in vivo* impairs ear swelling responses

To determine if inhibiting T-cell migration into the ear by antichemokine antibodies is associated with a reduction in the inflammatory cascades that lead to ear swelling, measurement of ear thickness was performed. Mice not receiving donor cells did not exhibit significant ear swelling after vehicle or oxazolone application (Fig. 7, left group, 1st and 2nd columns). Ear swelling was observed in mice that had received donor T cells and were applied with oxazolone (left group, 4th column), but not in those applied with vehicle (left group, 3rd column). Mice that had been pretreated with either anti-CCL17 plus anti-CCL22 or anti-CCL27 exhibited increased ear swelling comparable to that of the mice that were left untreated or treated with isotype controls (middle two groups of columns). In contrast, simultaneous administration of all three antichemokine neutralizing antibodies led to a significant reduction in skin swelling in the oxazolone-treated ears (right group columns).

Discussion

T cells are essential participants in pathological cutaneous inflammatory reactions. Using an oxazolone ACD mouse model,¹⁹ we demonstrated that T cells with the skin homing receptors CCR4 and CCR10 are increased in the affected skin and draining LNs, and that these cells are effectively attracted by their specific chemokines CCL17, CCL22 and CCL27 *in vitro*. Using *in vivo* confocal and wide-field fluorescence microscopy, we demonstrated that oxazolone exposure increases migration and extravasation of sensitized T cells in skin, and that this can be blocked by simultaneous neutralization of CCL17, CCL22 and CCL27. These results were supported by the impaired *in vivo* ear swelling through simultaneous inhibition of CCR4 ligands (CCL17 and CCL22) and CCR10 ligand (CCL27). Neutralization of CCR4 ligands or CCR10 ligand alone was not sufficient to block the transmigration of T cells into the tissue, indicating a redundant and compensatory mechanism. The results suggest that these tissue-selective adhesion molecules and

chemokine–receptor pathways may act in concert to attract specialized subpopulations of T cells to mediate cutaneous inflammation. Although the reduction in T-cell transmigration was statistically significant, it was not completely blocked when both CCR4 and CCR10 ligands were neutralized. This can be explained by incomplete neutralization of CCL17/22/27 by antibodies *in vivo*, the presence of other chemokines reacting to CCR4 and/or CCR10, the presence of other chemokine receptors in skin homing or complexity of extravasation steps.

Recruitment of T cells into an inflammatory site is crucial in the pathogenesis of skin disorders. It is mediated by a multistep process involving activation through chemoattractant–receptor interactions, as well as attachment and rolling through selectin–carbohydrate interactions.²⁰ Analysis of T-cell migration *in vivo* enables direct visualization of migrating T cells and evaluation of these complex interactive effects of several chemokines in the tissue microenvironment. The unique intravital confocal microscopy technique described here provides the means to image the behaviour of effector T cells in response to subsequent stimuli or inhibitors in its microenvironment, and was used for the evaluation of selectin-dependent T-cell migration in an ACD mouse model.²¹

In vivo imaging has not only been proven to be an important technique in basic science research, but has recently been explored as a potentially powerful tool in the clinical setting,²² especially to assist differentiating allergic from irritant contact dermatitis.^{23–25} Reflectance confocal microscopy used in patients with various cutaneous conditions may provide tissue resolution comparable to that of standard histology and allows repeated monitoring of the dynamic cell changes, demonstrating useful translation of scientific research into clinical applications.

Another important clinical application of modern technology in basic science research is the use of biological blocking agents. Blocking cytokines or inflammatory receptors has shown significant therapeutic potential in a number of inflammatory diseases such as psoriasis, rheumatoid arthritis and cancer.²⁶ Dual CCR4 and CCR10 antibody blockage could potentially be used in cutaneous diseases where memory T-cell trafficking plays a major role in the pathogenesis. The relative requirement (or redundancy) of CCR4 and CCR10 during skin lymphocyte homing may vary under different circumstances and requires further investigation to optimize the therapeutic potentials.

Although we propose that *in vivo* results from the oxazolone-treated animal model are more informative than *in vitro* studies, caution should be exercised in attempting any extrapolation of the findings to human diseases. Animal models of cutaneous diseases may not precisely mimic skin diseases observed in humans,²⁷ and T-cell skin homing receptors may have different roles in different species. The exact role of these chemokine receptors in T-cell homing to the skin and the translation of current animal studies into human therapeutic drugs awaits further elucidation.

