Imaging of Effector Memory T Cells during a Delayed-Type Hypersensitivity Reaction and Suppression by Kv1.3 Channel Block

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SUMMARY

Effector memory T (Tem) cells are essential mediators of autoimmune disease and delayed-type hypersensitivity (DTH), a convenient model for two-photon imaging of Tem cell participation in an inflammatory response. Shortly (3 hr) after entry into antigen-primed ear tissue, Tem cells stably attached to antigen-bearing antigen-presenting cells (APCs). After 24 hr, enlarged Tem cells were highly motile along collagen fibers and continued to migrate rapidly for 18 hr. Tem cells rely on voltage-gated Kv1.3 potassium channels to regulate calcium signaling. ShK-186, a specific Kv1.3 blocker, inhibited DTH and suppressed Tem cell enlargement and motility in inflamed tissue but had no effect on homing to or motility in lymph nodes of naive and central memory T (Tcm) cells. ShK-186 effectively treated disease in a rat model of multiple sclerosis. These results demonstrate a requirement for Kv1.3 channels in Tem cells during an inflammatory immune response in peripheral tissues. Targeting Kv1.3 allows for effector memory responses to be suppressed while central memory responses remain intact.

INTRODUCTION

Costimulation-independent CCR7−CD45RA− effector memory T (Tem) cells are essential mediators of numerous chronic inflammatory autoimmune diseases including rheumatoid arthritis (RA), multiple sclerosis (MS), type 1 diabetes mellitus (T1DM), and psoriasis (Beeton et al., 2006; Conrad et al., 2007; Ellis and Krueger, 2001; Haegel et al., 2007; Kivisakk et al., 2004; Krakauer et al., 2006; Rus et al., 2005; Wulff et al., 2003b). Tem cells are a tissue-resident subset of memory T cells that display immediate effector function at the site of antigen deposition (Sallusto et al., 2004). Tem cells respond in nonlymphoid tissues, where they initiate a localized inflammatory immune response. Upon activation, CD4+ Tem cells give rise to T helper 1 cells (Tem effectors) that produce interferon gamma (IFN-γ), interleukin-2 (IL2), tumor necrosis factor alpha and beta (TNF-α and TNF-β), all potent mediators of the inflammatory response that recruit and activate macrophages, which, in turn, secrete TNF-α and interleukin-1 (IL1). Together, these events inaugurate the self-propagating localized inflammatory immune response that is typical of delayed-type hypersensitivity (DTH) and autoimmune diseases. DTH in rats, as in humans, is characterized by tissue swelling and infiltration in the subcutaneous layer and demis by IFN-γ- and TNF-α-expressing Tem cells (Gaga et al., 1991; Hancock et al., 1994).

Fluorescence microscopy and single-cell patch-clamp studies show that quiescent human peripheral blood CD4+ and CD8+ naive, central memory T (Tcm), and Tem cells have similar channel phenotypes expressing ~300 voltage-gated Kv1.3 potassium channels per cell and ~10 calcium-activated KCa3.1 potassium channels per cell. Upon activation, naive and Tcm cells upregulate KCa3.1 channels, whereas Tem cells upregulate Kv1.3 channels when they change into Tem effectors (Wulff et al., 2003b). In Tem cells, Kv1.3 localizes at the immune synapse during antigen presentation and regulates the membrane potential of these cells, maintaining the driving force for influx of Ca2+ ions during cell activation (Beeton et al., 2005; Chandy et al., 2004; Panyi et al., 2004; Rus et al., 2005). Genetic silencing of Kv1.3 in human T cells leads to an expansion of Tcm cells and a depletion of Tem cells, highlighting the functional importance of the Kv1.3 channel in the Tem population (Hu et al., 2007). Specific Kv1.3 inhibitors preferentially suppress calcium flux, cytokine production, and proliferation in vitro of CCR7− Tem effector cells without affecting the function of naive and Tcm cells (Beeton et al., 2005; Beeton et al., 2006; Wulff et al., 2003b).

Disease-associated autoreactive T cells from the blood of patients with MS, RA, or T1DM display the Tem-effector-specific
phenotype of Kv1.3hi in the blood, whereas T cells specific for disease-irrelevant antigens from the same patient populations or T cells specific for autoantigens in control populations are CCR7−/CD45RC−, characteristic of Tem cells (solid line = resting Ova-GFP+ T cells, dotted line = background staining; gray = activated).

Figure 1. Characterization of Ova-GFP T Cells

(A) Flow-cytometric analysis of the indicated cell-surface proteins on Ova-specific GFP+ rat T cells. The cells are CCR7−/CD45RC−, characteristic of Tem cells (solid line = resting Ova-GFP+ T cells, dotted line = background staining; gray = activated).

