Supplementary material:

Video legends

Video 2Ai. Migration of resting naïve and memory T cells

OT1 memory (green) and naïve (red) T cells migrate randomly and without prolonged stopping in resting lymph nodes. Projection of a time lapse series imaged in intact lymph nodes is shown. Dimensions: 128 um x128 um x 22 um x 27 min.

Video 2Aii. Migration of memory T cells 5 hours after challenge with *T. gondii* expressing or not expressing OVA

Memory T cell migration 5 hours after infection with T. gondii + OVA. Most OT1 memory T cells (green) migrate rapidly while a proportion engage in prolonged interactions and clustering around infected cells (yellow circle). Projection of a time lapse series taken in the draining lymph nodes 5 hours after ear-flap infection with *T. gondii*+OVA (red). Dimensions: 126 um x 125 um x 28 um x 31 min.

Memory T cell migration 5 hours after infection with T. gondii (no OVA). OT1 memory T cells (green) migrate rapidly without making stable contacts with infected cells. Projection of a time lapse series taken in the draining lymph nodes 5 hours after ear-flap infection with *T. gondii* (no OVA, red). Dimensions: 125 um x125 um x 82 um x 30 min.

Video 2Aiii. Migration of memory T cells one day after challenge with *T. gondii+OVA*

Memory T cell migration 20 hours after infection with T. gondii + OVA. OT1 memory T cells (green) slow down and engage in prolonged interactions (yellow circles) with *T. gondii*+OVA invaded cells (red). Projection of a time lapse series taken in the draining

lymph nodes 20 hours after ear-flap infection with *T. gondii*+OVA (red). Dimensions: 191 um x 191 um x 82 um x 31 min.

Memory T cells dividing in the draining lymph nodes 26 hours after infection. Yellow circles indicate dividing OT1 memory T cells (green) after infection with *T. gondii*+OVA (red). Dimensions: 82 um x 82 um x 32 um x 26 min.

Video 2B-C. Examples of T cell clusters around infected cells 5 hours after infection with *T. gondii*+OVA

An example of a stable cluster that does not break up. OT1 memory T cells (green) clustering around a non-labeled cell harboring *T. gondii* (red). T cells move in a confined space around the infected cell throughout the movie. Projection of a 3-D time-lapse image sequence. Dimensions: 44 um x 44 um x 24 um x 31 min.

A cluster that breaks up during imaging. OT1 memory T cells (green) clustering around a non-labeled cell harboring *T. gondii* (red). In this example T cells disperse as parasites become motile, presumably as the target cell lyses. Projection of a 3-D time-lapse image sequence. Dimensions: 69 um x 69 um x 8 um x 23 min.

Naïve T cells also participate in clusters. OT1 memory (green) and naïve (magenta) T cells clustering around a non-labeled cell harboring *T. gondii*+OVA (red). Both naïve and memory T cells stay in a confined area in close proximity to the parasites. Projection of a 3-D time-lapse image sequence. Dimensions: 54 um x 54 um x 82 um x 36 min.

Video 2D. T cell clusters around OVA only and OVA and non-OVA infected cells

An example of OVA-specific OT1 T cells (green) clustering around OVA(red) rather than non-OVA infected cells (blue). T cells move in a confined space around a cell infected with two OVA-expressing parasites. Projection of a 3-D time-lapse image

sequence. Dimensions: 65 um x 65 um x 34 um x 24 min. Second video is an example of OVA-specific T cells (green) clustering around an infected cell containing OVA(red) and non-OVA (blue) parasites. T cells remain in a confined space in the proximity to the parasites throughout the movie. Dimensions: 36 um x 36 um x 14 um x 23 min. Note that spectral overlap caused between YFP (blue) and RFP (red) caused some YFP parasites (blue) to appear to have red centers.

Video 3A. T cells interacting with CD11c YFP high and low cells

OT1 memory T cells (blue) form long-lasting interactions with infected and uninfected, CD11cYFP (green) high DCs and also with YFP low cells after infection with *T. gondii*+OVA (red). White track follows an OT1 T cell engaged in prolonged interaction with an infected DC. Uninfected DC engaged in lasting interactions with T cells is circled in yellow. An infected YFP low cell interacting with OT1 T cells is highlighted by a yellow circle. Projections of 3-D time-lapse image sequences are shown. Dimensions: OT1 memory T cells interacting with an infected CD11c YFP DC: 54 um x 54 um x 48 um x 30 min; OT1 memory T cells interacting with an uninfected CD11c YFP DC: 59 um x 59 um x 40 um x 34 min, OT1 memory T cells interacting with a CD11c YFP low cell: 31 um x 31 um x 24 um x 34 min.

Video 3Bi. OT1 T cells form clusters within the layer of CD169 SCS macrophages

Two-photon time-lapse images of SCS region of a lymph node showing OT1 memory T cells (yellow) forming long-lasting interactions with CD169 cells (orange), 5 hours after infection with *T. gondii*+OVA (red), naïve OT1 T cells and second harmonic signal are in light blue. Left side shows the entire imaging volume at a single time point projected along the x-axis. Three right panels show time-lapse images of 20 um slices

corresponding to the positions indicated by brackets and projected in the z-axis. Circles indicate the positions of T cell clusters. Dimensions of the original imaging volume: 143 um x 143 um x 80 um x 36 min.

Video 3Bii. Examples of OT1 T cells forming clusters around infected CD169 cells

Two-photon time-lapse images of OT1 memory T cells (green) in long-lasting interactions with CD169 (yellow), 5 hours after infection with *T. gondii*+OVA (red), second harmonic signal is in blue. T cells (green) can be seen contacting an infected CD169 cell (yellow). Projection of a 3-D time-lapse image sequence. Dimensions: 72 um x 72 um x single optical section x 39 min.

Second time-lapse sequence shows a cluster consisting of a naïve T cell (blue) and a memory T cell (green) around an infected CD169 cell (yellow) 5 hours after infection with *T. gondii*+OVA (red). Approximately 15 minutes into the imaging run CD169 cell appears to lyse which corresponds to parasites egress and T cell dispersal. Dimensions: 64 um x 68 um x 18 um x 21 min.

Video 4A. Three examples of T cell invasion during contact with infected APC

Time lapse images of two-photon data sets showing memory OT1 GFP T cells (green) being invaded by parasites (red) while contacting an infected target cell. Parasites can be seen to relocate from an unlabeled infected cell into the green T cell. In examples 1 and 3 T cells quickly migrate away after invasion, parasites are circled in yellow. Examples 1 and 2 include both a 2-D projection of 3-D volume (left) and a single optical section (right). Third example shows 2 OT1 T cells getting invaded as the infected cell lyses. The images were generated in draining lymph nodes of immunized mice 5 hours after challenge with *T. gondii* + OVA. Dimensions of volume projections: Example 1: 43 um x

43 um x 42 um x 8 min, example 2: 32 um x 32 um x 24 um x 23 min, example 3: 52 um x 52 um x 26 um x 9 min.

Video 4D-F. T cell invasion occurs in different organs and infection settings

T cell invasion in the mesenteric lymph node after oral infection. OT1 T cells (green) clustering around an infected cell getting invaded by *T. gondii*+OVA (red) in the mesenteric lymph nodes 5 days after oral infection. Parasite (circled in yellow) moves from an unlabelled infected cell into the green T cell which then migrated away. Projection of a 3-D time-lapse image sequence. Dimensions: 54 um x 54 um x 32 um x 8 min.

A motile infected OT1 T cell in the brain. Projection of a time lapse series obtained by TPSLM from a brain slice of a mouse 13 days post-infection with *T. gondii*+OVA (red). The OT1 T cell (green) containing a parasite is circled in yellow. Dimensions: 85 um x 84 um x 48 um x 19 min.

An OT1 T cell being invaded in the brain and migrating away. Projection of a time lapse series obtained by TPSLM from a brain slice of a mouse 39 days post-infection with *T. gondii*+OVA (red). An OT1 T cell (green) slows down in an area containing parasites and moves away containing a parasite. This T cell is circled in yellow. Dimensions: 79 um x 79 um x 60 um x 20 min.

Video S3. Motile and stationary T cell conjugates

Examples of OVA-specific OT1 T cells (green) forming tight conjugates with both stationary and motile infected cells, indicated by *T. gondii*+OVA (red). T cells can be seen moving coordinately with the motile parasites (yellow circle) or staying in close proximity to stationary parasites (white circle). Projection of a 3-D time-lapse image

sequence. Images on the right show paths for T cells (green and blue) and *T. gondii*+OVA (red). Example 1 dimensions: 86 um x 83 um x 62 um x 31 min, example 2 dimensions: 63 um x 63 um x 38 um x 31 min.

Supplementary figure legends.

Figure S1. **OT1 T cells mount antigen-specific primary and recall responses to *T. gondii* expressing OVA**

Mice containing naive OT1 cells were immunized by IP injection of 10^6 irradiated *T. gondii* expressing OVA. A) Primary response. Splenocytes were analyzed by flow cytometry 3 days later after IP injection with irradiated parasites. The % of gated OT1 T cells expressing the activation marker CD69 (top panels) or expressing the cytokine IFN-gamma (bottom panels) are shown. Data from mice infected with parasites that did not express OVA, and non infected mice are shown for comparison. IFN-gamma expression was analysed after 4 hour of in vitro restimulation with SIINFEKL peptide in the presence of a transport inhibitor.

