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Dynamic Imaging of T Cell-Parasite Interactions in the Brains of Mice Chronically Infected with Toxoplasma gondii

Marie Schaeffer,* Seong-Ji Han,* Tatyana Chtanova,* Giel G. van Dooren,† Paul Herzmark,* Ying Chen,‡ Badrinath Roysam,‡ Boris Striepen,‡† and Ellen A. Robey‡*‡

The intracellular parasite Toxoplasma gondii can establish persistent infection in the brain of a mammalian host, a standoff that involves the active participation of host CD8 T cells to control infection. CD8 T cells generally protect against intracellular pathogens by local delivery of effector molecules upon recognition of specific pathogen Ags on invaded host cells. However, the interactions between CD8 T cells, T. gondii, and APCs in the brain have not yet been examined. In this study we have used a mouse infection model in conjunction with two-photon microscopy of living brain tissue and confocal microscopy of fixed brain sections to examine the interactions between CD8 T cells, parasites, and APCs from chronically infected mice. We found that Ag-specific CD8 T cells were recruited to the brains of infected mice and persisted there in the presence of ongoing Ag recognition. Cerebral CD8 T cells made transient contacts with granuloma-like structures containing parasites and with individual CD11b+ APCs, including some that did not contain parasites. In contrast, T cells ignored intact Ag-bearing cysts and did not contact astrocytes or neurons, including neurons containing parasites or cysts. Our data represent the first direct observation of the dynamics of T cell-parasite interactions within living tissue and provide a new perspective for understanding immune responses to persistent pathogens in the brain. The Journal of Immunology, 2009, 182: 6379–6393.

Immune responses to pathogens typically take place within highly organized, tightly packed tissue environments, a fact that has limited our ability to directly observe interactions between host immune cells and pathogens. Recently, the use of two-photon microscopy, together with the development of genetically encoded fluorescent reporters, has made it possible to examine the behavior of cells within living tissues (1). This approach has been particularly informative in the study of the immune system and has been extensively used to examine immune responses to model Ags within lymph nodes (2–5). Some recent imaging studies have examined innate immune responses during infection (6–9), but very little is known about the behavior of pathogens in the face of an ongoing adaptive immune response and the cellular dynamics of adaptive immune cells, such as T cells, during their interactions with pathogens within infected tissues.

Toxoplasma gondii infection in mice provides an excellent experimental model system to investigate the cellular dynamics of host-pathogen interactions in vivo. T. gondii is a protozoan parasite that infects a wide variety of warm-blooded species, including mice and humans. During the initial acute phase of the infection, rapidly dividing tachyzoites hijack host cellular machinery to spread throughout the body but are eventually brought under control by a strong adaptive immune response (10–13). Some parasites convert into slow-growing bradyzoites, which form cysts in brain and muscle that persist for the lifetime of the host and apparently effectively evade destruction by the immune system (14, 15). T. gondii infections in humans are generally asymptomatic, although reactivation of intracerebral cysts can lead to toxoplasmic encephalitis in immunocompromised hosts (16).

Immune responses to pathogens are finely balanced to allow for control of infection while limiting damage to host tissues. Nonetheless, this balance is more crucial than in the brain, a tissue that is essential, fragile, and has a limited capacity to regenerate (17). Control of T. gondii in the brain during chronic infection requires an ongoing adaptive immune response, with CD8 T cells playing a key role (18–22). In many settings effector CD8 T cells can form lasting contacts leading to the killing of target cells, although during T. gondii infection the production of proinflammatory cytokines such as IFN-γ appears to be more important for CD8-mediated protection (22–24). Given that cytokines could act in a paracrine fashion to stimulate other cell types, such as macrophages and astrocytes, to control T. gondii growth (11–13, 25, 26) it is conceivable that CD8 T cells could provide protection without the need to stably contact invaded host cells. Studies of leukocytes isolated from brains of chronically infected mice have provided important information about CD8 T cells (24, 27–29); however the nature of interactions between CD8 T cells and brain APCs has not yet been explored.

A related question concerns how CD8 T cells detect the presence of T. gondii in the brain. CD8 T cells typically recognize peptides derived from intracellular pathogens presented by MHC class I molecules on infected host cells. Parasites convert into bradyzoites within living host cells (30, 31), and cysts within the...
brains of chronically infected mice reside within intact cells (32). However, whether CD8 T cells can recognize and respond directly to parasite Ags on the surface of cyt-containing cells is not known.

To address these issues, we have used a mouse infection model and two-photon microscopy to examine the interactions between fluorescently labeled parasites, Ag-specific T cells, and potential APCs within the living brain tissue of chronically infected mice. We found that Ag-specific CD8 T cells were recruited to the brains of infected mice and persisted there in the presence of ongoing Ag recognition. Cerebral CD8 T cells underwent Ag-dependent transient arrest near parasites, but seldom formed stable contacts with APCs and did not form large T cell clusters. Isolated parasites were primarily found within CD11b⁺ APCs, and T cell contacts occurred both with invaded host cells and with host cells that were near parasites but did not themselves harbor parasites. We also observed T cells making transient contacts with granuloma-like structures that encased parasitized cells. In contrast, T cells ignored intact Ag-bearing cysts and did not contact astrocytes or neurons, including neurons containing parasites or cysts. Our data represent the first direct observation of the dynamics of T cell-parasite interactions within living tissue and provide a new perspective for understanding immune responses to persistent pathogens in the brain.

Materials and Methods

Parasites and cell culture

In all experiments, *T. gondii* of the Prugniaud strain were used. To generate Prugniaud parasites expressing tandem dimeric tomato red fluorescent protein (33), we transfected parasites with the pCRTCt2 vector (7) using standard protocols (34). To enrich for parasites stably expressing td-Tomato, we subjected transfected parasites to several rounds of cell sorting using a MoFlo cytometer (DakoCytomation) with an Enterprise 631 laser tuned to 488 nm for excitation and an emission filter with a band pass of 570/40 nm. During the final round of cell sorting we deposited parasitized into 96-well plates to obtain clonal lines. To generate a Prugniaud/tdTomato expressing secreting OVA, we digested the pthubP30OVA/sagCAT vector (35) with *HindIII* and *PacI* to remove the CAT cassette. We digested pBSK+ *SAG1/Bte/SAG1* (a gift from D. Sibley, Washington University, St. Louis, MO) with *HindIII* and *PacI* and ligated the excised Bte cassette into the p300OVA-expressing vector. This generated the vector pB7/P30OVA, which expresses secreted P30-OVA from the α-tubulin promoter and contains a Ble gene for plasmocin selection in *T. gondii*. We transfected this construct into Prugniaud/tdTomato parasites and subjected them to two rounds of plasmocin selection as described previously (36) before cloning into 96-well plates. Clonal lines stably expressing secreted OVA were identified by immunofluorescence assays. Tachyzoites were cultured on monolayers of human foreskin fibroblasts and purified immediately before mouse infection as described (7).

Mice and in vivo infections

All mice were bred and housed in pathogen-free conditions at the American Association of Laboratory Care-approved animal facility at the Life Science Addition, University of California, Berkeley, CA. All animal experiments were approved by the Animal Care and Use Committee of the University of California Berkeley. C57BL/6, C57BL/6-CD4⁻⁻, and C57Bl10 ↔ Rag2⁻⁻, and BALB/c mice were purchased from The Jackson Laboratory. Ubiquitin-GFP mice (39) were a gift from B. Schaefer (Uniformed Services University, Bethesda, MD). F5 transgenic mice (40) were a gift from D. Kioussis (National Institute for Medical Research, London, U.K.). OT-1 Rag2⁻⁻ (41) transgenic mice were from Taconic Farms. CD11c-YFP transgenic reporter mice (42) and actin promoter cyan fluorescent protein (CFP)⁺ transgenic mice (43) were provided by M. Nussenzweig's laboratory (The Rockefeller University, New York, NY). Mice were bred to generate OT-1 Rag2-GFP, F5 Rag2-GFP, and OT-1 Rag2-CFP mice. For all experiments, mice were infected with freshly purified tachyzoites i.p. (400 tachyzoites for infections in C57BL/6 and 4000 for infections in BALB/c mice). Mice were sacrificed between 9 and 80 days postinfection.

*Ex vivo* analysis of tissue samples

To determine the number of intracellular cysts, brains were homogenized and resuspended in 10 ml of PBS (Invitrogen). Five hundred microliters of brain suspension was analyzed microscopically for the presence of cysts. RFP fluorescence from the cysts was used for counting on a Nikon Eclipse TS100 upright microscope with a ×20 air objective (Nikon ×20/0.12; working distance = 3.1 inches) using a mercury lamp (X-cite 120) and a Texas Red filter.

Intracellular IFN-γ staining, cell suspensions were incubated for 5 h at 37°C in the presence of brefeldin A (1 µg/ml), and C57BL6 spleenocytes were pulsed with the OVA peptide (SIINFEKL) for 1 h at 37°C at 2 × 10⁶ cells/ml in complete RPMI 1640 medium. After incubation, the cells were washed in FACS buffer and permeabilized using a Cytofix/Cytoperm kit (BD Pharmingen) and stained before analysis by flow cytometry. The following Abs (from eBioscience) were used for flow cytometric analysis: FITC-conjugated anti-CD45 (clone 30-F11); anti-B220 (clone RA3-6B2); anti-CD4 (L3T4, clone RM4-5); anti-IFN-γ; anti-CD45.2 (clone 104); PE-Cy5-conjugated anti-CD8α (Ly-2, clone 53-6.7); PE-Cy5-conjugated CD45.1 (A20); PE-Cy5-conjugated anti-CD11b (clone M1/70); and PE-Cy5-conjugated anti-CD11c (clone N418). PE-Texas Red anti-CD8α (clone 5H10) was from Caltag Laboratories. Acquisitions were performed with a Coulter Epix XL-MCL flow cytometer (Beckman-Coulter), and data were analyzed with FlowJo software (Tree Star).

Mononuclear cells from the brain were the damaged using a protocol adapted from Reichmann et al. (44). Briefly, mice were digested 1 h at 37°C with 1 mg/ml collagenase type IA (Sigma-Aldrich) and 0.1 mg/ml DNase I (Roche) in complete RPMI 1640. Brains were dissociated and filtered through 70-µm cell strainers (BD Biosciences) before centrifugation for 10 min at 200 x g. Cells were resuspended in 600 µl Percoll (GE Healthcare), overlaid with 30% Percoll, and centrifuged for 20 min at 1000 x g. The infiltrating mononuclear cells were collected from the gradient interface, the remaining RBCs were lysed using 0.83% ammonium chloride (Sigma-Aldrich), and cells were washed twice before analysis. Cell suspensions from spleen, lymph nodes, and brain were filtered, stained in FACS buffer (PBS, 2% FBS, and 2 mM EDTA) and analyzed by flow cytometry. For CFSE labeling, spleenocytes from OT-1 Rag2-CD45.1 mice were made and labeled with 8 µM CFSE (Invitrogen) in PBS at 10⁷ cells/ml for 7 min at room temperature. Cells were washed in medium with 10% FBS. A total of 1–1.5 × 10⁵ cells were injected in the tail veins of CD45.2 mice infected with OVA-expressing parasites 3–4 wk previously. CFSE dilution in various organs was analyzed by flow cytometry with gating on CD45.1⁺CD8⁺ cells 2–5 days posttransfection.

Generation of hematopoietic chimeric mice

To generate mice containing labeled OT-1 CD8 T cells, we injected bone marrow from OT-1 Rag2-GFP mice into newborn C57BL/6 or CD11c-YFP mice as described (45). This resulted in mice with ~0.2% of spleenocytes corresponding to donor cells before infection. After infection, the vast majority of GFP-labeled cells of donor origin in the brain corresponded to OT-1 T cells due to the selective expansion and recruitment of these cells (data not shown). For production of irradiation chimeras, a 1/10 mixture of Rag2-GFP and CD45.1⁻⁻ wild-type bone marrow was used to reconstitute sublethally irradiated (300 rad, 3 Gy) CD45.2 C57BL/6 mice by i.v. injection. Mice were infected at least 6 wk after reconstitution. Naive OT-1 Rag2-CFP cells (1.5–1.5 × 10⁶/mouse) were transferred by tail vein injection just prior to infection (1–5 h; 0.7–1.3% CD8⁺ CFP⁺ cells in the spleen at the time of infection). Flow cytometric analysis of leukocytes from the brains of infected chimeric mice confirmed that >98% of GFP⁺ cells were CD11b⁺ (data not shown).

In vitro activation of CD8 T cells

CD8 T cells from OT1 or F5 TCR transgenic mice were isolated by dissociation followed by filtration through 70-µm cell strainers (BD Biosciences). Mice were sacrificed between 9 and 80 days postinfection. To generate mice containing labeled OT-1 CD8 T cells, we injected bone marrow from OT-1 Rag2-GFP mice into newborn C57BL/6 or CD11c-YFP mice as described (45). This resulted in mice with ~0.2% of spleenocytes corresponding to donor cells before infection. After infection, the vast majority of GFP-labeled cells of donor origin in the brain corresponded to OT-1 T cells due to the selective expansion and recruitment of these cells (data not shown). For production of irradiation chimeras, a 1/10 mixture of Rag2-GFP and CD45.1⁻⁻ wild-type bone marrow was used to reconstitute sublethally irradiated (300 rad, 3 Gy) CD45.2 C57BL/6 mice by i.v. injection. Mice were infected at least 6 wk after reconstitution. Naive OT-1 Rag2-CFP cells (1.5–1.5 × 10⁶/mouse) were transferred by tail vein injection just prior to infection (1–5 h; 0.7–1.3% CD8⁺ CFP⁺ cells in the spleen at the time of infection). Flow cytometric analysis of leukocytes from the brains of infected chimeric mice confirmed that >98% of GFP⁺ cells were CD11b⁺ (data not shown).

In vitro activation of CD8 T cells

CD8 T cells from OT1 or F5 TCR transgenic mice were activated in vitro based on a protocol from Purbhoo et al. (46). Briefly, splenic leukocytes from TCR transgenic mice were isolated by dissociation followed by filtering through 70-µm cell strainers (BD Biosciences). RBCs were lysed using 0.83% ammonium chloride (Sigma-Aldrich). C57BL6 spleenocytes were used as stimulator cells and were resuspended in complete RPMI 1640 medium at 2 × 10⁶ cells/ml and pulsed with peptide (the OVA peptide SIINFEKL or the A/NT/60/68 influenza virus nucleoprotein peptide ASNENMDAM (NP366–374) were used at 100 nM for 1 h at 37°C). Stimulators were then washed and irradiated at 2000 rad. A total of 2 × 10⁵
OT-1 or F5 responder cells were mixed with $5 \times 10^7$ peptide-pulsed stimulator cells in a volume of 1 ml in 24-well plates and incubated at 37°C in the presence of 50 U/ml IL-2 (National Cancer Institute Preclinical Repository, Frederick, MD) for 5–7 days, until use. For i.v. injection, CTLs were washed twice and resuspended in PBS. A total of 1.5–2 $\times 10^6$ cells/mouse were transferred.

**Fluorescence microscopy**

Mice were infected with fluorescent parasites, sacrificed at various times after infection, and brains were immediately removed and prepared for imaging. Confocal microscopy was performed using 20-μm frozen brain sections and projections of the entire section are shown unless otherwise indicated. For two-photon microscopy, brains were sliced using a vibrotome in oxygenated artificial cerebrospinal fluid (125 mM NaCl, 2.5 mM KCl, 25 mM glucose, 25 mM NaHCO3, 1.25 mM NaH2PO4, 2 mM CaCl2, and 1 mM MgCl2). The 1-mm-thick transverse sections were maintained in position using a 1-mm slice-holder (Warner Instruments) while being superfused with oxygenated artificial cerebrospinal fluid medium at 35°C and imaged by two-photon microscopy using a custom built microscope with a 20 × 0.95 numerical aperture water dipping objective as described previously (7, 47). Imaging volumes (255 × 212 × 30–90 μm or 175 × 142 × 30–90 μm) corresponded to regions of the brains at least 20 μm below the cut site and extending up to 200 μm below the surface of the slice and were scanned every 13–37 s for 20–40 min. Two-photon excitation was achieved using a Spectra-Physics MaiTai laser tuned to 920 or 900 nm. GFP and tdTomato, CFP and GFP, and GFP and YFP emission signals were separated using 560, 495, and 510 dichroic mirrors and collected using three nondescanned detectors.

For confocal fluorescence microscopy, brains of infected mice were fixed with 4% formalin and 10% sucrose in PBS for 1 h, sequentially submerged in 10%, 20%, and 30% sucrose for 18–24 h each, and finally stored in OCT. Twenty-micrometer serial sections were generated by cryosectioning (Microm H550; Microm) and stored at −80°C. For CD11b staining, sections were brought to room temperature, fixed with cold 4% paraformaldehyde for 20 min, air dried, and incubated with 10% mouse serum in Fc blocking buffer (2.4G2 supernatant) for 2 h at 37°C. The tissue was stained with biotin-conjugated anti-CD11b (R&D Systems) for 2 h at 37°C. Mouse biotinylated Abs to OVA (Sigma-Aldrich), glial fibrillary acid protein (GFAP) (DakoCytomation), microtubule-associated protein (MAP) 2 (Thermo Scientific), and Dolichol lectin (Vector Laboratories), sections were fixed with 100% methanol for 5 min, washed three times in PBS, and blocked with 10% goat serum in Fc blocking buffer for 2 h at 37°C. Goat anti-rabbit Alexa Fluor 647 (Invitrogen) secondary was used for OVA and GFAP staining, and goat anti-mouse IgG-alkaline phosphocyanin (Molecular Probes) secondary was used for MAP2 staining. Slides were then washed four times in PBS and incubated with streptavidin-Alexa Fluor 633 (Invitrogen) for 2 h at 37°C. Sections were then washed four times in PBS and cover-slipped using Vectorshield (Vector Laboratories) mounting medium. Stained sections were visualized on Zeiss 510 Axioplan META NLO upright microscope with a ×10 air objective (Plan-Neofluar ×100/0.3) and a ×40 oil objective (Plan-Neofluar ×40/1.3 oil; working distance = 0.17 mm) using 488–545- and 633-nm laser beams. Images were analyzed using Imaris Bitplane.

**Data analysis**

The $x$, $y$, and $z$ coordinates of individual cells over time were obtained using Imaris Bitplane software. Motility parameters were calculated using Matlab (code available upon request). The parameters reported here include average speed (defined as pathlength over time), arrest coefficient (percentage of time points of the total time points for the track for which the speed was lower than 2 μm/min), instantaneous speed (pathlength between two time points averaged over three successive time points), and displacement rate (straight line distance between first and last time points divided by time). To measure the cell density, regions of 80 × 80 × 30–90 μm (imaging volumes obtained by two-photon microscopy) or regions of 80 × 80 × 20 μm (imaging volumes obtained by confocal microscopy) were centered on parasites or cysts or in areas without red fluorescence and the number of OT-1-GFP T cells was counted using Imaris Bitplane software and normalized per unit of volume (106 μm3). For some data sets a spectral unmixing procedure was used to overcome the spectral cross talk in the image data (Y. Chen, unpublished observations). Briefly, we average all voxel intensities in small spatial neighborhoods (5 voxels wide) to make the procedure robust to imaging noise and compute the Otsu automatic threshold for the YFP channel are considered dendritic cell voxels. A similar method is used to identify voxels corresponding to the RFP (parasite) channel.

**Statistical analysis**

Values were expressed as mean ± SEM. Levels of significance were calculated by the Mann-Whitney U test using the GraphPad Prism program. Differences were considered significant at $p < 0.05$ (***, $p < 0.005$; ***, $p < 0.0005$).

**Results**

An experimental system to examine T cell responses to Toxoplasma gondii in the brain during chronic infection

*Toxoplasma* Ags and the T cell Ag receptors that recognize them are just beginning to be characterized (48, 49). Therefore, to track Ag-specific T cell responses we generated an engineered version of the cyst-forming Pru5 strain of Toxoplasma that expresses the model Ag chicken OVA, thus allowing us to use OT-1 OVA-specific TCR transgenic mice (41) to track Ag-specific CD8 T cell responses. The OVA peptide was expressed as a protein fusion that was secreted into the parasitophorous vacuole, a form that can be recognized in vivo by CD8 T cells (28, 35). We also engineered the parasites to express a RFP derivative to facilitate imaging of the parasites.

The course of Toxoplasma infection in mice is highly dependent on both the parasite and the mouse strains. We therefore began our study by characterizing our infection model. Upon injection with a low dose of the engineered Pru5 strain parasites, C57BL/6 (B6) mice developed a chronic progressive disease characterized by higher leukocyte recruitment and higher cyst burden compared with BALB/c mice (Fig. 1, A and B), consistent with previous reports using the ME49 Toxoplasma strain (50, 51). The brains of infected mice contained large numbers of leukocytes that consisted largely of T cells with CD11b+ cells making up most of the remainder (Fig. 1, C and D), which is in line with previous reports (52). In contrast, very few leukocyte-like cells could be isolated from the brains of uninfected B6 mice (Fig. 1, A and D).

These were predominantly intermediate or negative for the blood cell marker CD45 and included CD11b+ CD45int (where “int” is intermediate) cells that corresponded to resident microglia (Fig. 1, C and D) (53). CD11b+ cells in the brains of infected mice included cells with surface marker phenotypes associated with microglia (CD45intCD11b+), macrophages (CD45b2CD11b+CD11c+), and dendritic cells (CD45b2CD11c+) (Fig. 1, C and D, and data not shown), consistent with previous reports from Toxoplasma infection and other models of brain inflammation (54). To confirm the importance of CD8 T cells in our infection model, we compared the survival of wild-type, CD4–/–, and CD8–/– mice to infection. Survival was strongly dependent on the presence of CD8 T cells, whereas the loss of CD4 T cells had only a minor effect on survival (Fig. 1E), consistent with previous studies pointing to the key protective role of CD8 T cells during *T. gondii* infection (18–22).

To track Ag-specific CD8 T cell responses in vivo, we generated mice containing a small number of labeled OT-1 CD8 T cells infected with a low dose of parasites and analyzed the mice at various time points postinfection. OT-1 T cells were found in large numbers in the brain by day 9 postinfection (~2 × 106 cells/brain) (Fig. 2A). OT-1 T cell recruitment was more rapid than the overall increase in leukocyte number, which did not peak until 3 wk postinfection (Fig. 1A). OVA-specific T cells represented ~17% of total leukocytes and 40% of CD8 T cells recovered from the brains of infected mice (Fig. 2B and data not shown). Similar expansion of OT-1 T cells was observed in spleen and lymph nodes of infected mice (Fig. 2B). In contrast, OT-1 T cells did not expand substantially in mice infected with parasites that did not express
OVA or in uninfected mice. OT-1 T cells purified from various organs of mice infected with OVA-expressing parasites expressed IFN-γ after a short in vitro restimulation with the OVA peptide, unlike OT-1 T cells from uninfected mice or mice infected with parasites that did not express OVA (Fig. 2C). The majority of splenic and cerebral OT-1 T cells from mice infected with OVA-expressing parasites lacked expression of programmed death-1 receptor, a marker of T cell exhaustion (55), and expressed high levels of the activation marker CD44, and the lysosomal marker CD107a (LAMP-1) (data not shown). Thus OT-1 T cells in the brains of infected mice have the phenotype and effector functions of activated CD8 T cells. Importantly, the overall course of infection, as measured by cyst burden, leukocyte recruitment to the brain, and survival, was similar whether or not mice received an OT-1 T cell transfer and whether or not parasites expressed OVA (data not shown). This implies that the endogenous CD8 T cell response is sufficient to provide substantial protection and that the frequency of Toxoplasma-specific naive T cells is not a limiting factor in this setting. Thus, the transferred OT-1 T cells did not appear to dramatically alter the overall CD8 T cell response to infection, and they provided a convenient means for monitoring the CD8 response in vivo.

To analyze Ag-specific T cell proliferation and trafficking to the brain, we transferred naive CFSE-labeled, CD45.1 congenically marked OT-1 cells into mice that had been infected with OVA-expressing T. gondii at least 3 wk earlier. Two days after transfer, donor T cells were not detectable in the brain, although they were readily detectable in the spleen and had already undergone some cell division as indicated by dilution of the CFSE label (Fig. 2, D and E). By day 3 after T cell transfer a small number of OT-1 T cells could be detected in the brain, and these cells had already undergone substantial CFSE dilution. By day 5, OT-1 T cells were
FIGURE 2. Ag-dependent responses of naive OVA-specific OT-1 CD8 T cells responses during chronic *T. gondii* infection. A–C, Chimeric mice containing small numbers of naive GFP-labeled OT-1 T cells were infected and cell populations were analyzed at the indicated times by flow cytometry. Data are represented as mean ± SEM. A, Number of OT-1 T cells recovered after Percoll purification of brains from uninfected mice (dashed line), mice infected with parasites expressing OVA (filled circles), or mice infected with parasites without OVA (open circles) as a function of time postinfection. Error bars are from at least three replicates. B, Expansion of OVA-specific T cells in different tissues. Percentages of OT-1 T cells from the indicated samples 24–39 days postinfection are shown. Brain, Percoll isolated brain leukocytes; Spl, spleen; DC, dorsal cervical lymph node; VC, ventral cervical lymph node; ing, inguinal lymph node; MLN, mesenteric lymph node. Error bars are from at least four replicates. Data are the percentage of OT-1 T cells from a live gated population and correspond to $1 \times 10^7$ total OT-1 T cells in the spleen, $1.5 \times 10^7$ in peripheral lymph nodes, and $8 \times 10^7$ in mesenteric lymph node samples. C, OT-1 T cells from infected mice (24–39 days postinfection) produce IFN-γ. Cells from the indicated tissues were stimulated with OVA peptide in vitro for 5 h and then stained for intracellular IFN-γ and analyzed by flow cytometry. Filled bars are samples from mice infected with OVA-expressing parasites, gray bars are samples from mice infected with parasites without OVA, and open bars are samples from uninfected mice. Percentages of IFN-γ-producing cells among gated OT-1-GFP CD8 T cells are shown. Brain, Percoll isolated brain leukocyte; Spl, spleen; DC, dorsal cervical lymph node; VC, ventral cervical lymph node; ing, inguinal lymph node; MLN, mesenteric lymph node. Error bars are from at least four replicates. D and E, Chronically infected mice (21–48 days postinfection) were injected with naive allelically marked (CD45.1) CFSE-labeled OT-1 T cells, and OT-1 T cells in brain and spleen were quantitated by flow cytometry at the indicted times posttransfer. D, Representative flow cytometry plots of gated on donor derived CD8⁺CD45.1⁺ cells are shown. Percentages of OT-1 cells from the total brain-derived leukocyte population (bottom row) or from the total splenocyte population (top row) are written underneath each plot. Numbers in gates represent percentages of gated OT-1 T cells that had diluted CFSE. E, Compiled data for the indicated times and tissues after transfer. DC, dorsal cervical lymph node; VC, ventral cervical lymph node; ing, inguinal lymph node; MLN, mesenteric lymph node; Spl, spleen. Data are represented as mean ± SEM based on at least three replicates. Data are the percentages of cells that have diluted CFSE as a percentage of the total transferred allelically marked OT-1 T cells (CD45.1⁺) and corresponds to $2 \times 10^6$ OT-1 T cells in the spleen and $1 \times 10^7$ in lymph node samples at days 2–5 posttransfer, 400 OT-1 T cells in the brain at day 3, and 8000 OT-1 T cells in the brain at day 5 posttransfer.
readily detectable in the brain and virtually all had extensively diluted the CFSE label. As expected, there was very little proliferation of OT-1 T cells in mice infected with parasites not expressing OVA or in uninfected mice. Interestingly, when in vitro activated OT-1 T cells were transferred into chronically infected mice, T cells could be detected in the brain as well as spleen by day 2, regardless of whether the parasites expressed OVA, although Ag was necessary for the retention/expansion of OT-1 cells in the brain 5 days after T cell transfer (Fig. 3). Together, these data suggest that there was ongoing Ag presentation in the periphery of chronically infected mice, that naive T cells underwent activation and proliferation in the periphery before they trafficked to the brain, and that the retention of T cells in the brain required ongoing Ag recognition.

**T cells ignored cysts but slowed and accumulated near individual parasites**

To examine the location and behavior of T cells and parasites in the brain, we used a combination of confocal microscopy of Ab-stained fixed brain sections and two-photon time-lapse microscopy of living brain tissue. For the two-photon imaging studies we chose to use vibratome-cut brain slices, a tissue preparation that has been extensively used by neurobiologists and provides a robust system in which cells remain viable and functional for extended time periods (56). This approach also has the advantage that is it possible to scan relatively large areas of the brain to locate foci of infection, which are relatively rare in infected mice (<2000 cysts distributed throughout the entire brain). The alternative approach of intravital imaging of the brain (57, 58) is not a feasible approach for this study, because fluorescent detection is limited to a depth of ~200 μm in brain tissue, and we have yet to observe a cyst or foci of infection sufficiently close to the skull to be accessible by this approach.

Parasites exist in the brain as bradyzoites that form tissue cysts within infected cells and as individual tachyzoites and bradyzoites, some of which may have emerged from ruptured cysts (59–61). To examine which of these forms of the parasites T cells recognized, we examined the location of OT-1 T cells relative to OVA-expressing parasites in the brains of chronically infected mice (Fig. 4). Parasite-containing cysts were readily identifiable by their large size and typical spheroid shape (Fig. 4A, dashed circle). A fluorescent signal was also detected in smaller particles whose size and shape suggested that they correspond to single parasites (Fig. 4, boxed areas labeled 1 and 2, and B, arrowheads). Importantly, the OVA Ag is present in cysts within the cyst wall, identified here by Dolichol lectin staining (Fig. 4C) as expected, given that the promoter used to drive OVA expression is active in both bradyzoites and tachyzoites. Time-lapse imaging using two-photon microscopy of vibratome-cut brain slices revealed a few examples of isolated parasites emerging from disintegrating cysts (Fig. 4B and supplemental video 1), indicating that at least some isolated parasites derived from ruptured cysts. Although OT-1 T cells were found throughout the brain, we noted that the density of OT-1 T cells was highest in regions containing isolated parasites. In contrast, T cell density around T. gondii cysts was similar to the density in regions that did not contain any visible parasite fluorescence (Fig. 4D). This trend was observed both at early time points (days

**FIGURE 3.** Activated CD8 lymphocytes can enter chronically infected brains in the absence of ongoing antigenic stimulation. A, OT-1 T cells were activated in vitro and then transferred into mice that had been infected 17–34 days earlier with OVA-expressing parasites (filled circles). T cell transfers into mice infected with parasites without OVA (open circles) and infected mice (x) are shown for comparison. OT-1 T cells were quantitated in the brain (left panel) and spleen (right panel) by flow cytometry at the indicated times after transfer. Data are represented as mean ± SEM based on at least three replicates. Data are the percentages of OT-1 T cells as a percentage of total brain leukocytes (upper left panel) or the percentages of OT-1 T cells as a percentage of total splenocytes (right panel). B, Mice were transferred with 2 × 10^6 in vitro activated OT-1 T cells (filled circles) or left untreated (open circles) and then infected with a high dose (10^6) of OVA-expressing tachyzoites. Mice were monitored daily and euthanized at the first signs of illness (n = 14 mice for each group).
postinfection when the number of OT-1 T cells in the brain had reached a plateau but total leukocyte numbers were still increasing, as well as at later time points (days 19–39) postinfection when overall leukocyte recruitment to the brain had also reached a plateau (Figs. 1A and 2A). These data suggested that isolated parasites, but not intact cysts, were visible to CD8 T cells.

The accumulation of CD8 T cells around isolated parasites suggested that they might be detecting Ag in these areas. If this were
Figure 5. T cells slowed and arrested near isolated parasites and during ongoing antigenic recognition. A, OT-1 T cell migration in brain slices from mice infected with OVA-expressing parasites. Graphs show speeds and arrest coefficients for individually tracked T cells from imaging volumes (typically 255 × 212 × 60 μm) that contained isolated parasites, intact cysts only, or no visible parasites as indicated. Data were compiled from six runs with isolated parasites, five runs with intact cysts, and seven runs without visible parasites. The samples were analyzed 10–39 days postinfection and no significant differences in speed or arrest coefficients were observed between early vs late time points (data not shown). *** p < 0.0005, indicating significant differences. Panels to the right show projections of three-dimensional imaging volumes representing a single time point from a time-lapse series obtained by two-photon microscopy. Left panel shows a run containing isolated parasites (red, indicated by arrows in enlarged inset). Rightmost panel shows a sample containing an intact cyst and no isolated parasites. The lines show the paths taken by individual OT-1 T cells over a 13-min time period for T cells around parasites (orange lines) or around cysts only (white lines). CD11cYFP reporter-expressing cells are shown in green. This corresponds to supplemental video 2. The impact of proximity to parasites within individual imaging volumes is presented in Fig. 6. B, T cell slowing and stopping induced by ongoing Ag recognition. In vitro activated OT-1 or F5 T cells were transferred into mice infected with OVA-expressing parasites and brains imaged 4–6 days posttransfer. As an additional control, OT-1-GFP blasts were transferred into mice infected with parasites not expressing OVA. Right panels show projections of representative imaging volume representing single time points from a time-lapse series obtained by two-photon microscopy. The lines show the paths taken by individual T cells over a 13-min time period with Ag-specific cell tracks in orange and the tracks of control T cells (F5 and OT-1 in mice infected with OVA parasites) in white. Arrows indicate the positions of individual RFP-labeled parasites (red). This corresponds to supplemental videos 3 and 4.

The case we would expect T cells to slow and stop in these areas, as has been observed for Ag recognition by T cells in other tissues (2, 3). To examine this question, we performed time-lapse imaging using two-photon microscopy (Fig. 5). Overall, the speed of OT-1 T cells (5–6 μm/min) was substantially lower than that previously reported for naive CD8 T cells in lymph nodes, but in line with that of previous reports of effector T cell migration within tissues (62, 63). Importantly, OT-1 T cells in imaging volumes that contained isolated parasites showed reduced speed compared with T cells in imaging volumes with only cysts or with no visible parasite fluorescence. (Fig. 5A, supplemental Fig. S1A, and supplemental video 2. Moreover, the arrest coefficient, defined as the proportion of time that T cells spend not moving, was significantly increased around isolated parasites (Fig. 5A), which is consistent with the hypothesis that T cells were actively recognizing Ag in these areas. The relationship between proximity to parasites and T cell motility could also be observed within a single 255 × 212 × 60-μm imaging volume with T cells that were relatively close to parasites showing reduced speeds and increased stopping relative to T cells in the field that were more distant from parasites (Fig. 6).

To test whether Ag recognition was required to induce T cell slowing and stopping in the brains of chronically infected mice, we compared OT-1 T cells to T cells expressing an irrelevant TCR (F5) (40). For this experiment, we took advantage of our observation that in vitro activated T cells trafficked to the brain of chronically infected mice and persisted there for up to 5 days in the absence of ongoing antigenic stimulation (Fig. 3A). Transfer of activated OT-1 T cells provided some protection against a high dose of OVA-expressing parasites, confirming that the transferred T cells were functional in vivo (Fig. 3B). We activated T cells from OT-1 and F5 TCR transgenic mice in vitro and then compared their behavior in the brain following transfer into mice that had been infected 2–4 wk earlier with OVA-expressing parasites. As an additional comparison, we also transferred in vitro activated OT-1 T cells into mice that had been infected with parasites that did not express OVA. Using both approaches, we showed that Ag-specific T cells moved more slowly and arrested more frequently than nonspecific T cells (Fig. 5B, supplemental Fig. S1B, and supplemental videos 3 and 4. Importantly, these differences were observed even when OT-1 and F5 T cells were tracked within the same imaging volumes (Fig. 5B, lanes labeled “co-transfer”). These data suggest that the slowing and stopping of Ag-specific T cells around isolated parasites were not due solely to changes in the tissue environment but rather were due to local Ag recognition.

T cell contacts with granuloma-like structures and individual APCs in the brain

The Ag-dependent slowing and stopping of CD8 T cells in the brains of chronically infected mice was consistent with their interaction with Ag-bearing cells. To characterize potential APCs, we examined fixed brain sections by confocal microscopy using Abs to CD11b, a marker expressed by monocyte/myeloid lineage cells and microglia that has been previously shown to be associated with parasites in the brains of chronically infected mice (64).
In the brains of chronically infected mice we observed spherical, tightly packed aggregates of CD11b-expressing cells that may correspond to the “nodules” of leukocytes described previously (32, 59) (Fig. 7Ai and Aii). Isolated parasites were often found within CD11b aggregates (Fig. 7Ai, arrowheads), but they were also found outside of the aggregates within nearby isolated CD11b⁺ cells (Fig. 7Aii, arrowheads). Approximately one-third (44/122) of isolated parasites appeared to be inside a CD11b⁺ cell or aggregate. Moreover, the majority of the CD11b aggregates observed contained one or more isolated parasites (20 aggregates with a total of 28 parasites from 12 different sections) and aggregates of CD11b⁺ cells were not seen around cysts (data not shown). This is consistent with previous light microscopy studies (32, 59) and implies that, like T cells, CD11b⁺ cells in the brains of chronically infected mice responded to isolated parasites rather than to intact cysts. CD11b⁺ aggregates were most prominent at late time points postinfection (19 days postinfection), whereas at earlier times postinfection parasite fluorescence was more often seen associated with isolated CD11b⁺ cells (Fig. 7Aiii, boxed area labeled 1). In these areas we also observed individual T cells contacting isolated CD11b⁺ cells, including those that did and did not contain visible parasite fluorescence (Fig. 7Aiii, boxed areas labeled 1 and 2). Ag-specific T cells were also found in contact with the aggregates of CD11b⁺ cells and sometimes inside them (Fig. 7A), in line with the higher density of T cells in areas with isolated parasites (Fig. 4A). Interestingly, T cells within CD11b⁺ aggregates did not preferentially cluster around the isolated parasites, but rather appeared to be evenly distributed throughout the entire aggregate (Fig. 7A).

To examine other potential APCs, we also stained brain sections from infected mice with Abs specific for an astrocyte marker, GFAP, and a neural marker, MAP2. We did not see any indication of OT-1 T cells clustering around astrocytes that were in the vicinity of parasites, nor did we observe any clear examples of GFAP⁺ astrocytes containing parasite fluorescence (Fig. 7B and data not shown). Although we did observe a small number of neurons containing parasites, these were rare and were not contacted by OT-1 T cells (Fig. 7C and data not shown). Previous electron microscopy studies indicated that cysts could be found within intact neurons (32), which is compatible with our observation that 25% of cysts (4/16) were surrounded by MAP2 staining (Fig. 7D and data not shown). These data suggest that astrocytes and neurons are not major APC types in the brain, although neurons can harbor parasites and cysts.

To allow for dynamic imaging of potential APCs in the brain, we made use of mice bearing a dendritic cell reporter consisting of YFP under the control of the CD11c promoter (42). YFP cells increased in number in the brains of chronically infected mice in parallel with the overall increase in leukocyte number, reaching a plateau of ~5 × 10⁷ reporter expressing cells per brain at 3 wk postinfection (Fig. 1 and data not shown). Flow cytometric analysis showed that the majority of YFP cells expressed CD11b, although only ~25% expressed surface CD11c (data not shown), consistent with previous reports (42). Confocal analysis of brain sections showed that a subset of cells within the CD11b aggregates also expressed the CD11cYFP reporter (Fig. 8A). Thus, the CD11cYFP reporter marks a subpopulation of cells within CD11b aggregates and could serve as a marker for identifying aggregates during dynamic imaging. Indeed, two-photon microscopy of brains slices from infected CD11cYFP reporter mice revealed spherical aggregates of YFP-bright cells containing isolated parasites and surrounded by Ag-specific T cells (Fig. 8B and supplemental video 5). Isolated parasites within aggregates were generally not enclosed by reporter bright cells (Fig. 8B, inset 1), although we occasionally observed individual reporter bright cells.
forming intimate associations with cysts (supplemental video 6). Time-lapse imaging of brain slices from CD11cYFP reporter mice revealed that OT-1 T cells migrated actively around and through aggregates of reporter-positive cells, whereas the reporter-positive cells were largely sessile (Fig. 8B, right panel, and supplemental video 5). These structures, which are composed of balls of static myeloid lineage cells enclosing isolated parasites and are surrounded by the motile T cells described here, were strikingly similar to granulomas observed in the liver upon infection of mice with the bacterium *Mycobacterium bovis* (8) and may serve to contain parasite growth during the chronic phase of infection.

To examine more closely the interactions of T cells, parasites, and reporter-expressing cells within the granulomas, we performed spectral unmixing of data sets and quantitated T cell motility inside and outside of the granulomas (Fig. 8, C and D, and supplemental video 7). OT-1 T cells within granulomas migrated more slowly and arrested more frequently compared with T cells that were just outside of the granuloma (Fig. 8D), which is consistent with the hypothesis that Ag recognition by T cells took place within these structures. Interestingly, T cells within the granuloma that were closest to the parasites did not show a further decrease in speed or an increase in stopping (Fig. 8D). These data suggest that Ag may be distributed throughout the granuloma-like structures rather than being localized to individual parasite-containing cells.

Because most parasites were found in CD11b+ but not CD11c YFP reporter-positive cells (Fig. 8B and data not shown), we developed an alternative approach for dynamic imaging of potential APCs in the brain. We generated hematopoietic chimeras reconstituted with mixtures of bone marrow from Rag2−/− GFP-labeled donor mice and bone marrow from unlabeled wild-type mice. The rationale behind this approach was 2-fold. First, because Rag2−/− bone marrow cannot give rise to T or B cells, the majority of GFP-labeled leukocytes in the brains of infected mice should be CD11b+ cells, based on our flow cytometric analysis (Fig. 1C and data not shown). In addition, wild-type unlabeled bone marrow donor cells would give rise to unlabeled T and B cells, providing a more normal immune response and making it easier to visualize individual GFP-labeled CD11b+ cells. Reconstituted mice were transferred with naive OT-1 CFP-labeled T cells and infected with OVA-expressing parasites. Brains were examined at relatively early times postinfection (10–13 days), times at which interactions with individual CD11b+ cells were prominent based on immuno-fluorescence analysis of fixed brain sections (Fig. 7 and data not shown). Two-photon microscopy of vibratome-cut brain slices...
revealed numerous GFP-labeled cells and CFP-labeled T cells in regions containing isolated parasites as well as occasional examples of GFP+ cells apparently engulfing parasites (Fig. 9A and supplemental video 8), which is consistent with a response to recent parasite release by cyst rupture or egress from an infected cell.

To examine the duration of T cell contacts with potential APCs in regions containing parasites, we tracked individual T cells and scored their interactions with GFP-labeled cells over time (Fig. 9, B–D, and supplemental videos 9–11). In some cases T cells interacted with several different GFP-labeled cells during the course of imaging (Fig. 9, B and D, and supplemental videos 9 and 10), whereas in other examples T cells remained in contact with a single labeled cell during an imaging run (Fig. 9D and supplemental video 11). Only five of 31 of the contacts observed occurred with GFP cells that contained visible parasite fluorescence (Fig. 9C, hatched bars). Although most contacts were brief (average duration 7.8 min; Fig. 9C), they coincided with a transient reduction in speed (Fig. 9D and supplemental videos 9 and 10). Compiled data for multiple T cell contacts over several runs (supplemental Fig. S1C) showed that the average speed of T cells for all time points...
in which visible contacts occurred was 3.39 μm/min, a value that was similar to the speed of Ag-specific T cells near isolated parasites in experiments in which APCs were not visualized (4.43 μm/min; supplemental Fig. S1A). To estimate the basal migration rate of T cells in the brain, we considered the speed of T cells during time points that did not have a visible contact with an APC (7.07 μm/min), the speed of Ag-nonspecific T cells (7.6 μm/min; supplemental Fig. S1B), and the speed of T cells in areas that did not contain parasite fluorescence (6.35 μm/min; Fig. S1A, open circles). Although each of the values is subject to caveats, altogether these data suggest that the basal migration rate of T cells in the brain in the absence of Ag recognition is ~7 μm/min and that frequent transient contacts with Ag-bearing cells lead to average T cell speeds of ~5 μm/min. Together, these data indicate that T cells formed relatively brief contacts with APCs that were in the vicinity of parasites, only some of which contained intact parasites. These contacts may serve to either stimulate T cell effector responses or allow T cells to activate macrophages or both and may represent an early response to cyst rupture preceding granuloma formation.

**Discussion**

The control of the parasite *T. gondii* during chronic infection in the brain depends on CD8 T cells, but the nature of Ag presentation to CD8 T cells and how their response protects against parasites in the brain is not clear. In this study we have directly visualized T cells, parasites, and potential APCs in living brain tissue to address these questions. We found that CD8 T cells ignored intact Ag-bearing cysts but slowed and transiently arrested near isolated parasites. We provided evidence that the slowing of T cells near parasites was Ag specific and could occur through interactions with granuloma-like structures as well as contacts with individual APCs, some of which did not contain parasites. These results provide an important new framework for understanding immune responses to persistent infections in the brain.

**FIGURE 9.** T cell contacts with potential APCs in the brain. Mice were irradiated and reconstituted with Rag2−/− GFP bone marrow together with unlabeled bone marrow to label a subset of the potential APCs in the brain. Reconstituted mice were transferred with a small number of CFP-labeled OT-1 T cells and infected with RFP OVA-expressing parasites and analyzed 10–13 days postinfection. A, Example of a GFP-labeled cell engulfing a parasite. Panels show projections of 20-μm imaging volumes from a time-lapse series obtained by two-photon microscopy. OVA-specific, CFP-labeled OT-1 CD8 T cells are in blue, GFP-labeled APCs are in green, and parasites are in red; left panel corresponds to a time point before engulfment (arrowhead indicates the parasite) and middle and right panels correspond to time points during and just after engulfment (circled). The corresponds to supplemental video 8. B, Examples of T cells contacting GFP-labeled cells. Red, *T. gondii*; green, GFP; blue, OT-1-CFP. Portions of the tracks spent in contact with a GFP-labeled cell are in pink, and portions of the track spent not making visible contact are in yellow. Left panel shows a T cell making two successive contacts with two different GFP-labeled cells. This corresponds to supplemental video 9. Middle panel represents a T cell making two successive contacts with two different GFP labeled cells, one of which contains parasite fluorescence (shown in inset). This corresponds to supplemental video 10. Right panel shows a T cell that remained in contact with the same GFP-labeled cell for the length of the run (18 min). This corresponds to supplemental video 11. C, T cell-APC contact durations. Plot summarizes the number of OT-1 cell contacts with GFP cells for the indicated durations. T cell contacts with GFP-labeled cells containing visible parasites are indicated by the shaded portions of bars. Data are pooled from three runs. D, Decrease in T cell speed correlates with contacts with GFP-labeled cells. Plots of the instantaneous speed as a function of time for individual T cells are shown. Shaded areas correspond to time points in which T cells made a visible contact with a GFP-labeled cell. Arrowheads indicate the time points shown on still images.
The issue of which host cells can present *T. gondii* Ags to CD8 T cells has been somewhat of a puzzle. CD8 T cells recognize peptide fragments of Ag bound to MHC class I on the surface of another cell, and these peptides are typically derived from the cytosol of a host cell that has been invaded by an intracellular pathogen. Although *T. gondii* is an intracellular pathogen, it resides within a parasitophorous vacuole that prevents parasite proteins from entering the cytosol (65), and thus it is unclear how parasite Ags gain access to the class I presentation pathway. In some settings class I MHC Ag presentation can occur by an alternative “cross-presentation” pathway in which Ag is produced in one cell but presented by a different host cell, and it seemed plausible that this pathway might operate during *T. gondii* infection (66). However, only invaded but not bystander dendritic cells isolated from spleens of acutely infected mice present parasite-encoded Ags to T cells, and Ag presentation requires components of the classical class I presentation pathway, indicating that direct presentation by invaded host cells is important, at least during acute infection (35, 49, 67). Thus, it was surprising that CD8 T cells in the brain made contacts not only with cells containing parasites but also with APCs that were close to parasites but did not themselves contain parasites. Moreover, many T cell contacts occurred with tightly packed aggregates of CD11b \(^+\) cells and with T cells distributed evenly throughout the aggregate and did not preferentially arrest near the parasites within these structures. These results suggest that CD8 T cells were detecting Ag on the surface of CD11b \(^+\) cells that did not contain parasites and suggest that cross-presentation pathways may operate in the brain during chronic infection. Alternatively, APCs may have initially harbored live parasites but eventually degraded the parasites (68), leading to the loss of the fluorescent signal. In either case, this distinct mode of Ag presentation may reflect the unique ability of the environment of the brain to limit Ag presentation. Moreover, it may be more accurate to think of CD8 T cells detecting Ag on aggregates of APCs rather than on individual cells.

The concept of APCs acting as aggregates of cells rather than individual cells during their response to *T. gondii* is also relevant to the issue of immune effector mechanisms. CD8 T cells can protect either by direct killing of target cells or by the release of cytokines, both of which can be triggered when effector CD8 T cells recognize Ag on a target cell. Although killing requires direct contact between a CD8 T cell and an invaded target cell to provide effective protection, this need not be the case for cytokine release. Given that the major mode of protection by CD8 T cells during *T. gondii* infection is cytokine production rather than direct killing (22–24), interaction with a group of APCs, only some of which contain parasites, would be compatible with the delivery of effective protection. Moreover, the physical “walling off” of parasites within the granuloma could provide a means of controlling parasite spread, perhaps in conjunction with IFN-\(\gamma\)-inducible GTPases (26), while avoiding a more potentially damaging effector mechanism such as inducible NO synthase production. The cytokines produced by CD8 T cells that surround the granulomas could serve to activate macrophages within these structures to make these protective mechanisms more effective.

In contrast to the response to individual parasites, we saw no evidence that CD8 T cells responded to cysts. This is despite the fact that cysts express the model Ag OVA and reside within intact host cells that could potentially present Ag to T cells (32). One possible explanation is that many cysts are found within neurons (Fig. 7D and Ref. 32), cells that express low levels of class I MHC. In addition, bradyzoites within brain cysts are surrounded by a cyst wall and, thus, very little parasite material may escape into the cytoplasm of the cyst-containing host cell. We also observed large aggregates of CD11b \(^+\) cells near isolated parasites, but not intact cysts, consistent with previous reports based on light and electron microscopy (32, 59). Interestingly, in those earlier studies examples were noted in which a small numbers of macrophages surrounded a cyst that was within a host cell with a disrupted membrane, suggesting an early macrophage response to cyst rupture (59). This may correspond to the occasional examples that we observed of CD11c FP reporter-expressing cells or GFP-labeled APCs making intimate contact with intact cysts (supplemental videos 1 and 6). Together, these observations suggest that cyst rupture may be initially detected by APCs of the brain followed later by a larger influx of macrophages and T cells, leading eventually to granuloma formation. Ag within cysts may contribute to this response by being released during cyst rupture and then taken up and cross-presented by APCs within the granuloma.

Prolonged T cell arrest and cluster formation are often correlated with T cell priming in lymph nodes and have also been associated with T cell effector responses in tissues (2, 63, 69–71). In contrast, in the current study we observed no large clusters of CD8 T cells, and T cells made only transient contacts with APCs. The motility of T cells and the nature of their interactions with APCs is influenced by many factors, including the differentiation state of T cells, the tissue environment, the amount of Ag, the nature of the APC, and the presence of immune-suppressive mechanisms such as regulatory T cells (reviewed in Ref. 3). The lack of T cell stopping and stable cluster formation in our study may reflect limiting levels of Ag presentation due to immune evasion by the parasites and/or the presence of immune regulatory mechanisms to limit T cell activation in the brain.

Regarding the functional consequences of these transient interactions, it is worth noting that while stable contacts often correlate with T cell activation, transient contacts are sufficient to activate T cells in a three-dimensional collagen matrix, and transient contacts have also been associated with effector responses in vivo (63, 71, 72). Although we cannot be sure that all of the contacts that we observe are a direct result of Ag recognition, several lines of evidence suggest that they do reflect functional interactions. These include the Ag dependence of T cell motility changes (Fig. 5B), evidence that Ag is required for T cell retention in the brain (Fig. 3), evidence that cerebral OT-1 T cells produce the protective cytokine IFN-\(\gamma\) (Fig. 2C), and indications from our study and others that CD8 T cells play a protective role in this setting (Figs. 1E and 3B and Refs. 18–22). Transient serial interactions with Ag-bearing cells would likely be sufficient to sustain the activation states of effector CD8 T cells and trigger the local release of cytokines near granulomas and foci of infection in the brain. Future studies using real-time markers for TCR signaling events and cytokine release, combined with two-photon imaging, should eventually provide a more detailed and complete picture of T cell effector release in the brain and other sites during infection.

In summary, the current study provides evidence for a surprising mode of Ag recognition in the brain in which T cells ignore Ag-bearing cysts but make transient contacts with APCs and granuloma-like structures near foci of infection. This mode of interaction likely reflects immune evasion by parasites as well as the ability of the unique environment of the brain to limit Ag presentation and modulate immune responses and is compatible with a mode of protection in which CD8 T cells provide cytokines to APCs rather than directly kill target cells. These data provide a new perspective on CD8 T cell responses to chronic infection in the brain.

*Note added in proof.* After this manuscript was submitted, another study was published describing T cell migration in the brains...

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Disclosures

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