Supplemental Data

Imaging of Effector Memory T Cells

during a Delayed-Type Hypersensitivity Reaction

and Suppression by Kv1.3 Channel Block

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Supplemental Experimental Procedures

Culture of Ova-GFP T Cells

Ova-GFP T cells were maintained in culture by alternating phases of rest (6 days) and activation (2 days). The resting medium contained DMEM + 100 U/ml penicillin, 0.1 mg/ml streptomycin, 4 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 1% RPMI vitamins, 50 μM β-mercaptoethanol supplemented with 10% heat-inactivated horse serum and 10% TCGF. Cells were activated with 10 μg/ml Ova in the presence of irradiated (30 Gy) Lewis rat thymocytes (0.3 x 10^6 T cells and 12 x 10^6 thymocytes/ml) in medium supplemented with 1% Lewis rat serum.

Preparation of T Cell Growth Factor (TCGF)

Suspensions of single cells were prepared from Lewis rat spleens. Erythrocytes were lysed with 0.15 M NH_4Cl for 3 minutes on ice and splenocytes were washed and seeded (2 x 10^6 cells/ml) in RPMI 1640 medium supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 4 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 1% RPMI vitamins, 50 μM β-mercaptoethanol, 10% heat-inactivated fetal calf serum, and 2 μg/ml concanavalin A (all from Sigma, St. Louis, MO). Supernatant was collected 48 hours later and 15 mg/ml α methyl mannoside (Sigma) was added.

Electrophysiology

Kv1.3 currents were elicited by repeated 200 ms pulses from a holding potential of –80 mV to 40 mV, applied every 30 seconds to measure the characteristic voltage-dependence of these channels (left and middle panels, Supplemental Fig. 1B online) or blocking by ShK-186, or every second to visualize Kv1.3's characteristic cumulative inactivation (right panel, Supplemental Fig. 1B online). Kv1.3 currents were recorded in normal Ringer solution with a Ca^{2+}-free pipette solution containing (in mM): 145 KF, 10 HEPES, 10 EGTA, 2 $MgCl_2$, pH 7.2, 300 mOsm. Whole-cell Kv1.3 conductances were calculated from the peak current amplitudes at 40 mV. Kv1.3 channel numbers per cell were determined by dividing the whole-cell Kv1.3 conductance by the single channel conductance value of 12 pS. Cell capacitance, a direct measure of cell surface area, was constantly monitored during recording.

Flow Cytometry

T cells were washed and ~~incubated~~ with primary antibodies: anti-CD3 conjugated to biotin, anti-CD4 conjugated to PE-Cy5, anti-CD45RC, anti-CD25 conjugated to PE, anti-CD11a, anti-CD29 conjugated to biotin, anti-CD49d (BD Pharmingen, San Diego, CA), anti-CCR7 (Epitomics, Burlingame, CA), anti-VLA-1 (Chemicon, Temecula, CA). When required, secondary goat anti-mouse IgG conjugated to PE or goat anti-rabbit IgG antibodies conjugated to Alexa 647-PE, or streptavidin conjugated to PE-Cy5 (Molecular Probes, Eugene, OR) were used. In other experiments, GFP^+ $CCR7^-$ effector T cells isolated from the ears of rats were visualized by GFP fluorescence. Cells were analyzed on a BD FACSCalibur with CellQuest software (BD Biosciences, San Jose, CA).

Isolation of GFP^+ Cells from DTH Ears

Rats were euthanized 3 and 24 hrs after challenge with Ova and ears were removed after cardiac perfusion with saline. Ears were split, placed in PBS + 0.5% trypsin for 40 min at 37°C, and incubated for 20 min at room temperature in PBS + 0.05% Dnase I. Tissues were teased apart in tissue culture medium supplemented with 10% FCS to

produce a single-cell suspension. Lymphocytes were enriched on a Histopaque-1083 gradient (Sigma).

Proliferation Assays

Ova-T cells were used after 5 days of rest in TCFG-containing medium. They were seeded in flat-bottom 96-well plates in 200 μl of culture medium (see culture of OVA-GFP T cells) supplemented with 1% Lewis rat serum, in the presence of irradiated (30 Gy) thymocytes as antigen-presenting cells (10 x 10^4 T cells and 2 x 10^6 thymocytes per well). The indicated amounts of ShK-186 were added to the wells 30 minutes before addition of 10 μg/ml Ova. The cells were cultured for 3 days and were pulsed with [^{3}H] thymidine (1 μCi/well) 16-18 hours before cell harvesting. Incorporation of [^{3}H] thymidine was determined using a β scintillation counter.