B) Recall response. Mice were immunized then challenged 5 weeks later by subcutaneous injection with 10^6 live, non-irradiated parasites. One day after challenge draining lymph nodes were analyzed by flow cytometry. The % of gated OT1 T cells expressing CD69 (top graph) and IFN-gamma (bottom graph) are shown. Data from mice challenged with parasites that did not express OVA are shown for comparison. IFN-gamma expression was analysed after 4 hour of in vitro restimulation with SIINFEKL peptide in the presence of a transport inhibitor.

Figure S2. **Parasites reside in parasitophorous vacuoles within SCS macrophages 5 hours after infection**. A) Frozen sections of lymph nodes 5 hours after ear-flap infection with RFP-*T. gondii* (red). CD169 (blue) staining was used to visualize subcapsular sinus macrophages, and the parasite dense granule protein GRA6 was used to visualize the

parasitophorous vacuole. Top panels shows an example of an infected macrophage with GRA6 staining (white arrow) adjacent to one without GRA6 staining (yellow arrow). Bottom panels show a cluster of OT1 T cells surrounding a GRA6-expressing infected macrophage. Z-stack projections are shown. Plot on the right shows the numbers of CD169+ cells containing parasites that did or did not stain with GRA6. GRA6+ cells with an OT1 T cell in contact are indicated by hatched segment. No T cells were observed contacting GRA6- infected macrophages. B) Frozen sections of lymph nodes of CD11cYFP (green) reporter mice 5 hours after ear-flap infection with RFP-*T. gondii* (red). CD169 (blue) staining was used to visualize subcapsular sinus macrophages. Higher magnification images of infected CD169+ CD11cYFP intermediate macrophages are shown on the right. Boxed areas have been enlarged. Single optical sections are shown.

Figure S3. **Additional quantitation of naïve and memory T cell localization relative to lymph node capsule in resting and challenged lymph nodes**. A) A representative infected and uninfected lymph node samples from Fig. 1C were divided into quadrants as indicated in the image on the right hand side. The quantitation of distance to capsule for individual T cells in each quadrant was performed as in Fig. 1C and the data was plotted. B)The graph in the bottom panel shows the percentages of naïve and memory T cells close to the SCS (falling inside the blue box on the graph in Fig. S3A) relative to the total cell number for each population for an infected and uninfected lymph nodes.

Figure S4. **Confinement ratio and arrest coefficient of T cells before and after challenge with *T. gondii***

Draining lymph nodes from immunized mice containing GFP-labeled OT1 memory T cells, and transferred with dye-labeled OT1 naive T cells were imaged using two-photon microscopy either in resting state or at indicated times after ear-flap challenge with *T. gondii* expressing OVA. A) Confinement ratio (maximum displacement/path length) of naïve (red) and memory (green) OT1 T cells in resting and challenged lymph nodes. B) Arrest coefficient (% of time during which a cell moved less than 2 um) of naïve and memory OT1 T cells in resting and challenged lymph nodes. Each dot corresponds to a tracked T cell, horizontal bars indicate the mean value for each condition.

Figure S5. **Examples of stable T cell conjugates with stationary (i) and motile (ii) infected cells**. 2-D projections of 3-D two-photon imaging volumes are shown at time frames indicated. Corresponds to video 2c. Graphs show the changes in x,y,z coordinates for the T cell in conjugate (green) and *T. gondii* (red). Red and green arrows indicate time points corresponding to sharp turns for the *T. gondii* and the T cell respectively.

Figure S6. **Comparison of antigen specific (OT1) and non-specific (P14) CD8 T cell expansion and infection rate following oral infection**. Naive mice were injected i.v. with mixtures of TCR transgenic T cells (2.4 x 10^6 OT1 T cells and 1.2 x 10^7 P14 T cells) one day prior to oral infection with Pru RFP OVA parasites (75 cysts) and mesenteric lymph nodes (MLN) and spleen were analyzed 7 days after infection by flow cytometry. A) Number of OT1 (red dots) or P14 (black dots) T cells as a % of total CD8

T cells. B) Number of infected OT1 as a % of total OT1 T cells (red dots), and number of infected P14 T cells as a percentage of the total P14 T cells (black dots). C) Number of infected OT1 (red dots) T cells or number of infected P14 (black dots) T cells as a % of total infected cells.