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Dermal Regulatory T Cells Display Distinct Migratory Behavior That Is Modulated during Adaptive and Innate Inflammation

Zachary Chow,* Scott N. Mueller,† James A. Deane,*1 and Michael J. Hickey,*1

Regulatory T cells (Tregs) are important in controlling skin inflammation, an effect dependent on their ability to home to this organ. However, little is known regarding their behavior in the skin. In this study, we used multiphoton imaging in Foxp3-GFP mice to examine the behavior of endogenous Tregs in resting and inflamed skin. Although Tregs were readily detectable in the uninflamed dermis, most were nonmotile. Induction of contact sensitivity increased the proportion of motile Tregs, and also induced Treg recruitment. This response was significantly blunted in mice challenged with an irrelevant hapten, or by inhibition of effector cell recruitment, indicating a role for T cell–dependent inflammation in induction of Treg migration. Moreover, induction of Treg migration was inhibited by local injection of a CCR4 antagonist, indicating a role for CCR4 in this response. Exposure of naive mice to hapten also induced an increase in the proportion of migratory Tregs, demonstrating that innate signals can also induce Treg migration. Simultaneous examination of the migration of CD4+ effector cells and Tregs in the same region of uninflamed skin demonstrated that effector cells behaved differently, being uniformly highly migratory. These findings indicate that Treg behavior in skin differs from that of CD4+ effector cells, in that only a low proportion of Tregs is migratory under resting conditions. However, in response to both adaptive and innate inflammation, the proportion of migratory Tregs increases, raising the possibility that this response is important in multiple forms of skin inflammation. The Journal of Immunology, 2013, 191: 3049–3056.
stock generously provided by Dr. Alexander Radensky (University of Washington, Seattle, WA, and Memorial Sloan–Kettering Cancer Center, New York, NY). All animal experiments were approved in advance by either the Monash Medical Centre Animal Ethics Committee “B” or the Melbourne Health Research Animal Ethics Committee.

Oxazolone-induced model of contact sensitivity

Contact sensitivity (CS) was induced as previously described (19, 22). Briefly, mice were sensitized by the application of 50 μl 5% oxazolone (4-ethoxyethylene-2-phenyl-2-oxazolin-5-one [Oxa]; Sigma-Aldrich, St. Louis, MO) in an acetonitrile/olive oil vehicle (4:1) to a shaved region on the back. Five to seven days later, a 10 × 20-mm area of the skin was challenged with 1% Oxa in vehicle. In control experiments, mice sensitized in the same manner were challenged with vehicle alone or 0.2% 1-fluoro-2,4-dinitrobenzene (DNFB; Sigma-Aldrich) as an irrelevant hapten.

Multiphoton microscopy of the flank skin

Flank skin was prepared for multiphoton microscopy using a modification of a previously published technique (22). Mice were anesthetized (ketamine hydrochloride, 150 mg/kg; xylazine hydrochloride, 10 mg/kg), and a jugular vein was cannulated for administration of fluorescein dyes and additional anesthetic. A heating pad was used to maintain body temperature. The hair was removed from the challenged area of flank skin via brief treatment with depilatory cream. To ensure analysis of representative portions of all layers of the skin via multiphoton microscopy, the skin was imaged in two steps. The undersurface of the skin (hypodermis) was prepared for microscopy as previously described (19). Following imaging of the hypodermis, the skin preparation was inverted such that the epidermis was uppermost, and this region was imaged via multiphoton microscopy. To enable visualization of the vasculature, tetramethylrhodamine isothiocyanate (TRITC) albumin (375 μg/mouse) was administered i.v.

Skin multiphoton microscopy for both regions was performed with a Leica SP5 multiphoton confocal microscope equipped with a 20 × 1.0 NA lens, four cascaded detectors, and a Spectra-Physics Mai Tai laser. Experiments were performed at 900 nm excitation. Images (6 μm z step size) were collected to a depth of 100–125 μm. In control experiments, mice sensitized in the same manner were challenged with vehicle alone or 0.2% 1-fluoro-2,4-dinitrobenzene (DNFB; Sigma-Aldrich) as an irrelevant hapten.