In vitro detection of β-1 integrin and Ca^{2+} levels

Activated cells were generated by culturing Ova-GFP T_{EM} cells 48 hours with cognate antigen-pulsed irradiated thymocytes. Resting Ova-GFP T_{EM} cells were maintained in culture for 6 days after antigen exposure allowing them to regain the resting phenotype of Kv1.3^{low}. T_{EM} cells were incubated in 0 mM Ca^{2+} with 1 mM EGTA (0 Ca^{2+} Ringer), 2 mM Ca^{2+} (control) or 2 mM Ca^{2+} with 100 nM ShK-186 (ShK) for 30 min at 37 °C. The three-step antibody labeling procedure was performed using monocolonal anti-CD29 (556048, BD-Pharmingen), secondary antibody biotin rat anti-mouse IgG_{2a} (550332, BD-Pharmingen), followed by streptavidin-PE (554061, BD-Pharmingen) in ice-cold 2% BSA, 2% FBS in either 0 Ca^{2+} Ringer, 2 mM Ca^{2+} (Ringer), or 2 mM Ca^{2+} + 100 nM ShK. Control labeling was carried out with secondary antibody and streptavidin-PE. For Ca^{2+} imaging, T_{EM} cells were labeled with 2 μM fura-2 AM for (30 minutes, 37 °C) and mounted in a chamber permitting solution exchange by a syringe-driven perfusion system and placed on the stage of an Axiovert 35 microscope (Carl Zeiss MicroImaging, Inc.) equipped with a 40x plan-Neofluar objective (NA 1.30 oil). Alternating 340 ± 5 or 380 ± 5 nm light controlled by a high speed wavelength switcher (Lambda DG-4; Sutter Instrument Co.), was used to excite fura-2. A 400-nm dichroic mirror and 480-nm long-pass emission filter (Chroma) supplied light to the Photometrics CoolSNAP HQ CCD

camera (Roper Scientific). Images were acquired under control of Metafluor software (Universal Imaging Corp.). Intracelluar Ca^{2+} concentration was estimated using the formula $[Ca^{2+}]_i = K_d (R- R_{min})/(R_{max} - R)$. Calibration R_{min} and R_{max} was done as previously described (Fanger et al., 2001).

Induction and Monitoring of Chronic-Relapsing EAE

CR-EAE was induced in 7-9 weeks old female DA rats by the subcutaneous injection of an emulsion containing whole rat spinal cord and complete Freund's adjuvant, as described (Beeton et al., 2007). Clinical scoring of CR-EAE: 0= no clinical signs, 0.5 = distal limp tail, 1 = limp tail, 2 = mild paraparesis or ataxia, 3 = moderate paraparesis, 4 = complete hind limb paralysis, 5 = 4 + incontinence, 6 = death. ShK-186 (100 μg/kg) or saline was administered by subcutaneous injection once daily in the scruff of the neck, the flank or the thigh, for thirty days, starting from the onset of disease (clinical score of 0.5).

Clearance of Rat-Adapted Influenza Virus

These studies were done under GLP conditions by Burleson Research Technologies, Durham, North Carolina. Sprague Dawley rats were infected intranasally with 2×10^5 PFU rat-adapted influenza virus (Burleson, 1995). Animals received daily doses of peanut oil (2 ml/kg by oral gavage), dexamethasone (2 mg/kg/day by oral gavage), PAP-1 (50 mg/kg/day in peanut oil by oral gavage), or ShK-186 (100 μg/kg/day in saline by subcutaneous injection in the scruff of the neck or flank) for 28 days, starting 7 days before infection. Rats were euthanized at the indicated time points and viral titers were measured in lung homogenates using the Madin Darby Canine Kidney cell plaque assay as described (Burleson, 1995).

Clearance of *Chlamydia trachomatis*

Six-8 week old Lewis rats were infected intranasally with 10^7 IFU *Chlamydia trachomatis* serovar MoPn (strain Nigg II; ATCC VR 123; also called *C. muridarum*). Rats received daily subcutaneous injections, in the scruff of the neck, flank or thigh, of saline, dexamethasone (2 mg/kg/day), or ShK-186 (100 μg/kg/day) for 31 days, starting 3 days

before infection. Rats were euthanized at the indicated time points and *C. trachomatis* titers were measured in lung homogenates as described (Pal et al., 1994).

Histology on Spinal Cords of Rats with EAE

Spinal columns were removed from rats with EAE after cardiac perfusion with saline, and fixed for 24 hrs in 10% buffered formalin. Spinal cords were carefully dissected and sent to American Histolabs (Gaithersburg, MD) for paraffin-embedding, sectioning, and staining with hematoxylin-eosin. Unstained slides were deparaffinized in xylene and rehydrated for staining with Luxol Fast Blue (LFB). A 0.1% LFB solution (Newcomer Supply, Middleton, WI) was preheated in a plastic coplin jar for 40 seconds in a microwave (1400W; 30% power). Slides were immersed for 10 minutes in the preheated stain solution, washed with 95% ethanol, and rinsed twice with distilled water. Slides were differentiated in 0.05% lithium carbonate solution (Newcomer Supply), were washed with 70% ethanol and with distilled water. They were then counterstained by immersing in 0.1% cresyl violet acetate (Sigma) 4-8 times. Slides were washed once with distilled water, then dehydrated and mounted with Permount (Fisher) for visualization with a Nikon Eclipse 50i microscope. Severity of inflammation and demyelination were scored blind.