(B) Kv1.3 channel expression in resting (mean = 461 ± 69 Kv1.3 channels/cell, n = 11 cells) and activated (1862 ± 208 Kv1.3 channels/cell, n = 23) GFP+ CD4+ Ova-specific rat T cells. Data were obtained by patch-clamp analysis.

(C) Kv1.3 channel expression in GFP+ Ova-specific T cells isolated from ears undergoing DTH, 3 hr (307 ± 74 Kv1.3 channels/cell, n = 7 cells) and 24 hr (1136 ± 162 Kv1.3 channels/cell, n = 9 cells) after challenge with Ova.

GFP+ Ovalbumin-Specific T Cells Display Tem Markers and Abundant Kv1.3 Channels

Ova-specific GFP+ CD4+ rat T cells (Flugel et al., 1999) lack surface expression of CCR7 and CD45RC (Figure 1A) and upon in vitro activation upregulate ovalbumin-specific CD4+CCR7−CD45RC−Kv1.3hi Tem cells induces a highly reproducible DTH response upon subsequent local challenge by subcutaneous injection of antigen. By combining this system with two-photon microscopy and peripheral tissue preparation for live-cell imaging, we imaged the activation and motility of Tem cells in inflamed tissue during a DTH reaction. In addition, we show that ShK-186 preferentially suppresses the in vivo motility and activation of Tem cells at the inflamed site without affecting the motility of naive and Tcm cells in lymph nodes. Therefore, we examined whether the selective suppression of Tem cells via Kv1.3 blockade effectively treats chronic EAE, a model of MS, and whether such therapy compromises the immune response to two common and medically important infectious agents—influenza virus and Chlamydia trachomatis.

RESULTS

Development of Adoptive DTH and Preparation of Tissue for Imaging

Ova-specific GFP+ Tem effector cells generated by antigen-specific activation in vitro were adoptively transferred intraperitoneally into Lewis rats that were subsequently challenged in one ear with ovalbumin conjugated to Texas red (Ova-TR) and in the other ear with saline (Figure S2). Substantial increases in ear thickness (>50% increase in thickness) were seen as early as 3 hr after antigen challenge (Figure 2A). The increase in ear thickness (>50% increase in thickness) was seen as early as 3 hr after antigen challenge (Figure 2A).
thickness was maintained through the 42 hr time point. Using an ear explant preparation, we removed the epidermis and some of the dermis along with hair follicles (Figure 2B) that produced image-obscuring autofluorescence. Extravascular cells were imaged in the remaining dermis and underlying subcutaneous tissue (Figure 2C).

Motility of Tem Cells and Tem Effectors during Development of DTH

Two-photon imaging (Figures 3A–3C and Movie S1) revealed Ova-specific GFP⁺ Tem cells (green), presumptive (antigen-presenting cells (APCs) that had taken up and concentrated Ova-TR (red), and collagen structures visualized with second-harmonic generation (blue). At the 3 hr time point after Ova-TR injection into the ear, the majority of GFP⁺ Ova-specific cells were immobile and usually maintained stable contact with red-labeled Ova-TR-bearing APCs (Figure 3A and Movie S2). At the 24 hr time point, enlarged and highly motile cells extended long membrane projections while migrating along collagen in distinct subcutaneous regions and paused but did not arrest on Ova-TR-bearing APCs (Figure 3B and Movie S3). The heterogenous localization of Tem effector cells was confirmed histologically (data not shown) and is consistent with previous histological studies of DTH in rats and humans (Gaga et al., 1991; Hancock et al., 1994). At the 42 hr time point, effectors remained enlarged and highly motile (Figure 5D) and continued to crawl along collagen-fiber bundles in the midst of Ova-TR-containing APCs (Figure 3C and Movie S4).

At the 3 hr time point, effector cells in contact with a local APC migrated with an average velocity of 3.9 μm/min, slower than cells in contact with collagen (5.3 μm/min) (Figure 3D). As DTH progressed, enlarged Tem cells migrated faster, with average velocities at 24 hr of 7.4 μm/min when in contact with APCs and 11.1 μm/min when in contact with collagen (Figure 3E). By 42 hr, the enlarged effectors maintained high velocities, moving at 7.0 μm/min when making short-lived contacts with APCs and 12.0 μm/min while crawling along collagen (Figure 3F). Interestingly, the size of the activated effectors correlated with their velocity; larger cells moved with higher average velocities than smaller cells (Figure 3G). At the 3 hr time point, ~70% of cells were in contact with Ova-TR-bearing APCs at any given time and, as the DTH reaction developed, the proportion of time that effector T cells were in contact with APCs decreased progressively (Figure 3H). Contact durations at 3 hr often exceeded the length of our imaging records (~30 min). Later in the DTH reaction, interaction times became progressively shorter, with mean durations of 7 min at 24 hr and 3 min at 42 hr (Figure 3I).