Flow cytometric analysis of T cells in skin and lymph node

T cell recruitment to inflamed skin was quantitated using a modification of a previously described technique (19). In brief, skin samples were weighed, finely macerated, and dissociated using collagenase D (3 mg/ml) in RPMI 1640 plus 10% FCS and 0.1 mg/ml DNase (1 h, 37˚C). Dissociated skin was filtered through a 70-μm sieve, washed with RPMI/FCS, and labeled with cell surface Abs in PBS containing 1% FCS for 30 min on ice. Abs against cell surface molecules used were CD3 e450 (2 μg/ml; eBioscience, San Diego, CA), CD4 FITC (2.5 μg/ml; eBioscience), or CD4 PE (4 μg/ml; BD Biosciences) and CD45 PE Cy5.5 (2 μg/ml; eBioscience). To quantify the total number of T cells present, 25 μl CountBright counting beads (Life Technologies, Carlsbad, CA), containing a defined number of beads, was added to samples, allowing the proportion of the sample examined to be determined, and referenced to the number of T cells present in the same sample. Labeled cells were analyzed using a FACSCan flow cytometer (BD Biosciences, North Ryde, NSW, Australia). In parallel, a sample of the same skin region was taken for wet weight/dry weight analysis. T cell recruitment data are expressed as CD3+CD4+ cells/g of extrapolated skin dry weight. The numbers of Treggs in lymph nodes were quantitated using a similar approach to analyze cells in macerated lymph nodes, in the absence of treatment with dissociation enzymes.

HSV-specific effector CD4+ T cell model

Naive DsRed gDT-II CD4+ T cells (1 × 105) were transferred into Foxp3-GFP mice, which subsequently underwent cutaneous HSV infection on the left flank, as previously described (17). Eight days later, skin on the contralateral uninfected flank was examined via multiphoton microscopy, as described (17).

Histology

Skin samples from Foxp3-GFP mice were immersed fixed in 4% paraformaldehyde, cryoprotected with 30% w/v sucrose in PBS, and frozen in TissueTek OCT compound, and 8-μm cryostat sections were prepared. Sections were either stained with H&E or were stained with Hoechst 33258 to label nuclei for assessment of Treg location. The latter sections were visualized on a Nikon C1 confocal microscope. GFP+ Tregs were counted and categorized according to their distance from the epidermis.

Statistics

Data were compared using one-way ANOVA and the Dunnett or Tukey post hoc test or using unpaired t tests. Data are shown as mean ± SEM. The p values < 0.05 were deemed significant.

Results

Skin-resident Tregs predominantly reside in the dermis

Because the ear is a commonly used site in the investigation of leukocyte function in skin, we first attempted to examine skin-resident Tregs via in vivo multiphoton microscopy of ear skin in Foxp3-GFP mice. However, GFP+ cells were extremely rare and difficult to visualize at this site (data not shown). We recently examined Treg recruitment in a CS model involving abdominal flank skin and detected skin-resident Tregs in the absence of inflammation, as well as recruitment of additional Tregs during the inflammatory response (19). Therefore, we used multiphoton microscopy to examine this area of skin in Foxp3-GFP mice. Because multiphoton imaging was unable to penetrate all layers of the skin in this region, we visualized the dermis and hypodermis in separate procedures in the same mice, imaging the dermis via the external surface and the hypodermis via the exposed undersurface of the skin (22) (Fig. 1). In each case, we were able to visualize 100–125 μm into the tissue. The dermis was collagen rich and relatively avascular (Fig. 1B), whereas the hypodermis contained postcapillary venules supporting interacting leukocytes (Fig. 1C).