Supplemental References

Beeton, C., Garcia, A., and Chandy, K. G. (2007). Induction and clinical scoring of chronic-relapsing experimental autoimmune encephalomyelitis. J Visualized Experiments *5*, 7/04/07.

Burleson, G. R. (1995). Influenza virus host resistance model for assessment of immunotoxicity, immunostimulation, and antiviral compounds. In Methods in Immunotoxicology, G. R. Burleson, D. J.H., and A. E. Munson, eds. (New York, Wiley-Liss, Inc.), pp. 181-202.

Fanger, C. M., Rauer, H., Neben, A. L., Miller, M. J., Wulff, H., Rosa, J. C., Ganellin, C. R., Chandy, K. G., and Cahalan, M. D. (2001). Calcium-activated potassium channels sustain calcium signaling in T lymphocytes. Selective blockers and manipulated channel expression levels. J Biol Chem *276*, 12249-12256.

Pal, S., Fielder, T. J., Peterson, E. M., and de la Maza, L. M. (1994). Protection against infertility in a BALB/c mouse salpingitis model by intranasal immunization with the mouse pneumonitis biovar of *Chlamydia trachomatis*. Infect Immun *62*, 3354-3362.

Table S1

Rats	Inflammation		Demyelination	
	Score	**Location**	**Score**	**Notes**
Saline #1	3	Widespread	2	No peripheral neuronitis
Saline #2	1	Scattered meningeal aggregates, seemed more diffuse than aggregated; rare white matter focus	1	White matter patches and subpial ovoids
Saline #3	1-2	More parenchymal than other	1-2	
Saline #4	1-2	Meningeal aggregates; parenchymal focus (with demyelination)	0-1	
Saline #5	2	Multiple meningeal aggregates, larger than others, focal perivascular	1	Perivascular
ShK-186 #1	0-1	Meningeal aggregrates	0-1	Rare myelin ovoids in vicinity of inflammation)
ShK-186 #2	1	Multiple meningeal aggregates	0	Also a single focus of peripheral neuronitis with demyelination
ShK-186 #3	1	Scattered meningeal, single perivascular	0	
ShK-186 #4	1	Meningeal aggregates; some perivascular, parenchymal	1-2	Large focus

