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Class I Major Histocompatibility Complex Presentation of Antigens That Escape from the Parasitophorous Vacuole of *Toxoplasma gondii*

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The intracellular parasite *Toxoplasma gondii*, the causative agent of toxoplasmosis, induces a protective CD8 T-cell response in its host; however, the mechanisms by which *T. gondii* proteins are presented by the class I major histocompatibility complex remain largely unexplored. *T. gondii* resides within a specialized compartment, the parasitophorous vacuole, that sequesters the parasite and its secreted proteins from the host cell cytoplasm, suggesting that an alternative cross-priming pathway might be necessary for class I presentation of *T. gondii* antigens. Here we used a strain of *T. gondii* expressing yellow fluorescent protein and a secreted version of the model antigen ovalbumin to investigate this question. We found that presentation of ovalbumin secreted by the parasite requires the peptide transporter TAP (transporter associated with antigen processing) and occurs primarily in actively infected cells rather than bystander cells. We also found that dendritic cells are a major target of *T. gondii* infection in vivo and account for much of the antigen-presenting activity in the spleen. Finally, we obtained evidence that Cre protein secreted by *T. gondii* can mediate recombination in the nucleus of the host cell. Together, these results indicate that *Toxoplasma* proteins can escape from the parasitophorous vacuole into the host cytoplasm and be presented by the endogenous class I pathway, leading to direct recognition of infected cells by CD8 T cells.

A number of important human diseases, including malaria, leishmaniasis, cryptosporidiosis, and toxoplasmosis, are caused by infection with protozoan pathogens that reside within the cells of their hosts. Many of these organisms produce long-lasting chronic infections due to the parasite's ability to modulate the host's immune response and achieve a balance that allows both the host and the parasite to survive (for a review see reference 39). This balance is exemplified by *Toxoplasma gondii*, the causative agent of toxoplasmosis. Infection by *T. gondii* causes disease primarily in individuals with suppressed or immature immune systems (29, 37). In immunocompetent hosts, infection leads to a balanced immune response that is sufficient to prevent pathology yet allows the parasite to establish a chronic infection. During the initial acute phase of infection, the rapidly dividing tachyzoite stage of the parasite disseminates throughout the body by invading and replicating within host cells. During the subsequent chronic phase of the infection, the slowly dividing bradyzoite persists for long times within cysts in tissues such as muscle and brain. The host immune response is crucial for controlling parasite growth during the acute phase of the infection and for preventing the reemergence of acute infection in chronically infected individuals (for reviews see references 6, 26, and 54).

Like many intracellular pathogens, *T. gondii* elicits a strong class I major histocompatibility complex (MHC)-restricted CD8 T-cell response. CD8 T cells play an important role in protection against the parasite, primarily through production of the cytokine gamma interferon (2, 9, 10, 50, 51, 53). The class I MHC presentation pathway is generally designed to sample pathogens found in the cytoplasm of host cells, such as viruses. Cytoplasmic antigens are degraded by proteosomes, and the resulting peptides are pumped into the endoplasmic reticulum by the transporter associated with antigen processing (TAP) (for a review see reference 55). However, *T. gondii* resides not in the cytoplasm of the host cells but in a specialized compartment known as the parasitophorous vacuole (PV) (27). The PV does not fuse with lysosomes or exchange with other host cellular compartments (19, 30, 44). In addition, the PV membrane acts as a molecular sieve that allows small molecules to pass through but restricts the movement of molecules larger than 1,300 Da (40). Thus, the PV might prevent *T. gondii* antigens from entering the endogenous class I MHC presentation pathway. Alternative class I MHC presentation pathways have been described, in which material from one cell is taken up and presented by another cell, a phenomenon known as cross-presentation or cross-priming (17, 55). Evidence that cytotoxic T-cell populations can kill target cells that have been exposed in vitro to *T. gondii* suggests that direct recognition of infected cells can occur (7, 21, 49). However, these experiments did not distinguish between killing of actively infected targets and killing of targets that had acquired antigens from neighboring cells. Thus, it is not clear to what extent the presentation of *T. gondii* antigens via class I MHC occurs via the endogenous pathway and to what extent it occurs via cross-presentation pathways (for a review see reference 28).

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One major obstacle to addressing these questions is the lack of well-defined *T. gondii* antigens for CD8 T cells. Although there is evidence that SAG-1, one of the major surface proteins of *Toxoplasma*, is an important antigen (21, 22, 32) and a number of CD4 T cell epitopes have been identified (34–36, 38), the antigens and epitopes recognized by CD8 T cells are largely undefined, and the T-cell antigen receptors expressed by CD8 T cells specific for *T. gondii* antigen have not been characterized. One way to circumvent this difficulty is to engineer expression of model antigens. This approach has been used successfully in the study of CD4 responses to *Trypanosoma cruzi* (8, 24) and in examining the effect of stage and compartmentalization of antigen on CD8 responses to *T. gondii* (25).