In order to confirm that cell motility and contact durations in the ear tissue were not significantly altered by the explant imaging preparation, we performed intravitral imaging of adoptive DTH in the rat paw 24 hr after antigen challenge (Figure S3 and Movie S5). Velocities (averaging 8.5 μm/min) and the average contact duration (7 min) of enlarged, activated Ova-specific GFP⁺ T cells in the rat paw were similar to those characteristics determined by imaging of ear tissue.

In addition, to examine the behavior of bystander T cells, we imaged motility of Ova-specific GFP⁺ T cells during a DTH response to an irrelevant antigen. After initiation of an active DTH response with hen-egg lysozyme (HEL), Ova-specific Tem effector cells infiltrated the inflamed ear tissue and migrated rapidly along collagen fibers with an average velocity of 10.4 μm/min (Figures 3J–3L; Movie S6), comparable to the velocities of antigen-activated Tem cells migrating on collagen (Figures 3B and 3E). Unlike Tem cell behavior in the DTH response to cognate antigen, Tem cells infiltrating the site of inflammation induced by irrelevant antigen failed to enlarge (Figure 3L), indicating that they did not reactivate in vivo.

Tem Effector Cell Movement along Collagen

We further analyzed the patterns of migration of highly motile Tem effector cells along collagen networks in inflamed tissue (24 hr time point) as they moved along collagen fibers and intersected with Ova-TR-bearing APCs (Figure 4). Effector T cells predominantly followed paths parallel to the larger collagen bundles, (white cell tracks, Figures 4A and 4B; Movie S7).
Figure 3. Activation and Motility of CCR7<sup>+</sup> Effector Cells at the Site of DTH

(A) GFP<sup>+</sup> Ova-specific T cells (green) in subcutaneous ear tissue 3 hr after antigen injection interacting with local APCs (red) among collagen fibers (blue). Major tick marks = 20 μm.

(B) Enlarged, highly motile CCR7<sup>+</sup> effector cells (green) 24 hr after antigen injection, imaged crawling along collagen-fiber bundles.

(C) Large, highly motile CCR7<sup>+</sup> effectors in the subcutaneous tissue 42 hr after antigen injection.

(D) Distributions of instantaneous velocities of CCR7<sup>+</sup> effector cells at 3 hr. Those in contact with an APC are shown by red bars (mean velocity 3.9 μm/min, n = 1722 measurements); those in contact with collagen are shown by blue bars (5.3 μm/min, n = 758; p < 0.05).

(E) Corresponding velocity distributions of CCR7<sup>+</sup> effector cells 24 hr after antigen challenge. CCR7<sup>+</sup> effectors in contact with collagen fibers were highly motile (blue bars, 11.1 μm/min, n = 1654), whereas those contacting antigen-bearing APCs showed lower velocities (red bars, 7.4 μm/min, n = 1568; p < 0.05).

(F) Velocity distributions of CCR7<sup>+</sup> effector cells contacting collagen (blue bars, 12.0 μm/min, n = 2213) or encountering an antigen-bearing APCs (red bars, 7.0 μm/min, n = 1359; p < 0.05) measured 42 hr after antigen challenge.
Although occasional cells crossed the collagen bundles, the overall displacement from origin/distance traveled (meander index) was consistent with movement directed by structural constraints (Figure 4C). At junctions within the local collagen scaffold, effector cells closely tracked along the collagen, often resulting in circuitous pathways in which the overall cell displacement was minimal (Figure 4D and Movie S8). Effectors were also able to change direction by traversing from fiber to fiber at junctions (red and gold tracks; Figure 4E). This pattern of directed migration of Tem effectors predominantly along collagen bundles contrasts sharply with the apparent random-walk migration of Tem effectors predominantly along collagen bundles (H). Percentages of time for which CCR7 was engaged as a function of the cross-sectional areas of bystander GFP+ Ova-specific T cells at different times during DTH. Values were expressed as mean ± standard error (SE). (G) Mean velocities of CCR7+ effector cells as a function of their cross-sectional areas at different times during DTH. Values were expressed as mean ± standard error (SE). (H) Percentages of time for which CCR7 was engaged as a function of the cross-sectional areas of bystander GFP+ Ova-specific T cells at different times during DTH. Values were expressed as mean ± standard error (SE). (I) Duration of CCR7+ effector T cell contacts with local APCs at varying times after antigen challenge; mean contact durations are represented by red bars at 3 hr (24 min, n = 54 contacts in 6 experiments), 24 hr (7 min, n = 77 contacts in 3 experiments), and 42 hr (3.5 min, n = 55 contacts in 3 experiments). (J) Bystander GFP+ Ova-specific T cells (green) among collagen fibers (blue) in subcutaneous ear tissue 24 hr after induction of active DTH by injection of hen-egg lysozyme (HEL). Development of the DTH response was measured by ear swelling (44% increase in ear thickness, n = 2). Major tick marks = 20 μm. (K) Velocity distribution of bystander GFP+ Ova-specific T cells (mean velocity 10.4 μm/min, n = 5222). (L) Mean velocities of bystander GFP+ Ova-specific T cells as a function of their cross-sectional area 24 hr after induction of active DTH. Values were expressed as mean ± SE.