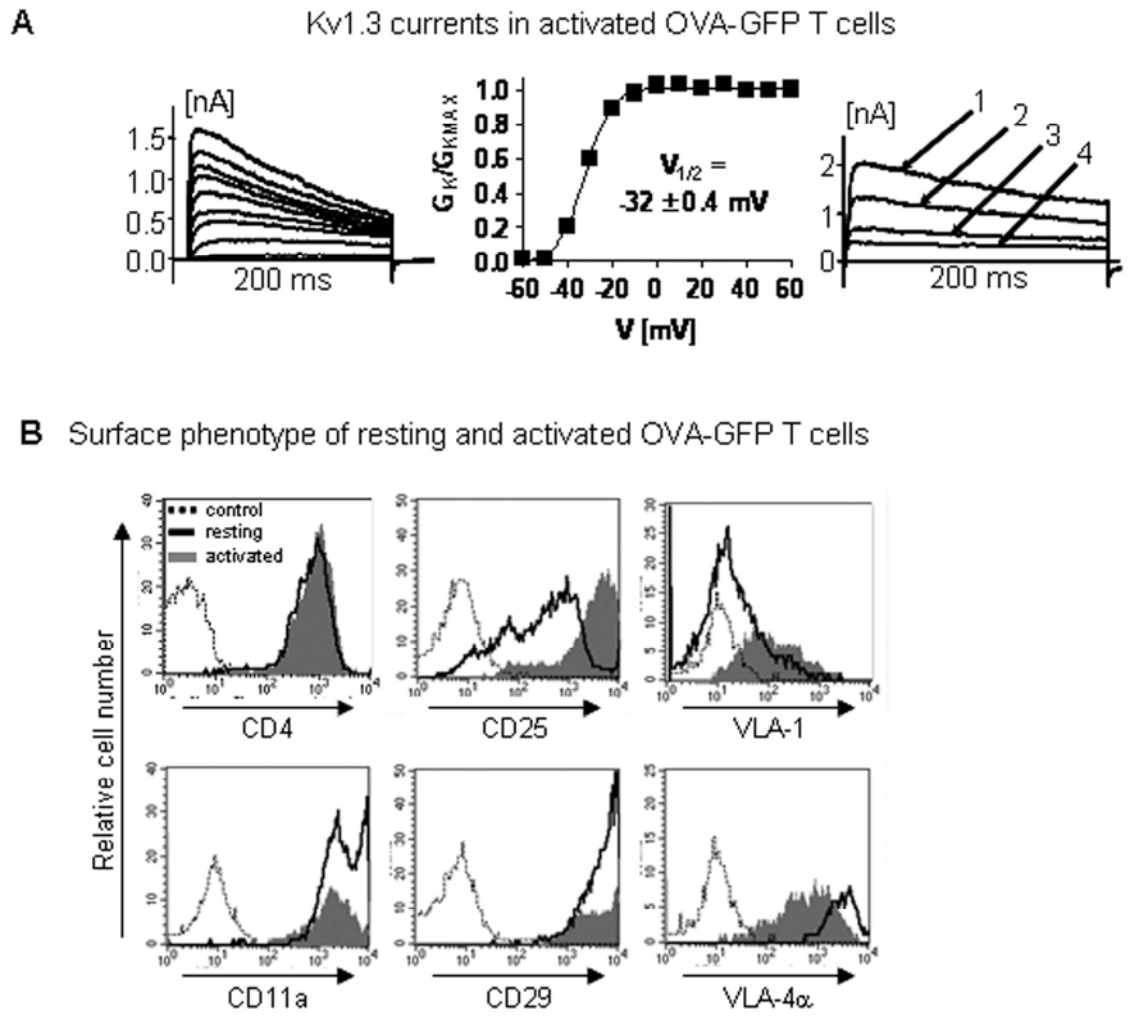

Figure S1. Phenotype of Resting and Activated Ova-GFP$^+$ T Cells

(A) Family of Kv1.3 currents. Test potential was changed from –60 to +60 mV in 10 mV increments every 30 s (left). The I/V curve showing the characteristic half-activation voltage for Kv1.3 of –32 ± 0.4 mV (middle). Kv1.3 displays use-dependent inactivation typical of this channel during successive 200-ms pulses at 1-s intervals to +40 mV (right). (B) Upon activation, CD4$^+$ CCR7$^-$ GFP$^+$ effector cells up-regulate CD25 and VLA-1, and down-regulate CD49d (a component of VLA-4) while levels of CD11a, and CD29 are unaffected by activation (solid line = resting T_{EM}; filled histogram = activated CCR7$^-$ effectors; dotted line = background staining).

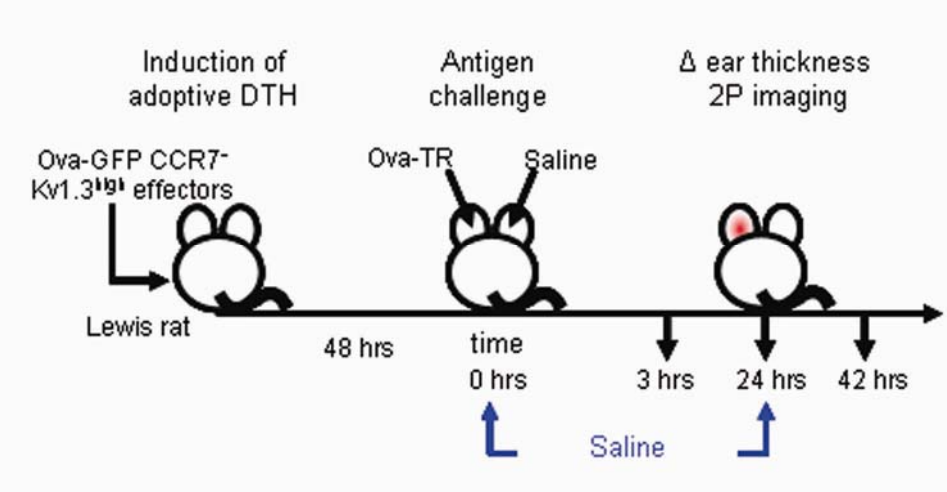

Figure S2. Time-line representing induction of adoptive DTH, antigen challenge, time of subcutaneous saline injection and post-challenge time points used for imaging.