In summary, our results using a unique *in vivo* imaging technique demonstrate that simultaneous inhibition of both CCR4 and CCR10 effectively blocks lymphocyte recruitment, supporting the concept of redundant and overlapping roles of CCR4 and

CCR10 in the T-cell recruitment into the inflamed skin in mice. Further investigation will be needed to determine if, under different physiological conditions, CCR4 and/or CCR10 receptors and their respective chemokines exert separate or additive biological effects. We believe the unique *in vivo* imaging technique can be applicable to other models of cutaneous diseases to help with better understanding of pathogenesis in a living system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Schematic diagram of the induction of oxazolone-induced allergic contact dermatitis mouse model and the T-cell adoptive transfer for *in vivo* imaging of T-cell migration. Balb/c female donor mice were sensitized with 3% oxazolone on the shaved abdomen on day 0 and were challenged with 1% oxazolone on the R ear on day 6, while the L ears were treated with vehicle alone. On day 7, LNs and ear skin were harvested for histological analysis and phenotypic analysis of lymphocytes by FACS. In addition, lymphocytes from R-side skin-draining LNs were used for *in vitro* chemotaxis analysis. CD4⁺ T cells purified from R-side skin-draining LNs were labelled with carboxyfluorescein succinimidyl ester (10 $\mu\text{mol L}^{-1}$) *in vitro*, and injected intravenously into unprimed recipient Balb/c-SCID mice. Immediately after the adoptive transfer, recipient mice were challenged with oxazolone or vehicle control on the R or L ear, respectively. Both ears of the anaesthetized recipients were then analysed by confocal microscopy at 0, 2, 12 and 48 h after the challenge. At each time point, ear thickness was also measured. In some experiments, the recipients received antichemokine antibodies (anti-CCL17, anti-CCL22 and anti-CCL27) or isotype control antibodies by intravenous injection 6 h and 2 h prior to donor cell transfer and challenge. FACS, fluorescence-activated cell sorting; IHC, immunohistochemistry; L, left; LN, lymph node; R, right

Fig. 2.

Cutaneous oxazolone challenge induces CD4⁺CD62L^{lo}CD44^{hi}CLA⁺ effector T cells and CCR4/CCR10 expression. Single-cell preparations of lymphocytes from draining LNs and the ear skin of the donor Balb/C mice were stained with anti-CD4, anti-CD8, anti-CD62L, anti-CD44, anti-CD3, and anti-CCR4 or anti-CCR10 and subjected to fluorescence-activated cell sorting analysis. Cells were gated on the T-cell memory phenotype (CD4⁺, CD62L^{lo} and CD44^{hi}), and CLA-expressing cells within this population were characterized by their staining with recombinant mouse E-Selectin/Fc chimera. The addition of ethylenediamine tetraacetic acid abolished binding of this chimera to the cells and served as a negative control (not shown). (a) Flow cytometry plots showing gated lymphocytes CD4⁺ and CD4⁺ memory population (CD4⁺CD62L^{lo}CD44^{hi}) from donor LNs. (b) Dot plots showing CLA vs. CD4 in the gated T-cell memory population CD4⁺CD62L^{lo}CD44^{hi}CLA⁺ from involved draining LNs or control LNs. (c) Dot plots showing CD4 vs. CCR4 (upper plot, CD4⁺CD62L^{lo}CD44^{hi}CLA⁺CCR4) or CD4 vs. CCR10 (lower plot, CD4⁺CD62L^{lo}CD44^{hi}CLA⁺CCR10) in the gated T-cell effector population from inflamed ear skin and control skin (left two panels) involved draining LNs and control LNs (right two panels). The differences in CCR4 and CCR10 expression observed in both skin and LN

between treated and control animals were statistically significant ($*P < 0.05$). LN, lymph node; CLA, cutaneous lymphocyte-associated antigen

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Fig. 3.

CD4⁺ T cells in oxazolone-induced ACD effectively migrate to CCL17, CCL22 and CCL27. Migration of purified CD4⁺ T lymphocytes obtained from skin draining LNs of donor mice compared with the migration of pan CD3⁺ T cells obtained from the same mice in response to optimal concentrations (100 nmol L⁻¹) of CCL17, CCL22 and CCL27 or the combination of all three through 3- μ m pores. Migration to CCL21 (SLC) served as a positive control. Migration was indicated as percentage of migrated T cells from upper chamber to bottom chamber. Background represents cell migration in response to the medium alone. The values represent the mean $\pm$ SEM. These results are representative of three independent experiments. The data were obtained from eight different mice in each group. * $P < 0.05$. ACD, allergic contact dermatitis; SLC, secondary lymphoid tissue chemoattractant

Fig. 4.