Suppression of DTH by Kv1.3 Channel Block
ShK-186, a synthetic analog of the ShK peptide isolated from the sea anemone Stichodactyla helianthus, is modified at the N terminus to increase specificity for Kv1.3 while retaining picomolar potency (Beeton et al., 2005, 2006). ShK-186 blocked the Kv1.3 current in the Ova-specific GFP+ Tem cells in a dose-dependent manner (Figure 5A). At concentrations that blocked the Kv1.3 channel, ShK-186 suppressed antigen-specific proliferation of the Tem cells (Figure 5B). ShK-186 (100 μg/kg) administered by subcutaneous injection in the scruff of the neck at the time of Ova-TR challenge and 24 hr later (Figure S4A) reduced DTH (smaller Δ ear thickness) at all time points compared to rats given saline injections (Figure 5C). The circulating levels of SHK-186 in injected rats (Figure S4B) were sufficient to suppress the proliferation of the Tem cells. The total number of extravascular GFP+ Tem effectors recovered from each ear was the same in rats administered saline or SHK-186 injections (Figure 5D), indicating that ShK-186 treatment did not prevent Ova-specific GFP+ Tem effectors from entering the ear tissue. By 42 hr the numbers of GFP+ cells in the ear had decreased in both saline- and ShK-186-treated rats (Figure 5D).

Skin-homing memory T cells involved in DTH express high levels of β1 integrins that facilitate the migration of these cells to a site of antigenic challenge via integrin interaction with matrix proteins (Andreasen et al., 2003; Ferguson et al., 1991; Mackay et al., 1992; Picker et al., 1990). To identify a mechanism that may underlie ShK-186’s activity, we examined the peptide’s effect on activated β1 integrin expression in Ova-specific Tem cells. Activation of β1 integrin in T cells is calcium dependent and is probably mediated by a concerted mechanism involving the increase of intracellular calcium and activation of PKC (Lim et al., 2000; Rowin et al., 1998). We have previously shown that ShK-186 treatment suppresses antigen-induced calcium signaling in Tem cells (Beeton et al., 2006). Therefore, we hypothesized that ShK-186 treatment immobilized Tem cells at the site of DTH by reducing intracellular calcium and thereby inhibiting the activation of β1 integrin. To mimic the conditions of the in vivo experiments, we incubated Ova-specific Tem cells in vitro with antigen-pulsed irradiated thymocytes for 48 hr to generate activated Tem effectors. In other experiments, Tem cells were “rested” in IL2-containing medium for 6 days (resting Tem cells). Intracellular calcium and activated β1 integrin expression were then measured in activated or resting Tem cells exposed to 100 nM ShK-186 or vehicle for 30 min. As expected, intracellular calcium was elevated in activated Tem effectors, compared to resting Tem cells (Figure S5). Acute exposure to ShK-186 significantly lowered intracellular calcium in activated Tem cells (p < 10^-6), but did not affect intracellular calcium in resting Tem cells. We also measured activated β1 integrin expression by staining with the HUTS-21 mAb (Luque et al., 1996) and gating on the GFP+ population. HUTS-21 staining was detected on activated but not resting Tem cells (Figures 6I and 6J). ShK-186 reproducibly minimized HUTS-21 staining in activated Tem cells to levels comparable to background staining (p < 0.01), paralleling its inhibitory effect on intracellular calcium. Confirming the calcium dependence of β1-integrin activation, acute removal of external Ca2+ brought HUTS-21 expression to baseline (p < 0.01). Our results thus suggest that ShK-186 immobilizes Tem cells at inflammatory sites by suppressing calcium signaling and thereby prevents β1 integrin activation.

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Motility of CCR7+ T Cells in the Lymph Node Is Unaltered by ShK-186 Treatment

The finding that ShK-186 dramatically reduces motility of Tem cells in ear tissue prompted us to determine its effects on resting T cells in lymph node. A pool of predominantly CD3+CCR7+ cells (Figure 7A) was purified from secondary lymphoid organs, labeled with CFSE, and transferred into Lewis rats, which received either ShK-186 or saline by subcutaneous injection at the time of cell transfer. CFSE-labeled T cells in both groups (saline- or ShK-186-treated rats) were highly motile, displaying no difference in cell localization velocities, directional preference, or meander index within the lymph node (Figures 7B–7F; Movies S12 and S13). Thus, ShK-186 does not affect the motility of lymph-node-resident T cells.