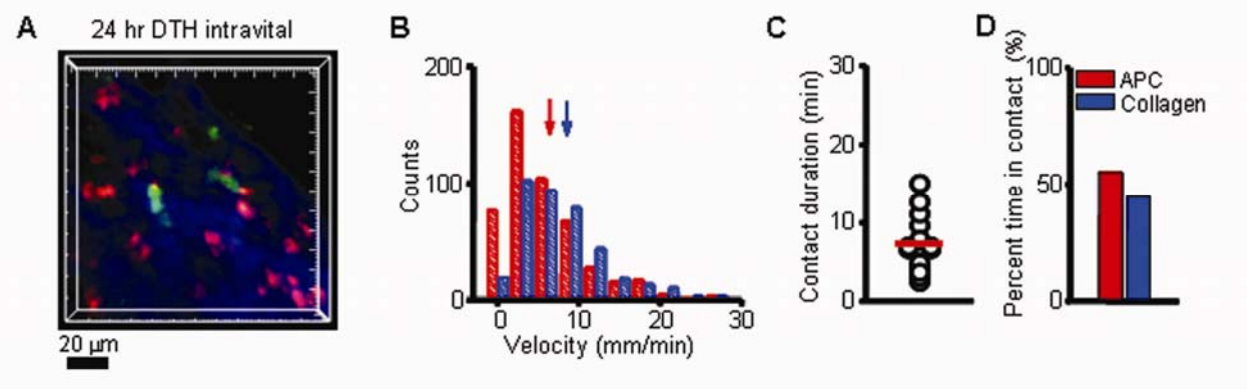

Figure S3. 24hr Intravital DTH

(A) GFP$^+$ Ova-specific T cells (green) interacting with local APCs (red) and collagen fibers (blue) in the intact ventral side of the rat paw 24 hr after antigen challenge. (B) Average velocities of CCR7$^-$ effectors when in contact with a local APC (red bars, 6.4 μm/min) and collagen (blue bars, 8.5 μm/min, n= 2 rats). Values were expressed as mean ± standard deviation (SD). (C) Duration of cell contact with APCs in the rat paw 24 hr after antigen challenge (mean contact duration = 7 min, n = 14 cells). (D) Percentage of time CCR7$^-$ effector cells are in contact with local APCs or collagen fibers in the intravital preparation at 24 hr (APC 55%, collagen 45%).