In vivo confocal microscopy allows real-time evaluation of T-cell migration in an ACD mouse model. CD4⁺ T cells (5×10^7) from donor mice were labelled with CFSE *in vitro* (a) and injected intravenously into recipient Balb/c-SCID mice. Both ears of the living recipients were imaged after donor cell transfer and ear painting with oxazolone or vehicle. Immediately after transfer, a 2-h time lapse series of single-plane, 0.3- μ m thick, confocal images taken 2 min apart, showed circulating CFSE-labelled T cells (green) in the blood vessels of the vehicle control ear (b) and of the oxazolone-treated ear (c). The natural autofluorescence of haemoglobin in the blood vessels was imaged in the red channel and merged with the green channel to show the path of T cells through the vessels. The resultant X-Y-T image series were respectively flattened into a single projection to show the total path of migrating T cells through the blood vessels with respect to time. This migration was not merely peripheral or tangential to the vessels, as the T cells were both in the very same

confocal plane of the vessels and corresponded spatially to the path of the vessels. Very few cells were seen incongruous to or outside of the vessels. After 12–48-h post-adoptive transfer, composite bright-field/wide-field fluorescence images, approximately 15- μ m thick, which show both green T cells and red blood vessels, exhibited higher numbers of extravasated T cells in the oxazolone-treated ear (e,g) in comparison to the vehicle control ear (d,f). Bar, 100 μ m. ACD, allergic contact dermatitis; CFSE, carboxyfluorescein succinimidyl ester

Fig. 5.

In vivo confocal microscopy allows real-time evaluation of T-cell attachment to blood vessels, rolling and extravasation into the skin in an allergic contact dermatitis mouse model. Still frames from a streaming video clip were imaged with wide-field fluorescence optics in the green channel, allowing visualization of CFSE⁺ T cells (arrows). Blood vessels are seen as dark, fluorescent negative channels. In these images, a T cell is seen initially moving within a blood vessel (a–d). Subsequently, the movement was progressively halted, and the T cell remained adherent to the endothelium for at least 20 s (e,f). It finally crossed the

vessel wall (f,g), and was seen in the skin tissue (h,i). Acquisition rate of 30 frames per second was used. CFSE, carboxyfluorescein succinimidyl ester

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Recipient mice (12 hrs after adoptive transfer and Ox challenge)

(g)

Fig. 6.

Neutralization of CCL17, CCL22 and CCL27 impairs lymphocyte recruitment to the inflamed ear skin. Neutralizing antibodies against CCL17, CCL22 and CCL27 were injected into recipient mice 6 h and 2 h before adoptive transfer of donor CFSE-labelled T cells and oxazolone challenge. Subsequent T-cell migration was assessed by fluorescence microscopy at 0, 2, 12 and 48 h after application of oxazolone to right ears. Cryostat sections of the ears were also processed for analysis CFSE⁺ T cells after being counterstained with Hoechst. Representative micrographs are shown from different skin areas of different mice. Wide-field fluorescence micrograph representing fields approximately 15- μ m thick was imaged in both green and red channels to show T cells and corresponding blood vessels. (a,b) Control ear and oxazolone-treated ear from mice were given relevant isotype control antibodies at 12 h after adoptive transfer and oxazolone challenge. Bar, 100 μ m. (c,d) Fluorescence images of control ear and oxazolone-treated ear from mice given neutralizing antibodies (anti-CCL17, anti-CCL22 and anti-CCL27). Bar, 100 μ m. (e) Cryostat section of oxazolone-treated ear from mice given isotype control antibodies. (f) Cryostat section of oxazolone-treated ear from mice given neutralizing antibodies. (g) Quantitative analysis of T-cell extravasation. At all time points (2h, 12h and 48h after oxazolone challenge), the entire recipient mouse ear was viewed by wide-field fluorescence microscopy, 10 nonconsecutive images were obtained, and the numbers of extravasated CFSE⁺ T cells in each field were counted in a blind fashion. The absolute number of total T cells in the whole ear was calculated. The data were obtained from four different mice in each group. Bars represent

mean $\pm$ SEM; * $P < 0.05$. CFSE, carboxyfluorescein succinimidyl ester, Ctrl, control; Ox, oxazolone

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Fig. 7.

Inhibition of CCR4 and CCR10 *in vivo* impairs swelling of the oxazolone-challenged ears in recipient mice that received sensitized T cells. Antibodies against chemokine receptor ligands for either CCR4 or CCR10 individually or combined were injected 6 h and 2 h before adoptive transfer and oxazolone challenge. Ear thickness was measured 2, 12 and 48 h after the challenge. Ear thickness at 12-h post-challenge is presented. Vehicle application and/or no donor cell transfer or appropriate isotype control antibody treatments were used as controls. The data were obtained from 12 different mice in each group. Mean $\pm$ SEM, * $P < 0.01$.