Evaluation of ShK-186 in Other Disease Models

We evaluated the therapeutic effectiveness of ShK-186 in a rat EAE model that closely follows the clinical course of human
MS. DA rats immunized with spinal cord homogenate and complete Freund’s adjuvant developed relapsing-remitting EAE about 10 days after immunization (Figure S6A). T cells in the central nervous system (CNS) of rats with EAE were initially a mixture of CD4+CCR7+ naive and Tcm and CD4+CCR7− Tem cells because of the adjuvant effect, but as the disease progressed, the majority of T cells in the rat CNS were CD4+CCR7− Tem cells (Figure S6B) similar to T cells in the CNS in human MS patients (Kivisakk et al., 2004; Rus et al., 2005). Daily subcutaneous administration of ShK-186 from disease onset had no effect on the initial phase of EAE, but produced a striking reduction in the clinical score, coincident with the emergence of the Tem population in the CNS (Figure 7G). Disease amelioration was accompanied by reduced demyelination and inflammatory infiltrate in the CNS (Figure S6C and Table S1). The presence of inflammatory cells in the CNS of treated rats is consistent with the two-photon DTH data, where Tem cells enter but are immobilized in inflamed tissues in ShK-186-treated rats.

Although Kv1.3 blockers have been previously shown to have a good safety profile in rats and monkeys (Beeton et al., 2006), the primary concern in development of an autoimmune therapy is whether it compromises the protective immune response. We examined whether ShK-186, at the dose that suppresses DTH and chronic EAE, would affect clearance of two medically important infections: influenza and chlamydia. In addition, we examined effects of another Kv1.3-selective blocker, PAP1 (Azam et al., 2007). As a control, we used dexamethasone, which broadly suppresses the immune response. Vehicle-treated Sprague Dawley rats cleared the influenza virus in 4 days, as did rats treated with ShK-186 or PAP-1, whereas dexamethasone significantly delayed clearance (Figure 7H and Figure S7A). In a parallel study, Lewis rats given saline or ShK-186 cleared a chlamydial infection equally rapidly, whereas rats treated with dexamethasone did not clear the infection during the 4 weeks of observation (Figure 7I and Figure S7B).

**DISCUSSION**

Delayed-type hypersensitivity is an inflammatory immune response initiated by the activation of CD4+ Tem cells, followed by recruitment of macrophages and other effector cells (Szabo et al., 2003). To image the cellular dynamics of this process, we developed a method enabling in situ two-photon microscopy in inflamed ear tissue. We provide the first visualization of Tem cell motility and interaction with APCs and collagen structural elements in peripheral tissue during a DTH immune response. Moreover, we report that a specific blocker of the voltage-gated Kv1.3 potassium channel (ShK-186) inhibits DTH and suppresses Tem effector cell activation in inflamed tissues, but does not alter acute immune clearance in two infectious-disease models.
At the onset of DTH, cytokine cross-talk between tissue-resident APCs and skin-homing T cells initiates an inflammatory cascade. Three hours after antigen challenge, we find that Ova-specific Tem cells are predominantly engaged in prolonged contacts with tissue-resident APCs in the subcutaneous tissue and display low overall motility. In contrast, antigen-irrelevant Tem cells are highly motile in the inflamed tissue. This mirrors the behavior of myelin basic protein-specific Tem cells invading the spinal cord, where many of the cells appeared tethered to a fixed point (Kawakami et al., 2005). The Ova-specific Tem cells used here were highly motile in spinal cord tissue, but became immobilized after intrathecal injection of Ova, presumably because of interaction with local APCs (Kawakami et al., 2005). Our results extend those observations by showing that during the course of an immune response, Tem effector cells arrest in contact with APCs enlarge and become highly motile through dense networks of collagen. Although less numerous from 24 to 42 hr, the Tem effector cells move rapidly, primarily tracking along bundles with apparently random changes in direction at scaffold junctions, but also occasionally traversing between bundles. Such directionality contrasts with the random motility of these same cells in spinal cord (Kawakami et al., 2005), and with the random motion of CCR7+ cells in lymph node (Miller et al., 2002).

When Tem cells encounter cognate-antigen-bearing APCs in the ear tissue, they enter an activation cascade that is mirrored by changes in cell dynamics. As the cells activate, Ca$^{2+}$ signaling is initiated; motility is arrested; and the cells enlarge, produce cytokines, and alter gene expression in preparation for cell

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**Figure 6. Inhibition of CCR7+ Effector Cell Motility and Activation by ShK-186**

(A–C) Maximum-intensity projections of two-photon image stacks acquired in the subcutaneous ear tissue at 3, 24, and 42 hr after antigen challenge and treatment with ShK-186. Ova-specific GFP+ CCR7+ effector cells (green), collagen-fiber bundles (blue), and APCs bearing Ova-TR (red) are shown. Major tick marks = 20 μm.