Tregs were rare in the hypodermis of uninflamed skin (Fig. 2A, 2C). In contrast, a consistently high number of Tregs was observed in the dermis (~2000/mm2) in the absence of inflammation (Fig. 2B, 2D). Intradermal Tregs were often observed in clusters around hair follicles (Fig. 1B). Flow cytometric analysis of T cells in dissociated skin samples confirmed that the GFP+ cells in these
FIGURE 2. Skin-resident Tregs are predominantly in the dermis and skin regions were CD4+ T cells (data not shown). These data are consistent with our recent work and other studies reporting the presence of Tregs in uninflamed skin (8, 9, 19). Moreover, LysM-eGFP mice (in which neutrophils express GFP) that underwent an identical multiphoton imaging protocol displayed no neutrophil infiltration (data not shown), indicating that exteriorization and imaging the flank were not inducing inflammation that may have been responsible for recruitment of Tregs to the dermis.

Following induction of the CS response, a significant increase in Treg abundance in the hypodermis was observed 24 h after hapten challenge, persisting until ≥48 h (Fig. 2C). However, the number of Tregs in the hypodermis remained an order of magnitude lower than in the dermis. Occasional Tregs were also observed adherent within the hypodermal microvasculature (data not shown). Similarly, in the dermis, Treg abundance was significantly increased above basal levels 24 h after hapten challenge (Fig. 2D), although no Tregs were observed within microvessels (data not shown).

Together, these data indicate that, under resting and inflammatory conditions, Tregs are more numerous in the superficial dermis than in deeper layers of the skin.

Dermal Tregs and effector CD4+ T cells display different patterns of migration in uninflamed skin

Given the relative abundance of Tregs in the uninflamed dermis, we next examined the migratory characteristics of Tregs in this region, in the absence of inflammation. To define a Treg as migratory, we established a threshold level of displacement of 10 μm, corresponding to cells that moved at least one cell width from their original position during the period of observation. In resting skin, Tregs were predominantly immotile, not moving from their original position (Fig. 3A–C, Supplemental Video 1). However, a small subset (7%) of the Tregs migrated >10 μm during the observation period (Fig. 3B). The immotile Tregs displayed occasional probing and elongation around their base position that resulted in measurement of an average velocity of ∼4 μm/min (Fig. 3C). These observations indicate that dermal Tregs exist in two populations: one sessile and the other undergoing active migration.

These findings contrasted markedly with previous studies in which effector CD4+ T cells in the dermis were uniformly highly migratory (17). It was conceivable that these divergent observations stemmed from differences between the inflammatory milieu in the two studies. Therefore, we next examined migration of Tregs and effector CD4+ T cells in the same region of uninflamed skin. This was achieved by transferring naive HSV-specific gDT-II CD4+ T cells into Foxp3-GFP mice and infecting one flank of the mouse with HSV. We then allowed 8 d for the HSV-specific T cells to undergo activation and subsequently migrate to the skin. At this point, examination of uninflamed skin remote from the site of HSV infection revealed that, as observed previously, effector CD4+ T cells were uniformly highly motile (Fig. 3D, 3E, Supplemental Video 2). In fact, >90% of the gDT-II cells in these areas of uninflamed skin were defined as migratory. In contrast, Tregs in the same skin regions remained predominantly immotile (Fig. 3D, 3E, Supplemental Video 2). These findings demonstrate that Tregs and effector CD4+ T cells display distinct migratory behavior in the skin.

Ag challenge induces migration of a subset of dermal Tregs

We next examined the effect of induction of T cell–dependent inflammation on the behavior of Tregs in the skin, concentrating on the more abundant dermal Tregs. Vehicle-treated control mice showed no significant changes in mean Treg displacement or velocity over a 72-h period (Fig. 4A, 4C, 4D). However, following oxazolone application to sensitized mice, Tregs gradually in-
increased motility, displaying significantly increased displacement and migration velocity within 24 h of challenge, persisting out to 48 h postchallenge, relative to vehicle-treated controls (Fig. 4B–D, Supplemental Video 3). Assessment of the directionality of Treg migration at the peak of the response (24 h post-Oxa challenge) demonstrated that migrating Tregs moved randomly in the XY plane but were confined to a 50-μm-thick zone of the dermis, as determined by assessment of migration in the XZ plane (Fig. 4E, 4F). This was further demonstrated by comparison of the displacement of migrating Tregs specifically in each of the X, Y, and Z planes. Displacement in X (14.2 ± 0.7 μm) and Y (13.3 ± 0.7 μm) planes were comparable, whereas migration in the Z plane (5.7 ± 0.3 μm) was significantly lower (Fig. 4G).