Another gap in our understanding of the relationship between *T. gondii* and its host is the tropism of the parasite for different cell types. While *T. gondii* is capable of infecting virtually any cell type in vitro, it is not clear which cell types are the major targets of infection in vivo. This information is particularly relevant for understanding the mechanism of antigen presentation, since cell types differ in the ability to use different antigen presentation pathways. For example, professional antigen-presenting cells, such as macrophages and dendritic cells (DC), are more efficient at cross-presentation than nonprofessional antigen-presenting cells.

Here we addressed these questions using genetically engineered strains of *T. gondii*. Using a fluorescent strain of *T. gondii* that also expresses a secreted form of the model antigen ovalbumin (OVA), we obtained evidence that class I presentation of this antigen is TAP dependent and occurs primarily in infected cells rather than in bystander cells. We also found that macrophages and dendritic cells are the major infected cell populations in lymphoid organs during the acute phase of infection in mice and that dendritic cells account for much of the antigen presentation activity in the spleen. Finally, we obtained evidence that a reporter protein secreted by the parasite (Cre recombinase) can activate expression of a reporter gene (floxed green fluorescent protein [GFP]) in the host cell nucleus. Together, our data indicate that proteins secreted by *T. gondii* can escape from the PV and be presented by the endogenous class I pathway, thus allowing direct recognition of infected cells by CD8 T cells.

MATERIALS AND METHODS

Plasmids. To express a secreted form of Cre, the coding sequence of Cre recombinase was cloned into ptubP30GFP/sagCAT; this resulted in translational fusion to the *T. gondii* surface protein P30/SAG1 and replacement of its GPI anchor signal sequence (ptubP30Cre/sagCAT). This arrangement resulted in a soluble fusion protein with a predicted molecular mass of 69 kDa, which is secreted into the parasitophorous vacuole via the dense granules (48). The Cre sequence was amplified by PCR by using plasmid pMC-Cre as the template (13) and the primer sequences ACTGAGATCTAAAATGCATCCCAAGAAGAAG and CAGTCCTAGGGGAATCGCCATCTTCCAG. This cassette also encodes an N-terminal simian virus 40 nuclear localization signal and a C-terminal cMyc epitope tag. The P30Cre gene is flanked by the promoter and 5′ untranslated region of the *T. gondii* α-tubulin gene and the 3′ untranslated region of the dihydrofolate reductase-thymidylate synthase gene (DHFR-TS) (see reference 48 for further details). Finally, the plasmid contained a chloramphenicol acetyltransferase cassette (sagCATsag) (46), which permitted drug selection of stably transformed parasites.

To express cytoplasmic Cre, plasmid ptubCre/sagCAT was constructed, which lacked the P30 sequence. A second plasmid with a weaker promoter and a different selectable marker (DHFR-TS) was constructed by introducing the Cre coding sequence (by BglII digestion, T4 polymerase end filling, and NotI digestion from ptubCre/sagCAT) into plasmid pRV-YFP (15). Subsequently, the gra1 promoter in this construct was replaced with the sag1 promoter (46) excised with XhoI, filled with T4 DNA polymerase, digested with NsiI, and introduced 5′ of Cre into the plasmid (opened with BstEII, filled with T4 DNA polymerase, and digested with NsiI; this construct was used to establish the cytoCre line).

Plasmid ptubP30OVA/sagCAT was constructed by cloning a fragment encoding amino acids 140 to 386 of chicken ovalbumin (amplified by PCR from cDNA with primers 5′-ATCGACCTAGGGATCAAGCCAGAGAGCTCATC-3′ and 5′-AAAACTGCAGTTAAGGGGAAACACATCTGCC-3′) into plasmid ptubP30GFP/sagCAT (48), replacing the GFP gene (C. Collazo, A. Sher, D. S. Roos, and B. Striepen, unpublished data). The predicted molecular mass of the secreted protein was 54 kDa.

Plasmid ptscABP-P30RFP/dhfrDHFR was constructed by BglII/AvrII replacement of FNR in ptubFNR-RFP/sagCAT (47) with P30 from ptubP30GFP/sagCAT (48). Subsequently, the P30-RFP-dhfr3′UTR segment of the plasmid was cloned by using BglII, end filling, and NotI into the pRV-YFP library plasmid (15) digested with EcoRV/NotI. The predicted molecular mass of secreted red fluorescent protein (RFP) is 53 kDa.

Cells and parasites. Wild-type and recombinant *T. gondii* strain RH tachyzoites were passaged in confluent human foreskin fibroblasts and transfected as described previously (48). In brief, 1×10^7 freshly harvested parasites were resuspended in 300 μl of cytomix and mixed with 50 μg of plasmid in 100 μl of cytomix. Electroporation was performed in a 2-mm cuvette (Genetronics, San Diego, Calif.) by using a BTX ECM 630 electroporator (Genetronics) set at 1,500 kV, 25 Ω, and 25 μF. Transfections with plasmids containing a CAT marker were selected for stable genomic integration with 20 μM chloramphenicol (Sigma, St. Louis, Mo.). Transfections with plasmids containing a DHFR-TSm2m3 marker were selected for stable genomic integration with 1 μM pyrimethamine (Sigma). Stable lines of parasites expressing cytoplasmic Cre could be established only by using the sag promoter-driven construct. This was most likely due to toxicity of overexpression of Cre (45). RH-YFP2 parasites have been described previously (14). RH secOVA/YFP2 was generated by stable transfection of ptubP30OVA/sagCAT, followed by a second transfection with ptubYFP-YFP/sagCAT selected for yellow fluorescent protein (YFP) expression by three rounds of cell sorting. All parasite lines were cloned by limiting dilution. GFP-STOP cells (based on the FBL-3 murine erythroblastic leukemia cell line) were a kind gift of Pandelakis Koni and Sunil Joshi, Medical College of Georgia (20) and contained a loxP site-flanked transcriptional terminator STOP cassette (pBS302; Gibco BRL) separating the CMV promoter from the GFP open reading frame.

Flow cytometry of infected GFP-STOP cells. GFP-STOP cells were grown to confluence in T25 tissue culture flasks (5×10^6 cells per flask) and infected with parasites (multiplicity of infection [MOI], 1 to 10) in Ed1 medium. After 24 h monolayers were washed with 10 ml of phosphate-buffered saline and trypsinized (1 ml of 0.25% trypsin [HyClone, Logan, Utah]). The trypsin was inactivated by addition of 10 ml of 10% serum in Dulbecco modified Eagle medium, and cells were passed through a 70-μm-mesh cell strainer (BD/Falcon, Franklin Lakes, N.J.). Cells were pelleted by centrifugation for 15 min at $1,500 \times g$ and resuspended in 500 μl of phosphate-buffered saline. An analysis was performed with a MoFlo cytometer (Cytomation, Fort Collins, Colo.). Cellular debris and free parasites were excluded from the analysis by gating on forward and side scatter, and 500,000 total events were recorded. GFP expressed in cells and YFP expressed in parasites were excited with a 488-nm air-cooled argon laser at 100 mW, and emission was measured with a 530/540-nm band pass filter. RFP was excited by a coherent I-90 water-cooled argon laser tuned to 514 nm at 350 mW, and emission was measured with a 570/40 nm band pass filter. In dual GFP-RFP experiments, 514-nm laser scatter in the GFP emission channel was filtered out by using a 540/30 band pass filter with a 514 block (Chroma Tech, Burlington, Vt.). Two thousand RFP- and GFP-positive cells were sorted in a single well of a 96-well plate with an optical bottom (BD/Falcon). Data were prepared by using the FlowJo v4.5.4 software (Tree Star Inc., Stanford, Calif.).

Microscopy. Sorted GFP- and RFP-positive GFP-STOP cells or infected GFP-STOP cells grown on a coverslip in a six-well plate were analyzed by using a DM IRB inverted microscope (Leica, Wetzlar, Germany) equipped with a 100-W HBO lamp. A fluorescein isothiocyanate filter set was used for GFP detection (excitation with band pass at 460 to 500 nm; emission with band pass at 491 to 551 nm), and a tetramethyl rhodamine isocyanate filter set was used for RFP detection (excitation with band pass at 515 to 560 nm; emission with long pass at >590 nm). Images were recorded by using a cooled charge-coupled device camera (Hamamatsu, Bridgewater, N.J.). Image processing and analysis were performed by using the Openlab 3.0.3 software (Improvision, Quincy, Mass.).

Mice and infections. C57BL/6 mice and TAP-1-deficient mice (C57BL/6 background; Jackson Labs) were bred and maintained in the University of California, Berkeley, mouse facility. Mice between 4 and 12 weeks of age were used for analyses. For in vivo infections, tachyzoites were isolated from partially lysed fibroblast layers by scraping cells, passing them several times through a 23-gauge needle, and then filtering them through a 2-μm-pore-size filter (Millipore) to remove residual fibroblasts. Tachyzoites (10^5 to 10^6 cells) were injected either intraperitoneally or subcutaneously into mice. After 3 to 6 days, mice were euthanized, and spleens and lymph nodes were removed for further analysis. For in vitro infections, splenocytes were cultured at 37°C in complete RPMI media at a concentration of 10^7 cells/ml with tachyzoites at a ratio of 1:1.

Flow cytometry and purification of cell populations. Lymph nodes or spleens were dissociated by collagenase digestion as previously described (1). Cells were filtered and then counted and analyzed by flow cytometry as described previously (4). The following antibodies were used: anti-CD4, anti-CD8, or anti-B220 directly conjugated to phycoerythrin-Cy5, anti-CD11b and anti-CD11c conjugated to biotin, and streptavidin conjugated to phycoerythrin-Texas Red (Caltag, Burlingame, Calif.; BD Pharmingen, San Jose, Calif.; and eBioscience, San Diego, Calif.). Cells were processed with a Coulter Epics XL-MCL and were analyzed by using the FlowJo software (Tree Star, Ashland, Oreg.). For cell fractionation, splenocytes from infected mice were incubated with anti-CD11c beads, and the positive and negative fractions were separated by AutoMacs magnetic bead separation by using the manufacturer's guidelines (Miltenyi Biotec, Auburn, Calif.). For the YFP^+/YFP^- sorting experiment, splenocytes were cultured at a concentration of 10^7 cells/ml with secOVA/YFP2 parasites at an MOI of 1. After 48 h, cells were sorted into YFP^+ and YFP^- fractions by using a Coulter Epics cell sorter. Dead cells and free parasites were excluded by PI gating together with a gate on forward and side scatter. To test whether ovalbumin from dead parasites could be presented, secOVA parasites were heat killed at 56°C for 50 min prior to incubation with splenocytes.

Ovalbumin presentation assays. B3Z assays were performed as previously described (42). Briefly, 10^5 B3Z cells were cocultured in flat-bottom 96-well plates with the number of antigen-presenting cells indicated below for 6 to 24 h. For most experiments pyrimethamine (1 to 10 μM) was included during the coculture to prevent parasite growth. The plates were spun, and the cell pellets were incubated with CPRG for detection of LacZ activity. Absorbance at 595 nm was measured by using an enzyme-linked immunosorbent assay plate reader. The assay was generally sufficiently sensitive to detect 10 pM $OVA_{257–264}$ peptide, based on control peptide titrations run in parallel.

RESULTS

Flow cytometric detection of cells infected by a fluorescent strain of *T. gondii*. While *T. gondii* is capable of infecting virtually any cell type in vitro, very little is known about its tropism for different cell types in vivo. To determine which cell types are the predominant in vivo targets of *Toxoplasma* infection, we used a strain expressing tandem copies of YFP (YFP2) (14). We infected mice with YFP2 tachyzoites, and 3 to 6 days after infection we removed the spleens or lymph nodes, dissociated them with collagenase, stained them with fluorescently labeled antibodies, and analyzed samples by flow cytometry (Fig. 1). In these analyses, free parasites could be readily distinguished from parasites within cells based on the difference in size (forward scatter) (Fig. 1A, lower right panel). In addition, staining for marker proteins allowed us to quantify infected cells within distinct cell populations, including dendritic cells ($CD11c^+$), macrophages ($CD11b^{high}$), B cells ($B220^+$), and T cells ($CD4^+$ or $CD8^+$). By dividing the percentage of marker-positive, YFP^+ cells by the total percentage of marker-positive cells in the sample, we could determine the proportion of each cell population that was infected.

Compiled data from nine experiments are presented in Fig. 1B. While the percentage of infected cells varied substantially between samples (0.11 to 3.1%), there was a striking and consistent trend for which cell types were preferentially infected. Specifically, the proportion of infected cells in the myeloid populations ($CD11c^+$ and $CD11b^+$) was consistently 10-fold higher than the percentage of infected cells in the lymphoid populations ($B220^+$, CD4, and $CD8^+$). We also analyzed the percentage infected cells following in vitro infection of dissociated splenocytes and lymph node cells (Fig. 1B). In agreement with previous studies (3), we found only a twofold preference for infection of myeloid cells over lymphoid cells following in vitro infection. The greater preference for infecting myeloid cells seen in in vivo infections suggests that anatomical factors may contribute to the tropism for myeloid cells seen in infected mice.

TAP-dependent presentation of *T. gondii* proteins via class I MHC. Studies of CD8 T-cell responses to *T. gondii* are hampered by the lack of well-defined class I MHC-restricted T-cell antigens. To circumvent this problem, we engineered strains expressing the model antigen chicken OVA. We used a secreted version of ovalbumin (secOVA), based on a previous study showing that there was a class I restricted T-cell response to a version of LacZ that was secreted into the parasitophorous vacuole but not to a version that was retained in the cytosol of the parasite (25). The predicted protein contains amino acids 140 to 386 of chicken ovalbumin and has a predicted molecular mass of 54 kDa. To confirm that the engineered ovalbumin is presented via class I MHC, we used a highly sensitive B3Z T-cell hybridoma assay (42). B3Z cells express a T-cell antigen receptor specific for a peptide derived from chicken ovalbumin, $OVA_{257–264}$, bound to class I MHC-K^b, and are engineered to express LacZ upon T-cell antigen receptor triggering. We infected mice with either secOVA parasites or the YFP2 strain as a negative control. Four days after infection, we isolated splenocytes, separated them into DC-enriched and DC-depleted fractions, and assayed for ovalbumin presentation using the B3Z assay (Fig. 2A). We found significant activity in spleen cells from secOVA-infected mice but not in spleen cells from control YFP2-infected mice. A comparison of CD11c-enriched and -depleted fractions indicated that DC were effective at presenting ovalbumin, although they did not account for all of the ovalbumin class I-presenting activity in vivo. These data indicate that ovalbumin expressed as an engineered protein in *T. gondii* can be presented via a class I MHC on the surface of host cells from infected mice.

Although *T. gondii* is an intracellular pathogen, its location in a specialized vacuole that sequesters it from the host cell cytosol raises the question of whether *T. gondii* antigens are presented via the endogenous class I presentation pathway. The endogenous class I presentation pathway requires TAP to transport peptides that are generated in the cytosol into the endoplasmic reticulum for loading onto class I proteins. To assess the TAP dependence of ovalbumin presentation in this system, we compared presentation from TAP-deficient mice and presentation from wild-type mice infected with ovalbumin-expressing parasites. For these experiments, we used parasites that were engineered to express both OVA and YFP (secOVA/YFP2). This allowed us to monitor the percentage of infected cells in antigen-presenting cell populations from infected mice. While ovalbumin presentation was readily detectable in antigen-presenting cells from wild-type infected mice, we were unable to detect significant activity from TAP-deficient infected mice (Fig. 2B). This was in spite of the fact that comparable percentages of cells were infected in samples from

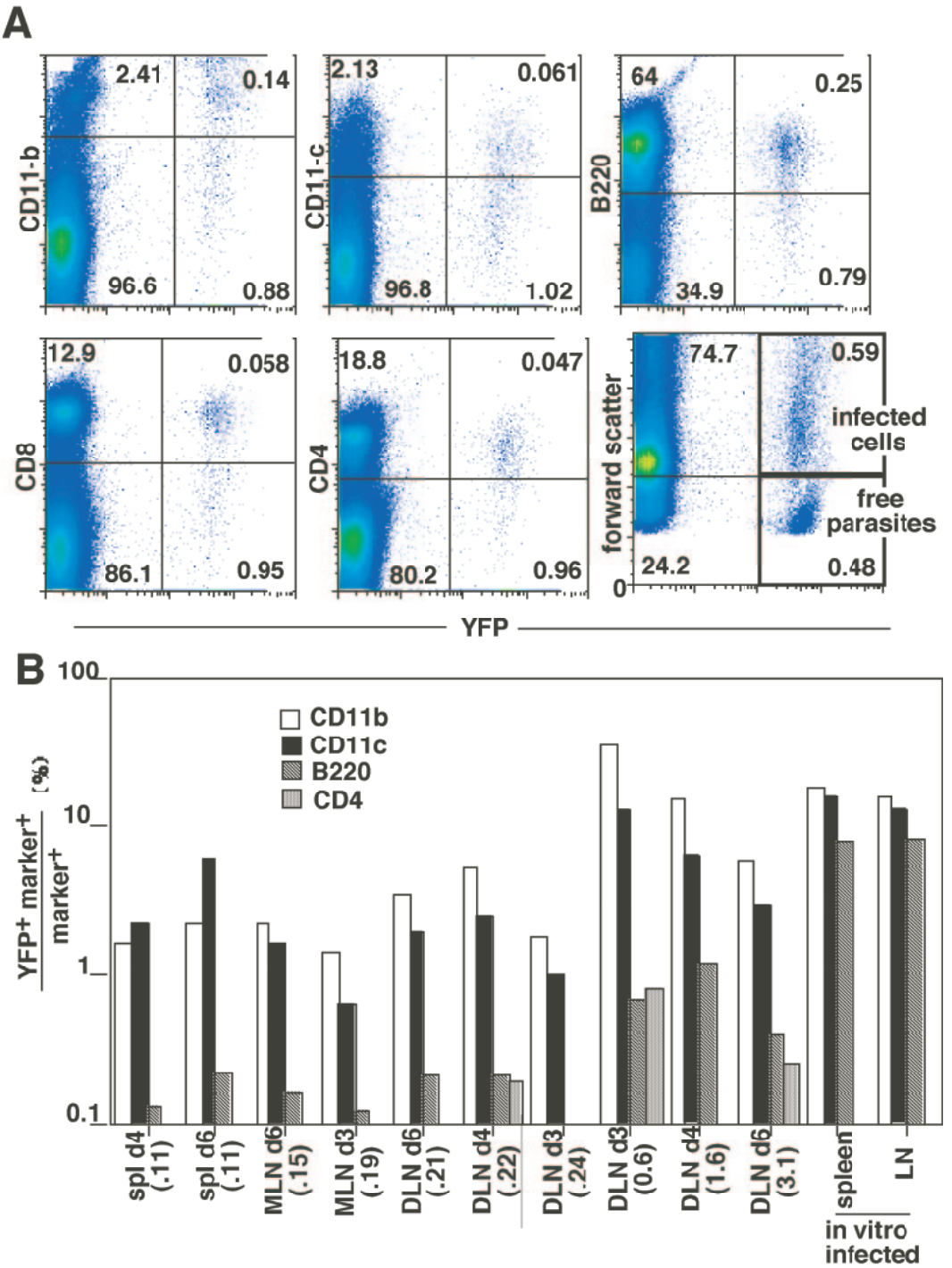

FIG. 1. Flow cytometric analysis of cell types infected by *T. gondii* following in vivo infection. Mice were infected with the RH strain of *T. gondii* expressing YFP (YFP2), and spleen and lymph nodes were analyzed by flow cytometry. (A) Representative flow cytometric analysis of draining lymph node sample on day 3 following infection. (B) Compiled data from 10 samples in nine different experiments. Each group of bars represents an individual sample and is identified by the tissue (spl, spleen; MLN, mesenteric lymph node; DLN, draining lymph node), the number of days following infection (d4, day 4; d6, day 6; d3, day 3), and the percentage of cells in sample that were YFP^+ (in parentheses). Infections were either intraperitoneal (for spleen and mesenteric lymph node samples) or subcutaneous (for DLN samples). The data are expressed as percentages of YFP^+ marker-positive cells based on the marker-positive subset. In a separate experiment, dissociated splenocytes (spleen) and lymph node cells (LN) were exposed to YFP2 parasites in vitro and analyzed by flow cytometry after 1 day.

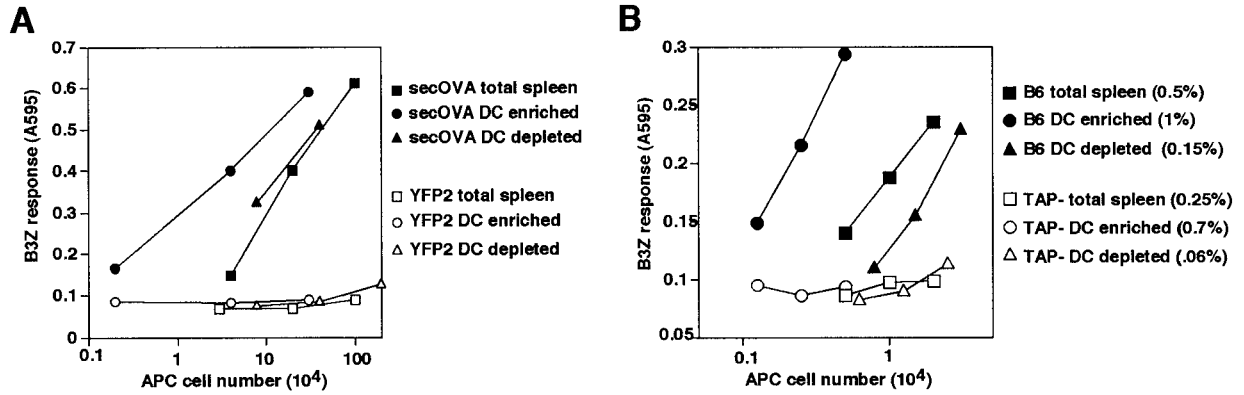

FIG. 2. TAP-dependent class I MHC restricted ovalbumin presentation by splenocytes of mice infected with secOVA parasites. Mice were injected with parasites (10^6 tachyzoites intraperitoneally) expressing a secreted version of ovalbumin (secOVA) or YFP (YFP2) (A) or with ovalbumin plus YFP (secOVA/YFP2) (B). After 4 days splenocytes were dissociated by using collagenase digestion and were fractionated by using CD11c magnetic beads to generate DC-enriched and DC-depleted fractions. Ovalbumin presentation activity was measured by using a LacZ B3Z hybridoma assay. In this assay a T-cell hybridoma that is reactive to an ovalbumin peptide ($OVA_{257–264}$) bound to class I MHC (K^b) and is engineered to produce LacZ upon stimulation (42) is used. (A) Comparison of secOVA-infected splenocytes (solid symbols) and YFP2-infected splenocytes (open symbols). For the secOVA samples, the total spleen sample was 7.4% $CD11c^+$, the DC-enriched fraction was 32% $CD11c^+$, and the DC-depleted fraction was <0.25% $CD11c^+$. For the YFP2 samples, the total spleen sample was 4.3% $CD11c^+$, the DC-enriched fraction was 45% $CD11c^+$, and the DC-depleted fraction was <0.4% $CD11c^+$. (B) TAP dependence of antigen presentation. Wild-type (C57BL/6) mice (B6) (solid symbols) and TAP-deficient mice (TAP−) (open symbols) were infected with parasites expressing both ovalbumin and YFP (secOVA/YFP2). For the C57BL/6 samples, the total spleen sample was 10% $CD11c^+$, the DC-enriched fraction was 64% $CD11c^+$, and the DC-depleted fraction was 1.0% $CD11c^+$. For the TAP-deficient samples, the total spleen sample was 12% $CD11c^+$, the DC-enriched fraction was 70% $CD11c^+$, and the DC-depleted fraction was 1.5% $CD11c^+$. The values in parentheses indicate the percentage of each population that were YFP^+ (actively infected). The data are representative data from one of three experiments. APC, antigen-presenting cells; A595, absorbance at 595 nm.

TAP-deficient and wild-type mice. The requirement for TAP suggests that presentation involves the transport of ovalbumin peptides from the cytoplasm to the endoplasmic reticulum. While these data are consistent with the possibility that presentation occurs via the endogenous class I pathway, they do not rule out cross-presentation, which in many systems also requires TAP (16–18, 33).

Class I MHC presentation can occur in actively infected cells. For certain antigens, notably tumor antigens, class I presentation can occur via an alternative pathway in which antigen produced in one cell is taken up and presented by a bystander cell, a phenomenon known as cross-presentation or cross-priming (17, 55). We used the strategy diagrammed in Fig. 3A to investigate whether the class I presentation of *T. gondii* antigens occurs within actively infected cells or from bystander cells. We cultured splenocytes in vitro with parasites expressing both ovalbumin and YFP (secOVA/YFP2) for 2 days and then sorted the infected cells into YFP^+ (actively infected) and YFP^- (bystander) fractions and assayed for ovalbumin presentation using the B3Z assay (Fig. 3B). We found that the ovalbumin presentation activity was predominantly in the YFP^+ fraction and that there was no detectable activity in the YFP^- fraction. We obtained similar results with sorted YFP^+ and YFP^- splenocytes from in vivo infected mice (Fig. 3C). We were also unable to detect ovalbumin presentation following incubation of splenocytes with heat-killed secOVA/YFP2 parasites (data not shown). Together, these results suggest that presentation by bystander cells makes little contribution to the class I presentation of *Toxoplasma*-encoded ovalbumin by infected splenocytes and that the majority of presentation comes from actively infected cells.

Interestingly, we consistently found that the ovalbumin presentation activity was higher from in vivo infected splenocytes than from in vitro infected splenocytes (Fig. 2 and 3 and data not shown). This is in spite of the fact that the percentage of infected cells was higher following in vitro infection. In addition, we found very low ovalbumin presentation activity in several class I MHC-K^b-expressing cell lines infected with secOVA/YFP2 parasites, including K^b-transfected L cells, RAW309 cells, and DC24.1 cells (data not shown). These observations suggest that different cell types may differ in the ability to present *T. gondii* antigens via class I MHC.

Cre-LoxP-mediated detection of protein escape from the parasitophorous vacuole. The TAP dependence of ovalbumin presentation, together with the observation that antigen presentation of in vitro infected splenocytes comes primarily from actively infected cells rather than bystander cells, suggests that ovalbumin produced by the parasite and secreted into the parasitophorous vacuole can find its way into the host cytosol. However, this scenario is at odds with studies showing that the parasitophorous vacuole membrane is impermeable to large molecules (>1,300 Da) (40). To further investigate this question, we devised a sensitive genetic assay to monitor the ability of *T. gondii* proteins to gain access to the host cell (Fig. 4A). In this system, host cells harbor a silent GFP gene, which can be activated upon Cre-mediated deletion of a transcriptional stop signal (20). The ability of parasite-encoded Cre to activate the GFP reporter in the host cells serves as an assay for the ability of parasite proteins to reach the host nucleus. Flow cytometric analysis showed that infection with parasites expressing a secreted version of Cre (secCre) (Fig. 4C) activated GFP expression in a small (1 to 2%) but significant proportion of host cells, while infection with wild-type parasites (Fig. 4B) failed to activate the GFP reporter. GFP activation in the host cell is

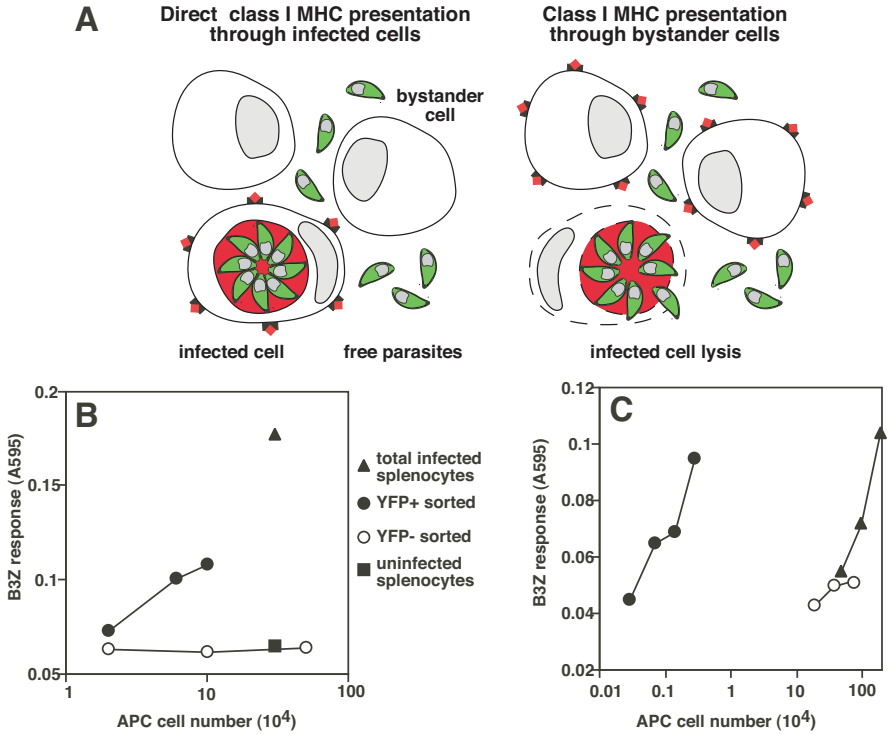

FIG. 3. Ovalbumin presentation by actively infected splenocytes. (A) Schematic diagram of experimental rationale. If the majority of presentation activity derives from actively infected cells, we would expect most activity to come from YFP^+ cells rather than bystander cells (YFP^-). If presentation occurs primarily via cross-presentation of *Toxoplasma* antigens via bystander cells, we would expect YFP^- cells to have antigen presentation activity similar to that of actively infected (YFP^+) cells. Note that these possibilities are not mutually exclusive. (B) YFP^+/YFP^- sorting of in vitro infected splenocytes. Splenocytes were infected in vitro with parasites engineered to express both ovalbumin and YFP (secOVA/YFP2). After 2 days the splenocytes were fluorescence-activated cell sorted into YFP^+ (●) and YFP^- (○) populations (excluding free parasites and dead cells). Unfractionated infected splenocytes (▲) were included for comparison, and uninfected splenocytes (■) were included as a negative control. Ovalbumin presentation in the populations indicated was read out by using the B3Z LacZ assay. The lower activity in the total spleen relative to sorted populations may have resulted from lysis of infected cells during the process of sorting. The starting population contained 3.4% infected cells (YFP^+ FS^{hi}), the sorted YFP^+ population contained 63% infected cells, and the YFP^- population contained less than 0.1% infected cells based on postsort analysis. The data are representative data from one of three independent experiments. (C) YFP^+/YFP^- sorting of in vivo infected splenocytes. Mice were infected with parasites engineered to express both ovalbumin and YFP (secOVA/YFP2). After 7 days splenocytes were fluorescence-activated cell sorted into YFP^+ (●) and YFP^- (○) populations (excluding free parasites and dead cells). The starting population contained 0.22% infected cells, the sorted YFP^+ population contained 83% infected cells, and the YFP^- population contained less than 0.01% infected cells based on postsort analysis. APC, antigen-presenting cells; A595, absorbance at 595 nm.

dependent on parasite infection, since both the infection rate and the frequency of GFP^+ cells vary linearly with the amount of inoculum (http://supplement.ctegd.uga.edu/). In addition, the activation of GFP appears to require that Cre is produced within the infected host cells, since supernatant from secCre parasite-infected cultures also failed to activate the reporter (Fig. 4E). Interestingly, parasites expressing a version of Cre that was retained in the parasite's cytoplasm (cytoCre) (Fig. 4D) also failed to activate the GFP reporter. This suggests that the parasite's plasma membrane is a more stringent barrier than the membrane of the parasitophorous vacuole.

Because infection with secCre parasites led to activation of the GFP reporter in only a few percent of the host cells, we considered the possibility that these GFP^+ host cells might have represented infected cells in which the parasitophorous vacuole membrane was no longer intact. To examine this possibility, we infected host cells with parasites expressing both secCre and a secreted version of red fluorescent protein (secRFP/secCre). As shown in Fig. 5A, infection of host cells with secRFP/secCre led to activation of GFP in 1.7% of infected (RFP^+) host cells. We did not observe any uninfected (RFP^-) cells that expressed GFP, confirming that activation of the reporter occurred only in infected cells. Examination of the GFP^+ RFP^+ infected cells by epifluorescence microscopy showed that all host cells expressing GFP had intact parasitophorous vacuoles, as indicated by the vacuolar localization of RFP (Fig. 5B to I) ($n > 100$). