Supplemental Methods.

Skin cell extractions. Dermal mononuclear cells were isolated as described[1]. Briefly, ears were incubated in dispase (Invitrogen, Carlsbad, CA) to separate the dermis and epidermis. The dermis was cut in pieces and incubated with collagenase IV (Sigma-Aldrich) for 2 hours.

OT-I effectors. To create OT-1 effectors, $1.5x10^5$ blood-derived dsRed+ RAG1$^{-/-}$ OT-1 were injected into B6 mice. Eight hours later, mice were injected with 50ug i.v. of a murinized anti-DEC205 antibody (gift of Ralph Steinman[2]) modified to express OVA with 50ug i.p. of anti-CD40 (clone FGK45). OT-I effectors were harvested 6 days later and isolated using the Easy Sep CD8 isolation kit (STEMCELL Technologies).

Immunohistology. Ears were fixed in 1% paraformaldehyde followed by dehydration in 10% to 30% sucrose and frozen in Tissue-TeK OCT compound (Sakura Finetek). Ten micron sections were incubated overnight with antibodies against CD5 (Alexa-647, clone 53-7.3, Biolegend) and FITC-labeled anti-GFP (Rockland Laboratories). This antibody also recognizes YFP but not dsRed. Sections were washed and incubated with alexa488-labeled anti-FITC (EbioscienceA). Sections were mounted onto slides with Anti-Fade reagent with or without Dapi (Invitrogen). Separate images for each fluorochrome were acquired with an automated Eclipse TI wide-field microscope (Nikon) and a Retiga CCD camera (QImaging,) with NIS Elements software (Nikon). DsRed$^+$ CD4 or CD8 cells were directly imaged without antibody labeling. Light was collected through 440/40 or 460/50, 525/50, 605/70, and 700/75 nm bandpass filters. Image processing was performed with ImageJ (NIH).

Image analysis. Cell tracking and velocities were measured with Imaris (version 5.3, Bitplane AG). T cells were tracked with the “spot tracking” function. Each cell was assigned a spot that was tracked over time; all tracked cells were manually vetted. CD11c-YFP$^+$ cells formed a dense network that prevented identification of individual cells. YFP$^+$ cells were therefore considered as a “surface” that was mapped with the surface function of Imaris.

Distance from individual T cells to the DC surface (shortest distance from centroid of the T cell “spot” to the edge of surface) was calculated over time with Imaris XT (Bitplane). We used Imaris tools to calculate the following parameters: mean speed (track length/time); total displacement (shortest distance between points at the beginning and end of imaging); sphericity (the ratio of the surface area of a sphere (with the same volume as the given object being tracked) to the surface area of the object).

Statistics that involved distance of T cells to DCs (such as “proportion of $CD4^+$ T cells in stable contact with DCs”) were calculated with Matlab (Matworks).

Definitions of motility categories. “Enter and stable”: cells that enter the imaged volume during imaging (i.e. not present when imaging began) and which have a speed of <5 μm/minute *and* are ≤2μm from the YFP surface for at least 10 continuous minutes. “Leave after stable contact”: cells that have a speed of <5 μm/minute *and* are ≤2μm from the YFP surface for at least 10 continuous minutes before leaving the imaged volume. “Enter and leave”: cells that enter then leave the imaged volume regardless of speed or distance from the YFP surface parameters. “Stable in black space, stays in volume”: cells that are present for the entire imaging time and have a speed of <5 μm/minute *and* are >2μm from the YFP surface for at least 10 continuous minutes. “Leave after stable contact with black space”: cells that have a speed of <5 μm/minute *and* are >2μm from the YFP surface for at least 10 continuous minutes before leaving the imaged volume. “Stable in contact, stay in volume”: cells that are present for the entire video, have a speed of <5 μm/minute *and* are ≤2μm from the YFP surface for at least 10 continuous minutes. “Enter and wander”: cells that enter the imaged volume during imaging and never have a speed of <5 μm /minute for at least 10 minutes. “In volume, other behaviors”: cells that do not meet the aforementioned criteria. These cells are in the volume for the entire image time and never have speeds <5 μm /minute speed for at least 10 minutes.

1. Ginhoux F, Collin MP, Bogunovic M, et al. Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. *J Exp Med*. 2007;204(13):3133-3146.
2. Hawiger D, Inaba K, Dorsett Y, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med*. 2001;194(6):769-779.

Supplemental Figure 1. GVHD in the B6 (H-2^b)→129 (H-2^b) model. Lethally irradiated 129 mice (1000cGy) were reconstituted with 6x10^6 B6 BM cells alone or in combination with 0.5x10^6 and 1x10^6 B6 CD4$^+$ and CD8$^+$ T cells, respectively. Shown are combined survival (A; P<0.0001) and weight loss (B; $P<0.05$ from day 5 and onward) data from 3 independent experiments. Approximately 28 days after transplant, ears were harvested for histopathology analysis. Representative 20x images of ear sections from BM alone (left) and BM + T cell (right) recipients are shown in (C). Pathology scores combined from two independent experiments are shown in (D). $P\leq 0.005$ for all tissues comparing BM alone to BM + T groups.

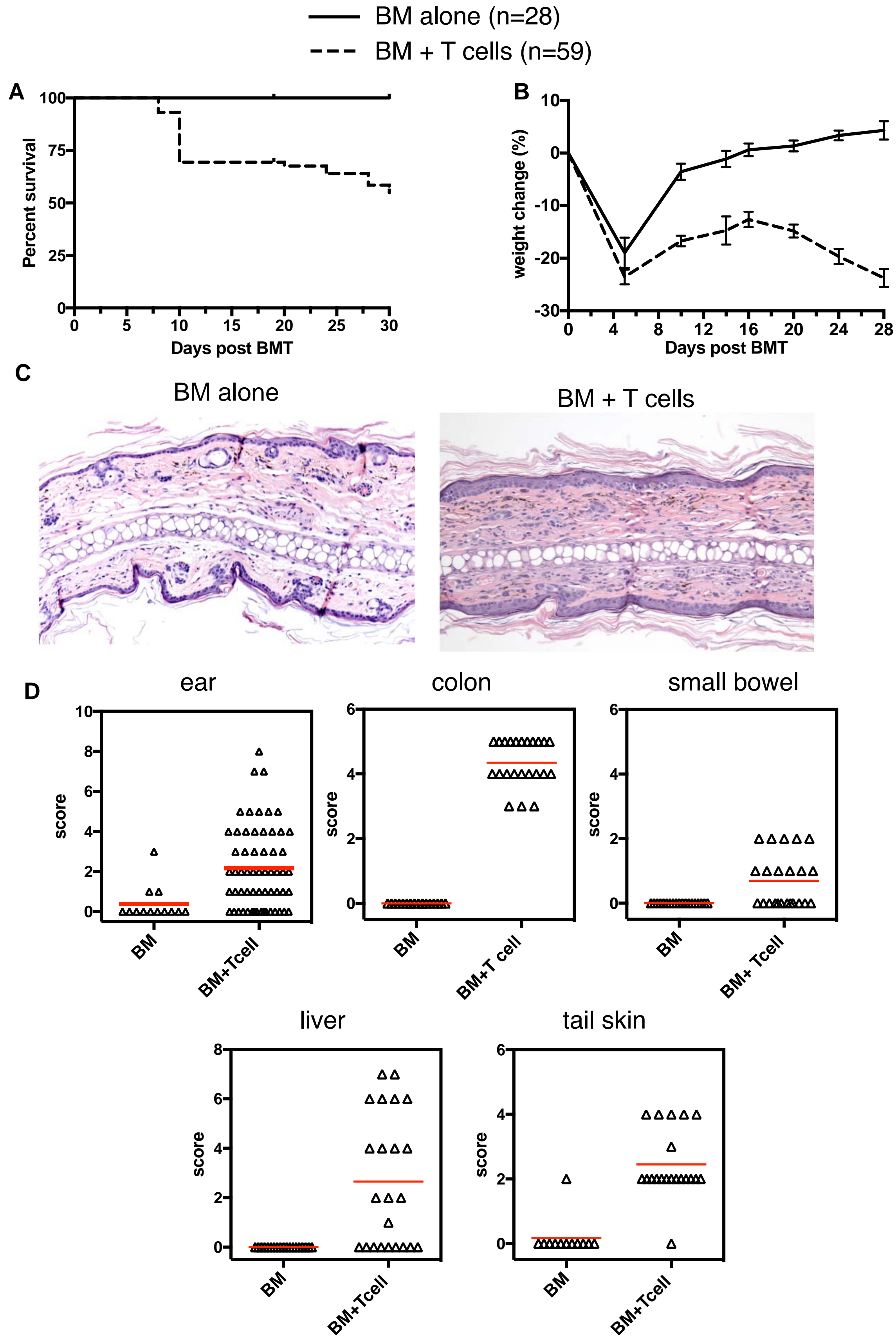
Supplemental Figure 2. Characterization of cutaneous GVHD. Shown are images of ears from mice transplanted with B6 CD11c-YFP BM with: RFP CD8$^+$ and wt CD4$^+$ T cells (A); RFP CD4$^+$ and wt CD8$^+$ T cells (B); and wt CD4 and CD8 T cells (C). Sections were stained with antibodies against YFP (A and B) and YFP and CD5 (C). YFP is rendered in green and CD5 and RFP in red, and DAPI in blue. Images are representative from 3 mice per group. (D and E) Flow cytometry of cells from digested ears harvested from B6→129 GVHD mice. (D) Cells were analyzed for the presence of CD4$^+$CD25$^+$FoxP3$^+$ cells. All panels are gated on live/CD4$^+$ cells. The first column shows control staining from spleen and pooled dermis and epidermis cells ("skin"). The right 3 panels show staining of cells isolated from spleen, epidermis and dermis. Note the clear FoxP3$^+$CD25$^+$ population in spleen cells, not present in either epidermis or dermis. (E) The majority of YFP$^+$ cells are MHCII$^+$ and therefore not NK cells.

Supplemental Figure 3. Second harmonic (SH) images from ears from GVHD mice. Shown are still images captured from videos rendering the second harmonic (blue) alone (left panels) and with YFP (CD11c) and CD8 cells (A; Video 21) or CD4 cells (B; video 22). Note gaps in the collagen identifying locations of hair follicles.

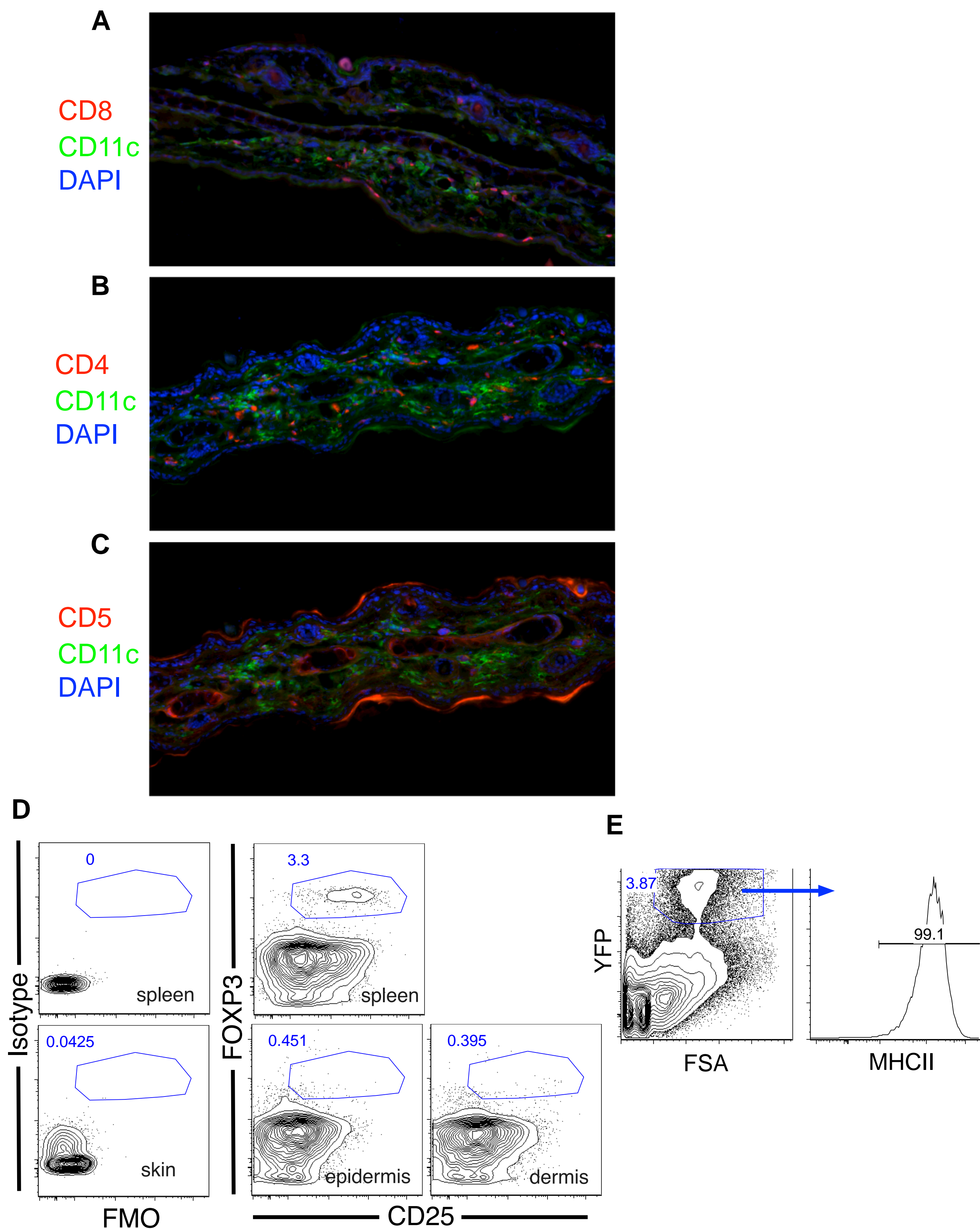
Supplemental Figure 4. Skin donor-derived hematopoietic cells are intertwined in mixed BM chimeras. Irradiated 129 mice were reconstituted with a 1:1 mix of CD11c-YFP BM and B6 dsRed BM cells with unlabeled B6 CD8 cells and GFP^+ CD4 cells. Mice were imaged by 2PIM at approximately day +28. Shown is a captured image showing CD11c-YFP cells intertwined with dsRed cells and infiltrating GFP^+ CD4 cells.

Supplemental Figure 5. Imaging data from all individual mice in the CD11c-DTR experiments. Speeds and displacements of $CD8^+$ cells from individual mice that were imaged pre and post DT (A) or mice that were imaged after PBS or DT injection (B) are shown. *=$P<0.05$.

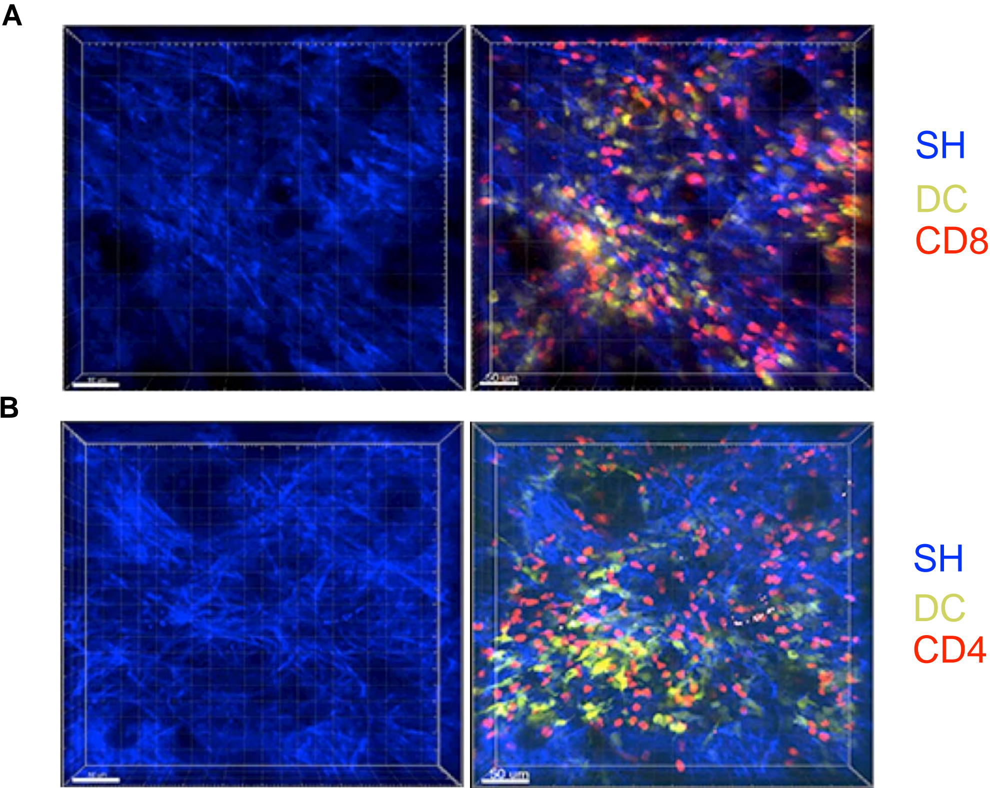
Supplemental Figure 1. GVHD in the B6 into 129 model.



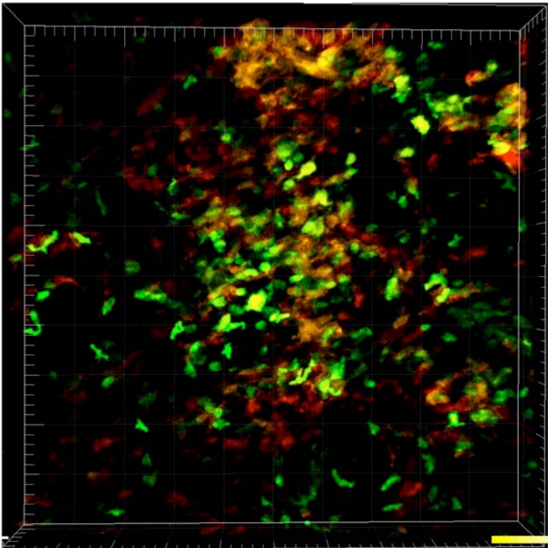
Supplementary Figure 2. Characterization of cutaneous GVHD.



Supplemental Figure 3. Second harmonic rendering



Supplemental Figure 4. Skin hematopoietic cells in mixed BM chimeras.



DsRed BM
CD11c-YFP
CD4

List of supplemental videos.

1. CD4 dynamics
2. CD8 dynamics
3. $CD8^+$ T cells in mice with CD11c-YFP $MHCI^+$ cells
4. Rendering of movie 3
5. $CD8^+$ T cells with CD11c-YFP $MHCI^-$ DCs ($\beta 2M^{-/-}$)
6. Rendering of movie 5
7. CD8 cell movements in mice transplanted with CD11c-YFPxmuLangerin-DTR BM with PBS treatment
8. CD8 cell movements in mice transplanted with CD11c-YFPxmuLangerin-DTR BM with diphtheria toxin (DT) treatment
9. CD4 movements before Y3P injection
10. CD4 movements after Y3P injection
11. CD4 movements before isotype injection
12. CD4 movements after isotype injection
13. CD8 movements in mice transplanted with CD11c-DTR prior to DT treatment
14. CD8 movements in mice transplanted with CD11c-DTR after DT treatment
15. OT-1 mouse 1 with DCs visualized
16. OT-1 mouse 2 with DCs visualized
17. OT-1 mouse 3 with DCs visualized
18. OT-1 mouse 1 without DCs visualized
19. OT-1 mouse 2 without DCs visualized
20. OT-1 mouse 3 without DCs visualized
21. Second harmonic in CD8 GVHD experiment showing: 3D rendering with only the second harmonic, 3D rendering with second harmonic and dsRed and YFP; movie with second harmonic, dsRed and YFP.
22. Second harmonic in CD4 GVHD experiment showing: 3D rendering with only the second harmonic, 3D rendering with second harmonic and dsRed and YFP; movie with second harmonic, dsRed and YFP.