We next examined the effect of CS challenge on the proportion of migratory Tregs and found that, 4 h after initiation of CS, there was no change in this parameter relative to vehicle-challenged mice (Fig. 5A, 5B). In contrast, 24 h after CS challenge, 40% of Tregs were migratory compared with 16% in vehicle-challenged mice (Fig. 5C, 5D). Notably, despite the difference in the relative proportions of migratory Tregs under these conditions, assessment of the migratory subsets separately demonstrated that the average velocity of migratory Tregs during the CS response did not differ from that in the absence of treatment (untreated: 8.4 ± 0.6 μm/s, n = 23; CS/24 h: 7.3 ± 0.1 μm/s, n = 249). This finding indicates that the measured increase in average migration velocity stemmed solely from an increase in the proportion of migrating Tregs. The increase in the proportion of migratory Tregs persisted 48 h after challenge (Fig. 5E, 5F), consistent with the observed significant increase in mean velocity (Fig. 4D).

Ag recognition and leukocyte recruitment contribute to induction of Treg migration

We next investigated the role of Ag recognition and leukocyte recruitment in induction of Treg migration. To assess whether increased Treg migration was a downstream response to recognition of the sensitizing Ag upon challenge, mice were sensitized to Oxa and then challenged either with Oxa or an irrelevant hapten (DNFB) (Fig. 6). Twenty-four hours after challenge, the numbers of Tregs in the hypodermis and the dermis were both significantly reduced in DNFB-challenged mice relative to Oxa-challenged mice (Fig. 6A, 6B), findings consistent with Treg recruitment during the
cells were defined as motile. Data were derived from incorporating 150–300 cells/group (vehicle) or 280–740 cells/group (Oxa).

Migration of dermal Tregs is reduced by CCR4 inhibition

We next assessed the role of chemoattractant pathways in induction of Treg migration. First, we used PTx as a tool to block chemoattractant receptor–dependent signaling in Tregs. PTx was injected intradermally, 20 h after induction of CS, allowing time for recruitment of leukocytes, including Tregs, into the skin as part of the normal CS response. Treg migration was then examined by multiphoton microscopy after an additional 4 h, 24 h after CS challenge, using 30-min recordings at 60-s intervals to increase the precision of the analysis (Fig. 8). In mice injected with PTx, Treg displacement and average velocity were significantly reduced relative to CS mice injected intradermally with saline (Fig. 8).

Given that CCR4 has been shown to play a key role in Treg homing to the skin (8), we next examined the role of CCR4 in CS-associated Treg migration. Using a similar approach to the PTx experiments, we injected C 021, a small molecule CCR4 inhibitor (25) intradermally, 20 h after Oxa challenge, and examined Treg migration after an additional 4 h. CCR4 inhibition resulted in significant reductions in Treg displacement and average velocity (Fig. 8, Supplemental Videos 4, 5). In addition, the percentage of motile cells (displacement > 10 μm) decreased from 47% in saline-injected mice to 25 and 27% in PTx- and C 021-treated mice, respectively. Together, these results indicate that a CCR4-dependent mechanism plays a key role in induction of intradermal Treg migration in CS.