(D–F) Distributions of instantaneous velocities of CCR7+ effector cells in ShK-186-treated animals at corresponding time points after Ova-TR challenge. Arrows mark mean velocities (3 hr: 2.7 ± 0.04 μm/min, n = 2397 measurements; 24 hr: 2.1 ± 0.05 μm/min, n = 2870; 42 hr: 1.2 ± 0.04 μm/min, n = 2227).

(G) Percentage of time effector cells contacted APCs (red bars) or collagen (blue bars) at different time points after Ova-TR challenge (n = 34–46 cells in 3 or 4 experiments).

(H) Sizes (cross-sectional area) of CCR7+ effector cells in saline-treated (open symbols) and in ShK-186-treated animals (filled symbols) at different times after Ova-TR challenge. Mean values are indicated by red bars (in saline-treated animals at 3, 24, and 42 hr after antigen challenge, respectively, 130 ± 9.6, 280 ± 11.0 μm², and 269 ± 11.6 μm²; in ShK-186-treated animals, 32 ± 2.5, 38 ± 4.1, and 44 ± 4 μm²).

(I) FACS plots of activated β1 integrin (exposed amino acids 355–425) on the surface of activated Tem cells with and without ShK treatment. Activated Tem cells have the activated form of β1 integrin on the cell surface (gray solid line), which is suppressed by 100 nM ShK-186 (black solid line) to levels similar to control staining (two overlapping dotted lines, left panel).

(J) Mean fluorescence intensity (MFI) ratios (HUTS-21 staining/secondary control) for activated Tem cells (1.8 ± 0.08 SE, n = 21) were significantly higher (p < 0.01) than in activated Tem incubated in 0 Ca$^{2+}$ solution (1.1 ± 0.07 SE, n = 12) or treated with 100 nM ShK (1.06 ± 0.03 SE, n = 17). Resting Tem cells (day 6) had low levels of activated β1 integrin that were not significantly different (p > 0.44) than in control conditions (2 mM Ca$^{2+}$; 1.2 ± 0.04, SE, n = 4), 0 Ca$^{2+}$ (1.2 ± 0.05, n = 4), or with ShK treatment (1.3 ± 0.12, n = 4).
proliferation. The enlarged T cell blasts later release from the APCs and migrate rapidly along collagen fibers, while continuing to have further encounters with APCs. In vitro studies have shown that Ca$^{2+}$ signaling is required for stable T cell-APC association and alterations in gene expression (Negulescu et al., 1996, 1994). If ShK-186 is present, Ca$^{2+}$ signaling is attenuated, resulting in reduced activation of $\beta 1$ integrin and suppression of cytokine production and cell proliferation (Beeton et al., 2006). Consequently, Tem cells do not reactivate at the site of DTH in rats treated with ShK-186, as indicated by their failure to form productive contacts with APCs or enlarge. The attenuated calcium signaling and reduced activated $\beta 1$ integrin expression probably contribute to the nonmotile state of the Tem cells. Integrins $\alpha 1\beta 1$ (VLA-1) and $\alpha 2\beta 1$ (VLA-2) enable lymphocytes to adhere to collagen in inflamed tissue (de Fougerolles et al., 2000), and in this study $\alpha 1\beta 1$ and $\alpha 4\beta 1$ (VLA-4) are both
upregulated by Ova-specific GFP+ Tem cells that are found tracking along collagen in ear tissue. Together, our results and those of others (reviewed in Dustin and de Fougerolles [2001]) indicate that normal Ca^{2+} signaling and maintenance of activated β1 integrin in Tem cells are necessary for Tem cell activation and subsequent motility in the collagen-rich tissue environment.

Altered motility and suppression of Ca^{2+} signaling provide a plausible mechanism for the selectivity and effectiveness of Kv1.3 blockers in preventing or treating disease in five distinct autoimmune models that are mediated by Tem cells: EAE in Lewis rats induced by adoptively transferred myelin-specific Tem cells (MS model) (Beeton et al., 2005; Beeton et al., 2001), chronic relapsing-remitting EAE in DA rats (MS model) (Figure 7G), pristane-induced arthritis in DA rats (RA model) (Beeton et al., 2006), experimental autoimmune diabetes mellitus in BB rats (T1DM model) (Beeton et al., 2006), and allergic contact dermatitis (Azam et al., 2007). Kv1.3 channel blockade during treatment of an autoimmune or inflammatory condition would reduce the ability of T cells to locate APCs in the tissue environment and Ca^{2+} signaling and downstream activation responses by T cells that do make contact with APCs. Currently available immunosuppressive agents, including natalizumab (Tysabri), a VLA4 antagonist that prevents effectors from entering tissues (Li et al., 2006), and S1P1-receptor-antagonists (e.g., FTY720) that regulate lymphocyte trafficking at vascular endothelial and lymphatic endothelial barriers (Rosen et al., 2007), are less selective for Tem cells.