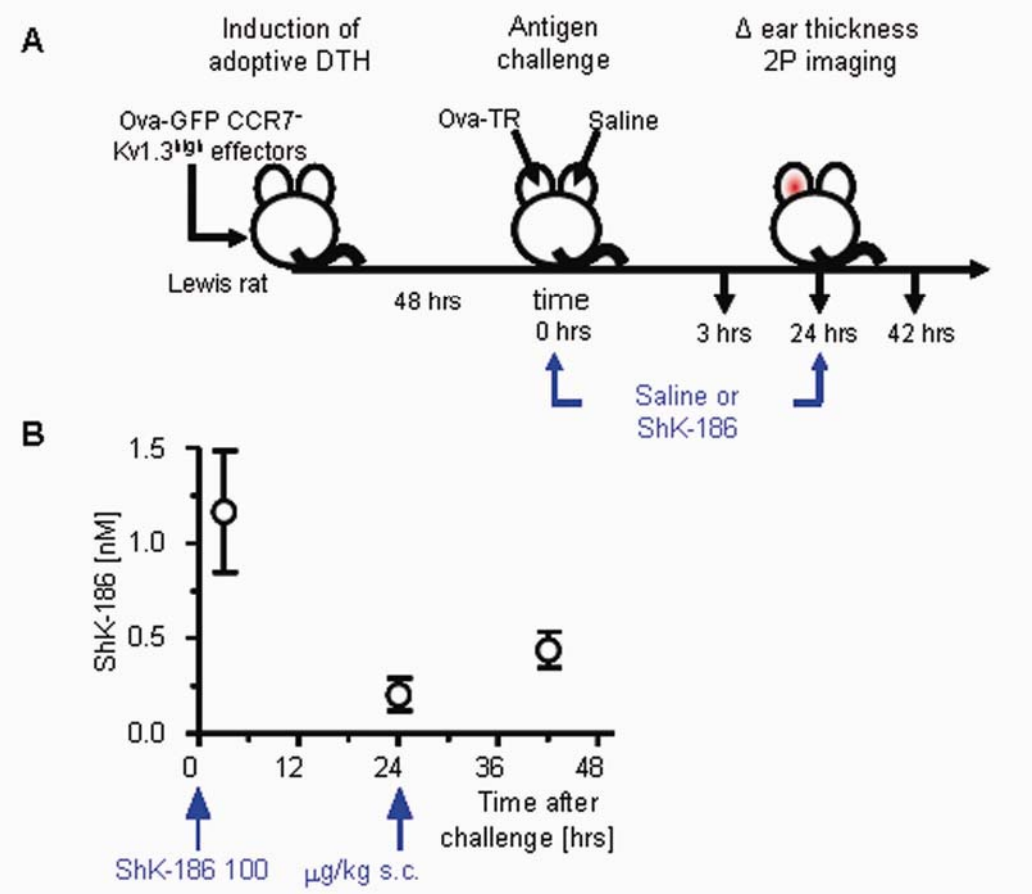

Figure S4. Experimental protocol and ShK-186 serum levels in DTH trial

(A) Time line representing induction of adoptive DTH, antigen challenge, time of subcutaneous injection of saline or ShK-186 (100 μg/kg), and time points used for imaging post-antigen challenge. (B) Serum levels of ShK-186 after subcutaneous injections of 100 μg/kg at times 0 and 24 hr. Known amounts of ShK-186 were added to Lewis rat serum and the blocking activity on Ova-effector cells Kv1.3 channels tested by patch-clamp to establish a standard dose-response curve. Serum samples from Lewis rats were taken 3 and 24 hr after the first ShK-186 injection and 18 hr after the second ShK-186 injection and tested for Kv1.3 blocking activity by patch-clamp. Levels of ShK-186 were determined from the standard curve. Serum levels were 1.17 ± 0.39 nM (n=4 rats) at 3 hr, 0.20 ± 0.08 nM (n=4) at 24 hr, and 0.44 ± 0.09 nM (n=4) at 42 hr. Values were expressed as mean ± SD.

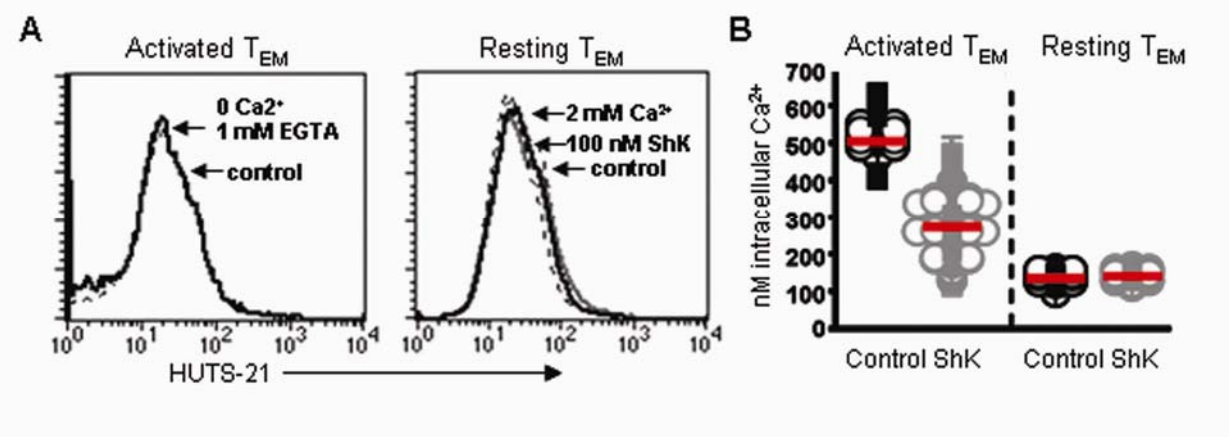

Figure S5. HUTS-21 staining and intracellular calcium levels

(A) FACS plot of HUTS-21 levels on activated cells incubated in 0 mM Ca^{2+} with 1 mM EGTA for 30 minutes at 37 °C (solid line) compared to secondary antibody control (dotted line, left). HUTS-21 staining above background was not detected. FACS plot of HUTS-21 levels on resting Ova-GFP T_{EM} cells (center) treated with 2 mM Ca^{2+} with 100 nM ShK-186 (solid line) or 0 mM Ca^{2+} with 1 mM EGTA (solid line). Resting cells had HUTS-21 staining similar to background staining (two dashed lines). (B) Intracellular Ca^{2+} in activated T_{EM} cells (509 nM ± 2.9 SE, n = 60) was significantly reduced by ShK treatment (274 ± 6.7 SE, n = 113) ($p < 0.01$). Resting T_{EM} cells had a lower overall average intracellular calcium (121 ± 2.1 SE, n = 42) which was similar to resting cells treated with ShK (141 ± 1.8 SE, n = 65).