To determine whether mast cell activation contributed to this response, we also examined Treg migration in CS mice treated with the mast cell stabilizer, sodium cromoglycate (cromolyn). In cromolyn-treated mice, Treg migration parameters did not differ from those in control mice (data not shown), indicating that mast cell degranulation was not required for induction of Treg migration in this response.
Finally, it was notable that following challenge with an irrelevant hapten, as well as in mice in which CS-associated effector leukocyte recruitment was inhibited, Treg migration remained above levels observed in uninfamed skin. One possible explanation for this observation is that dermal hapten exposure alone was sufficient to induce Treg migration. Haptens are capable of causing inflammation in nonsensitized animals, demonstrating their capacity to induce innate responses (17, 24, 28). Therefore, we next assessed whether dermal exposure to Oxa was capable of inducing Treg migration in the absence of prior sensitization. Unexpectedly, 24 h after exposure of naïve mice to 1% Oxa, Treg numbers (as detected via multiphoton imaging) were unaltered in the hypodermis, but were significantly reduced in the dermis, relative to untreated mice (Supplemental Fig. 1A, 1B). In contrast to this finding, flow cytometric analysis of the Treg content of the full-thickness skin and draining lymph nodes showed no significant alteration in Treg abundance in either location (Supplemental Fig. 1C, 1D), indicating that Tregs were not migrating from the skin to the draining lymph nodes. However, histological analysis of skin sections demonstrated that hapten-exposed skin was significantly swollen (Supplemental Fig. 1E–G), and the proportion of skin-resident Tregs (detected via GFP expression) present in the outermost ~125 μm accessible to multiphoton microscopy was significantly reduced relative to that in untreated skin (Supplemental Fig. 1H–J). The latter findings explain the reduction in dermal Tregs detected via multiphoton imaging (Supplemental Fig. 1B).

In Oxa-treated mice, analysis of migration of the dermal Tregs detectable via multiphoton imaging revealed that displacement was significantly increased above that in untreated controls (Fig. 9A). As seen in CS, this increase stemmed from an increase in the proportion of motile cells (Fig. 9B) above the level in untreated skin (7%, Fig. 3B). These experiments reveal that dermal Treg behavior can also be modulated by an innate inflammatory stimulus.

**Discussion**

Despite studies demonstrating that Tregs must home to peripheral organs to provide effective regulation of local inflammation (5–8), the actions of Tregs in these sites are poorly understood. In this study, we show that, under both resting and inflammatory conditions, skin-resident Tregs exist in two populations: nonmotile or actively migrating throughout the dermis. In the absence of inflammation, the migratory population represents <10% of Tregs. However, upon inflammatory stimulation, the proportion of migratory Tregs increases. Interestingly, this response occurs to varying degrees both upon exposure of sensitized mice to the sensitizing hapten, as well as in mice in which CS-associated effector leukocyte recruitment was inhibited, Treg migration remained above levels observed in uninfamed skin. One possible explanation for this observation is that dermal hapten exposure alone was sufficient to induce Treg migration. Haptens are capable of causing inflammation in nonsensitized animals, demonstrating their capacity to induce innate responses (17, 24, 28). Therefore, we next assessed whether dermal exposure to Oxa was capable of inducing Treg migration in the absence of prior sensitization. Unexpectedly, 24 h after exposure of naïve mice to 1% Oxa, Treg numbers (as detected via multiphoton imaging) were unaltered in the hypodermis, but were significantly reduced in the dermis, relative to untreated mice (Supplemental Fig. 1A, 1B). In contrast to this finding, flow cytometric analysis of the Treg content of the full-thickness skin and draining lymph nodes showed no significant alteration in Treg abundance in either location (Supplemental Fig. 1C, 1D), indicating that Tregs were not migrating from the skin to the draining lymph nodes. However, histological analysis of skin sections demonstrated that hapten-exposed skin was significantly swollen (Supplemental Fig. 1E–G), and the proportion of skin-resident Tregs (detected via GFP expression) present in the outermost ~125 μm accessible to multiphoton microscopy was significantly reduced relative to that in untreated skin (Supplemental Fig. 1H–J). The latter findings explain the reduction in dermal Tregs detected via multiphoton imaging (Supplemental Fig. 1B).

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Ag and in nonsensitized mice in response to an innate inflammatory stimulus. This suggests that increased Treg migration is a fundamental component of the response of skin to inflammatory stimulation.

Although Tregs have been shown to be important in suppression of inflammation in this model of CS, the role of increased Treg motility in Treg-mediated suppression of inflammation is unknown (29). One hypothesis to explain the anti-inflammatory effects of Tregs is that this occurs via bystander suppression, as a result of nondirected release of broadly acting anti-inflammatory mediators (1, 2). It is conceivable that induction of Treg migration allows soluble mediators released by Tregs to affect a greater expanse of tissue, potentially achieving a more effective bystander suppression effect. This behavior may also increase the probability of Tregs encountering other immune cells with roles in skin inflammation. The finding that the majority of Tregs are sessile in uninfamed skin may indicate that these putative functions are minimally required in the absence of an overt inflammatory stimulus.

Previous studies demonstrated that Tregs represent a substantial proportion of the CD4+ T cell population that undergoes constitutive migration from the skin to the draining lymph node under steady-state conditions (30). These findings are somewhat difficult to reconcile with the present finding that only a small proportion of the dermal Tregs are actively migratory. Recent studies in humans and mice provide evidence that the skin is a major reservoir of nonrecirculating, resident effector memory T cells (9, 31–33). These resident cells are thought to serve important functions in controlling local immune responses. Given the present findings, it is conceivable that the sessile Tregs make up the major Treg population that is resident in the skin, whereas the migratory Tregs may represent a subpopulation of cells, potentially of a different phenotype, which are in the process of trafficking to the local lymph nodes (30). It will be important to determine whether the migratory Tregs are recent immigrants into the skin or resident cells that have been stimulated to mobilize.

The finding that Oxa induced Treg migration in nonsensitized animals (i.e., via an innate pathway) suggests that an element of the innate response to this irritant is sufficient for induction of Treg migration. This is supported by recent observations of induction of Treg migration in the skin of naive mice within 5–20 min following intradermal exposure with infection by Staphylococcus (34). The finding that the presence of Tregs may represent a subpopulation of cells, potentially of a different phenotype, which are in the process of trafficking to the local lymph nodes (30). It will be important to determine whether the migratory Tregs are recent immigrants into the skin or resident cells that have been stimulated to mobilize.

In conclusion, these data indicate that increased Treg migration in response to an innate stimulus, the Treg migration response induced following re-exposure to sensitizing Ag was substantially more robust and resulted in recruitment of additional Tregs. Our studies using an alternative hapten in the challenge phase indicate that Ag recognition by the adaptive immune system contributed to the increase in Treg migration. Limiting leukocyte recruitment during the effector phase also resulted in a reduction in the extent of alteration in Treg migration. Although these studies emphasize the importance of the recognition of the hapten by the adaptive immune system, they do not demonstrate that the Tregs are responding via Ag recognition. Subtracting the proportion of Tregs migrating in response to cognate Ag exposure (Oxa, 41%) from that of Tregs migrating in desensitized mice in response to an irrelevant hapten (DNFB, 23%) indicates that Ag recognition contributes to altered migration ~18% of skin Tregs. This is conceivable, but unlikely, that 18% of the dermal Tregs in sensitized mice are specific for Oxa. An alternative explanation is that Ag recognition drives induction of inflammation; subsequently, the proportion of Tregs induced to undergo migration is relative to the intensity of the inflammatory response, with the Ag-induced inflammation being more intense than the innate response to Oxa.

In conclusion, these experiments examined the compartmentalization of Tregs within the skin; they were abundant in the dermis, whereas they were relatively scarce in the deeper layers. Because the dermis is more likely to be exposed to inflammatory stimuli from the external environment, it is possible that the inflammation-suppressing actions of Tregs are more critical in this region. However, it was notable that induction of CS resulted in increased numbers of Tregs in the hypodermis. In addition, Tregs were observed undergoing intravascular adhesion in vessels in the hypodermis but not in the more superficial layers. Because adhesion is required for delivery of circulating leukocytes to sites of inflammation, these findings led us to speculate that the hypodermal vasculature is an important route of delivery of Tregs into the inflamed skin and that, upon exiting the vasculature in this location, Tregs subsequently migrate to the more superficial dermis. More work is required to determine the validity of this hypothesis. An additional consideration emerging from the analysis of Treg compartmentalization in the skin in these experiments is that inflammation-associated swelling impacts the capacity of multiphoton imaging to detect intradermal Tregs. This observation illustrates that experiments using imaging to examine Treg recruitment to the inflamed skin need to be supported by alternative approaches, such as histology and flow cytometry, as we did in this study and previously (19).

In conclusion, these data indicate that increased Treg migration is an inherent element of the response of the skin to inflammation. Indeed, it is notable that the number of Tregs stimulated to undergo this response was proportional to the level of inflammation affecting the skin. Although it is tempting to speculate that induction of migration is indicative of activation of Treg suppressor function, it remains unclear whether this migration is critical to the ability...
of Tregs to limit dermal inflammation. Future studies will aim to determine whether this behavior contributes to Treg-mediated suppression of skin inflammation.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplement

Dermal Regulatory T cells display distinct migratory behavior that is modulated during adaptive and innate inflammation

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1. Video Descriptions

2. Supplemental Figure 1
Video Descriptions

**Video 1: Tregs in uninflamed skin:** Multiphoton microscopy recording showing Tregs (green) visible in the dermis of an uninflamed mouse, undergoing minimal migration. Dermal collagen (blue) is visible via detection of the second harmonic signal, and the vasculature (red) has been labeled with TRITC albumin. Maximum projection image of ~100 µm depth. Duration = 5 min.

**Video 2: Comparison of migration of Tregs and CD4⁺ effector T cells in same region:** Area of dermis in uninflamed skin of a Foxp3-GFP mouse, in which DsRed-expressing, HSV-specific CD4⁺ (gDT-II) effector T cells are present in addition to endogenous GFP-expressing Tregs. All of the effector T cells present are rapidly migrating throughout the dermis. In contrast, the Tregs are immotile. Dermal collagen (blue) is visible via detection of the second harmonic signal. Maximum projection image of ~100 µm depth. Duration = 25 min.

**Video 3: Migratory Tregs in inflamed skin:** Area of dermis in a sensitized mouse, 24 h after challenge with oxazolone. Most Tregs are undergoing migration – colored tracks indicate the paths of migration. Dermal collagen (blue) is visible via detection of the second harmonic signal, and the vasculature (red) has been labeled with TRITC albumin. Maximum projection image of ~100 µm depth. Duration = 5 min.

**Video 4: Migratory Tregs in inflamed skin, following intradermal injection of saline:** Area of dermis in a sensitized mouse 24 h after challenge, following intradermal injection of saline 4 h
earlier. Most Tregs are undergoing migration. Dermal collagen (blue) is visible via detection of the second harmonic signal, and the vasculature (red) has been labeled with TRITC albumin. Duration = 28 min.

**Video 5: Effect of intradermal CCR4 antagonist on Treg migration in inflamed skin.** Area of dermis in a sensitized mouse 24 h after challenge, following intradermal injection of CCR4 antagonist, C021, 4 h earlier. Most Tregs are static. Dermal collagen (blue) is visible via detection of the second harmonic signal, and the vasculature (red) has been labeled with TRITC albumin. Duration = 28 min.
Supplemental Figure. 1: Effect of innate hapten application on Treg numbers and location in skin and draining lymph nodes. A, B: Treg numbers detectable in skin regions (A – hypodermis, B – dermis) via multiphoton microscopy, in untreated mice (Un), and 24 h after application of oxazolone (Oxa). C, D: Flow cytometric analysis of total Treg content in full-thickness skin (C), and in skin-draining lymph nodes (D) of untreated (Un) mice, and 24 h after application of oxazolone (Oxa). E, F: Histological (H&E) assessment of untreated skin (E), and hapten-treated (24 h) skin (F) demonstrating increase in skin thickness resulting from hapten exposure. G: Quantitative data for skin thickness measured in histological sections from the same animals. H, I: Fluorescence microscopy assessment of location of Tregs in skin of Foxp3-GFP mice, under resting conditions (H), and following hapten application (I). Tregs are visible via GFP expression (green, some cells also indicated by arrows). Nuclei were stained with Hoechst 33258 (blue). Epidermis is uppermost in sections. In untreated skin, most cells are within ~125 µm of epidermis, while in hapten-treated skin, Tregs are located further from the epidermis. J: Percentage of Tregs with 125 µm of the epidermis in untreated mice (Un) and 24 h after application of oxazolone (Oxa). Data represent mean ± sem of n=6 mice in each group. ** p<0.01 & *** p<0.001 versus untreated mice.