The Kv1.3 channel in Tem cells is an important molecular target in inflammatory and autoimmune disorders. Tem cells are implicated in the pathogenesis of MS, and therapies that selectively target Tem cells, while sparing naive and Tcm cells, may have utility in MS therapy (Beeton et al., 2006; Haegеле et al., 2007; Hu et al., 2007; KiviSakk et al., 2004; Krakauer et al., 2006; Rus et al., 2005; Wulf et al., 2003a). The normal timeline to clearance of influenza virus and *Chlamydia trachomatis* in rats treated with a DTH-suppressing dose of ShK-186 is probably the result of redundancy in the multiple types of immune cells and cellular mechanisms involved in the clearance of these infectious agents. Preferential suppression of Tem cells in psoriasis patients by Alefacept (Ellis and Krueger, 2001) does not enhance the frequency of infections, and patients receiving Alefacept mount adequate vaccine antibody responses (Goffe et al., 2005; Gottlieb et al., 2003). In addition, Kv1.3 blockers are not expected to compromise anti-tumor immune responses (Klebanoff et al., 2005), or responses to chronic infections (Hedtmann et al., 2006; Stubbe et al., 2006), which are more effectively mediated by Tcm cells than by Tem cells.

In summary, our two-photon imaging methodology reveals the Tem cell dynamics underlying the normal immune response in peripheral tissues and provides mechanistic insights into the therapeutic potential of immunosuppression by targeting the Kv1.3 channels on Tem cells. The profound effects of Kv1.3 blockers on the motility and activation of Tem cells at the tissue site of antigen challenge suggest a promising strategy for treatment of chronic autoimmune diseases mediated by autoreactive Tem cells, a therapy based upon prolonged immobilization of Tem effectors at sites of inflammation and by preventing effectors from making contact with APCs and receiving activation signals.

**EXPERIMENTAL PROCEDURES**

**Animals**

Female inbred Lewis and DA rats were purchased from Harlan-Sprague Dawley (Indianapolis, IN, USA), and female inbred Sprague Dawley rats were purchased from Charles River (Raleigh, NC, USA). Animals were housed under clean conditions with irradiated rodent chow and acidified water ad libitum. All procedures were in accordance with National Institutes of Health (NIH) guidelines and approved by the University of California, Irvine, Institutional Animal Care and Use Committee.

**Cells**

Lewis rat Ova-specific CD4+ T cells transduced with GFP (Flugel et al., 1999) were maintained in culture by alternating phases of expansion in medium containing T cell growth factor (TCGF) and phases of stimulation with Ova (Sigma, St. Louis, MO, USA) in the presence of irradiated thymocytes as antigen-presenting cells, as described (Beeton et al., 2001). See Supplemental Experimental Procedures for additional details. T cells were isolated from Lewis rat spleens and lymph nodes with a MACS separation column (Miltenyi Biotec, Auburn, CA, USA). Purities of CD3+ and of CD4+ T cells were >95%, as indicated by flow cytometry.

**Potassium-Channel Blockers**

ShK-186 was synthesized as described (Beeton et al., 2005), PAP-1 (Schmitz et al., 2005) was a kind gift from H. Wulff (UC Davis, CA).

**Delayed-Type Hypersensitivity and Administration of ShK-186**

Adaptive DTH was induced in 7- to 9-week-old Lewis rats by the intraperitoneal transfer of 10 × 10^6 GFP+ Ova-activated effector T cells (Beeton and Chandy, 2007). Rats were challenged 48 hr later with 20 μg Ova-TR in the pinna of the right ear and saline in the pinna of the left ear. Rats received subcutaneous injections in the scurf of the neck of ShK-186 (100 μg/kg/day) or vehicle (PBS + 2% Lewis rat serum) at the time of challenge and 24 hr later. Induction of active DTH with the nonrelevant antigen HEL was initiated by priming 7- to 9-week-old Lewis rats with an intradermal injection of emulsion of HEL (100 μg/rat) in complete Freund’s adjuvant (Difco, Detroit, MI, USA). Five days later, 10 million activated Ova-GFP T cells were injected intraperitoneally. Two days later, the rats were challenged with an injection of HEL dissolved in saline in the pinna of one ear (20 μg/20 μl) and saline in the other ear. Ear thickness was measured at the site of injection 3, 24, and 42 hr after challenge with a spring-loaded micrometer (Mitutoyo, Spokane, WA, USA) and the difference in ear Ova-TR versus saline-challenged ears was calculated. Statistical analysis was carried out with the Mann-Whitney U test.

**Tissue Preparation**

DTH in rat ears is characterized by effector T cell infiltration of the subcutaneous tissue and dermis (Hancock et al., 1994). After determining that autofluorescence and second-harmonic signals from hair follicles obscured visualization of inflammatory cells and antigen-bearing APCs, we developed a method to expose the subcutaneous tissue of the ear for imaging during DTH. Lewis rats with DTH were euthanized 3, 24, or 42 hr after challenge, and after cardiac perfusion, the ears were removed by a single angled slice and kept in C02-independent media on ice. Under a 10× dissecting microscope, edges of the ear (~2 mm of tissue on each side) were removed to aid in separation of the epidermis and dermis together with the hair follicles. Tissue separation began at the proximal end of the ear with iridectomy dissection scissors (3 mm Vannas style, Fine Science Tools, Foster City, CA, USA) used to cut small extensions of connective tissue between the two layers. The preparation was then immediately mounted under the two-photon microscope, with the exposed subcutaneous tissue on the dorsal side of the ear facing the objective lens. Subsequent hemotoxylin and eosin staining of sections from imaged tissue confirmed tissue integrity.

**Footpad DTH and Intravital Imaging**

Adaptive DTH in the footpad of 7- to 9-week-old Lewis rats was induced by the intraperitoneal transfer of 10 × 10^6 GFP+ Ova-activated effector T cells (Beeton and Chandy, 2007), followed by antigen challenge 48 hr later with 20 μg Ova-TR in the right front paw. Twenty-four hours after antigen challenge, animals were anesthetized with 100 mg/kg ketamine, 10 mg/kg xylene, and...
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1/2 dosages were given every 45 min after initial anesthesia dose. The imaged paw was immobilized in a liquid-tight chamber filled with prewarmed RPMI for two-photon imaging of the ventral surface. DTH reaction was assessed by paw swelling and visualization of inflamed tissue marked by redness and observation of animal preference for the alternate forepaw.

Two-photon Imaging and Data Analysis
Two-photon imaging was performed as described previously (Matheu et al., 2007; Miller et al., 2002), with an upright Olympus BX50 microscope fitted with a 20x water-immersion objective (numerical aperture 0.95), a Spectra-Physics Tsunami femto-second laser, a resonant-mirror scan head, and a motorized focus controller (Prior Scientific, Rockland, MA, USA) (Nguyen et al., 2001). Images were acquired under software control by METAMORPH (Universal Imaging, Downingtown, PA, USA). Three-dimensional (3D) image stacks in ear tissue (x, y, z) were acquired at 18 s time intervals through an imaging volume 50 μm deep. Images and movies were processed with the 3D analysis software IMARIS 5.5.3., 64-bit version (Bitplane AG, Saint Paul, MN, USA). An excitation wavelength of 890 nm gave an optimal second-harmonic generation from collagen together with two-photon excitation of GFT and TR, and these signals were imaged with three photomultiplier channels (respectively, blue, green, and red) in conjunction with 500 nm and 560 nm dichroic mirrors and a 510 nm long-pass filter. Cell size was measured as cross-sectional (x-y) area, with an ellipsoid shape approximation. Data are presented as mean ± standard error of the mean (SEM). Tests of statistical significance were performed with a two-tailed Student’s t test, or Mann-Whitney U test where indicated. Significance is denoted as follows: * = 0.05; ** = 0.01; *** = 0.001; **** = 0.0001.

Calcium Imaging
Ratiometric calcium imaging was performed with Tem cells loaded with Fura-2 as described (Fanger et al., 2001), with cell-based calibration using ionomycin and EGTA or 10 mM external Ca²⁺ to achieve Rmin and Rmax, respectively.

In Vitro Assessment of Activated β1 Integrin
In the adoptive DTH model, Ova-specific Tem cells were activated in vitro for 48 hr before adoptive transfer into rats. Therefore, for the HUTS staining experiment, we activated the cells in vitro for 48 hr before assessing the effect of ShK-186 on HUTS expression. Cells were stained with monoclonal HUTS-21 (BD Biosciences), which recognizes an epitope among amino acids 355–425 that is exposed upon activation of β1 integrin (Luque et al., 1998). FACS analysis of HUTS-21 staining was performed by gating on the GFP⁺ cell population. The observed shift in mean fluorescence ratio of activated cells is consistent with previous studies (Gomez et al., 1997; Levite et al., 2000; Luque et al., 1998). See Supplemental Experimental Procedures for additional details.

Proportion Assays
Effect of ShK-186 on the proliferation of Ova GFP⁺ Tem cells was determined with [³H]-thymidine incorporation assays as described (Beeton et al., 2005; Beeton et al., 2001). See Supplemental Experimental Procedures for additional details.

Supplemental Data
Supplemental Data include Supplemental Experimental Procedures, one table, seven figures, and thirteen movies and can be found with this article online at http://www.immunity.com/cgi/content/full/29/4/ --- DC1/.

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