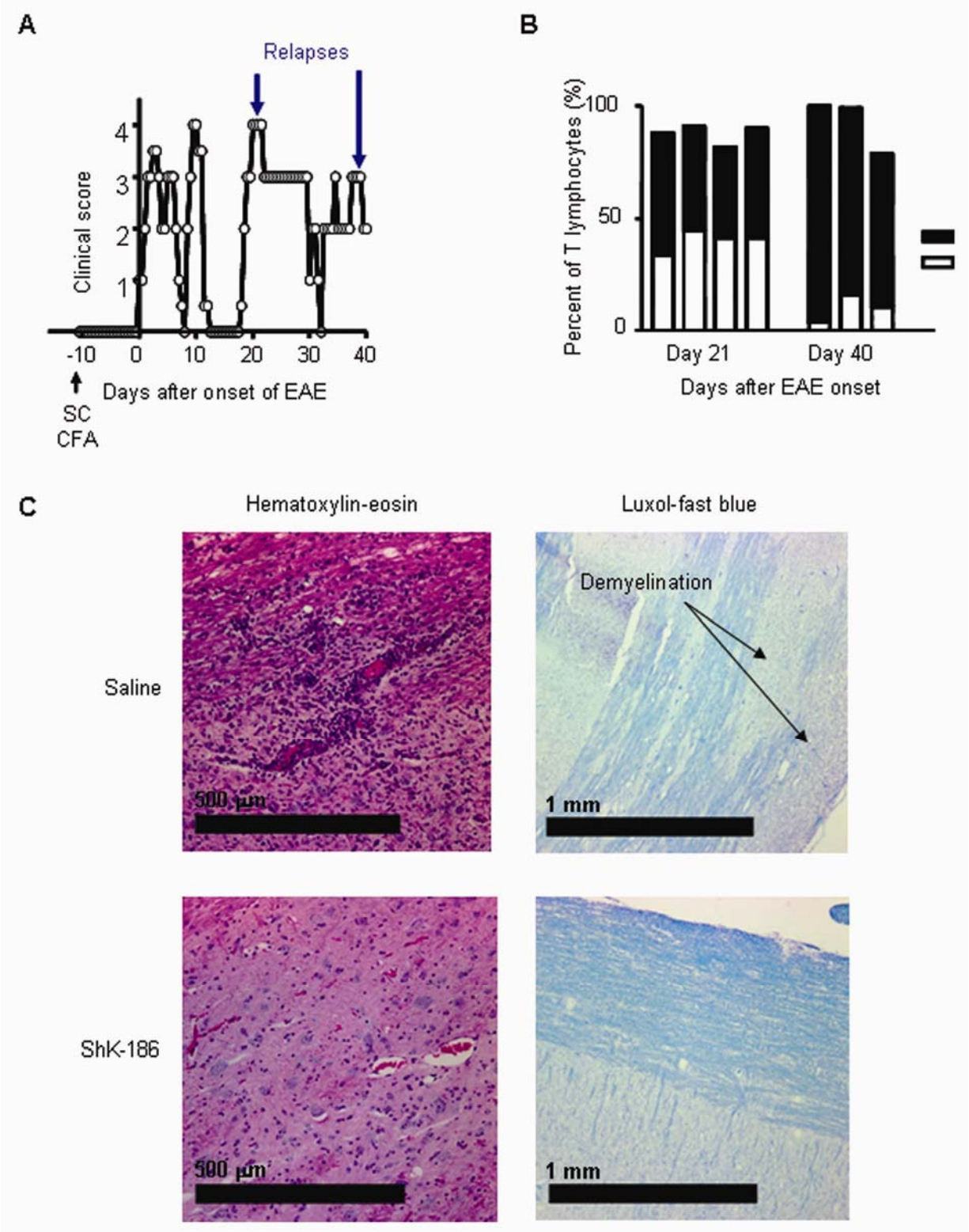

Figure S6. Characterization of CNS-infiltrating T cells in DA rats with CR-EAE.

(A) Representative clinical course of CR-EAE induced in a DA rat by the injection of spinal cord (SC) and complete Freund's adjuvant (CFA). Clinical scoring: 0, no disease;

1, limp tail; 2: mild paraparesis, ataxia; 3: moderate paraparesis; 4, complete hind limb paralysis. (B) Percentage of $CCR7^{+}$ T_{CM} (white) and $CCR7^{-}$ T_{EM} (black) lymphocytes in the CNS of CR-EAE rats 21 and 40 days after onset of clinical signs. Each bar represents one rat. (C) Representative photos of spinal cord slides from CR-EAE rats treated with saline (top) or 100 μg/kg ShK-186 (bottom), stained with hematoxylin-eosin (left) or luxol-fast blue (right).

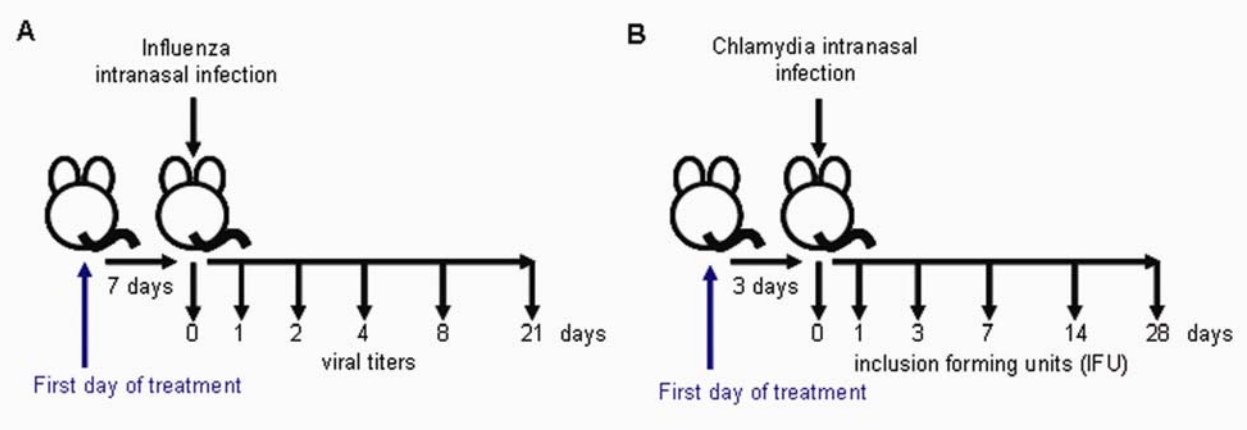

Figure S7. Experimental protocol for influenza and *Chlamydia* trials

(A) Timeline of the trial with rat-adapted influenza virus. (B) Timeline of the trial with *Chlamydia trachomatis*.

Supplemental Movie Legends

Movie S1. Two-photon imaging in subcutaneous tissue of rat ear during DTH

Time-lapse video illustrates GFP^+ $CCR7^-$ T cells (green) together with APC that had taken up Ova-TR (red), and collagen second-harmonic signal (blue). The video represents DTH at 42 hr in a 87 x 89 x 5 μm volume. A rotation about the y-axis is shown as cells are moving. Large tick marks = 20 μm. Time is represented in min:sec.

Movie S2. DTH at 3 hours

$CCR7^-$ effector cells are non-motile and in contact with APC. Large tick marks = 20 μm; time represented in min:sec.

Movie S3. DTH at 24 hours

Enlarged, highly motile effector cells (green, $CCR7^-$ effectors) moving rapidly through the collagen-rich tissue (blue) and pausing to interact with antigen-bearing APCs (red). Large tick marks = 20 μm. Time represented in min:sec.

Movie S4. DTH at 42 hours

Enlarged $CCR7^-$ effector cells are still highly motile along the collagen fiber bundles in the subcutaneous tissue, similar to the 24-hr time point. Large tick marks = 20 μm. Time represented in min:sec.

Movie S5. Intravital rat paw imaging

$CCR7^-$ effector cells are found in the rat paw 24 hrs after antigen challenge. In the intravital preparation cells are motile and crawl along collagen and make contact with local APCs in a similar manner to the in situ ear preparation. Large tick marks = 20 μm; time represented in min:sec.

Movie S6. Bystander Ova-specific T_{EM} cell motility during DTH response to HEL

GFP$^+$ Ova-specific T cells are found at the site of inflammation 24 hrs after induction of active DTH with nonrelevant antigen hen egg lysozyme (HEL). Bystander T lymphocytes do not enlarge and are highly motile. Large tick marks = 20 μm; time represented in min:sec.

Movie S7. T cells crawling on collagen at 24 hours

A region with long parallel collagen fibers, selected to demonstrate the directed motion of effector cells along the collagen matrix and interactions with antigen bearing APCs. White tracks denote the previous 6 minutes of cell movement. Large tick marks = 20 μm. Time is represented in min:sec.

Movie S8. T cell at a collagen junction at 24 hours

Circuitous path taken by a CCR7$^-$ effector T cell at a junction of collagen fiber bundles. Large tick marks = 20 μm. Time is represented in min:sec.

Movie S9. ShK-186-treated animal at 3 hours

Similar to animals injected with saline, CCR7$^-$ effector T cells imaged in subcutaneous ear tissue 3 hr after antigen challenge and ShK-186 treatment were non-motile. Large tick marks = 20 μm. Time is represented in min:sec.

Movie S10. ShK-186-treated animal at 24 hours

CCR7$^-$ effector T cells remain small and immotile, whereas, in the absence of ShK-186 treatment (see Movies S2, S4, and S5), they are enlarged and highly motile. Large tick marks = 20 μm. Time is represented in min:sec.

Movie S11. ShK-186-treated animal at 42 hours

In contrast to the highly motile cells found in animals treated with saline (see Video 3), CCR7$^-$ effector T cells remain small and immotile 42 hr after antigen challenge. Large tick marks = 20 μm. Time is represented in min:sec.

Movie S12. CCR7$^+$ T cells in the lymph node of saline-treated animal

Adoptively transferred $CD3^+$ $CCR7^+$ CFSE-labeled T cells are highly motile in the rat lymph node. Tracks represent the 6 previous minutes of T cell movement. Rotation during the video facilitates visualization of motion in x, y, and z axes. Large tick marks = 20 μm. Time is represented in min:sec.

Movie S13. $CCR7^+$ T cells in the lymph node of ShK-186 treated animal

The motility of adoptively transferred $CD3^+$ $CCR7^+$ CFSE-labeled T cells was not altered by treatment with ShK-186. Tracks represent the 6 previous minutes of T cell movement. Rotation during the video facilitates visualization of motion in x, y, and z. Large tick marks = 20 μm. Time is represented in min:sec.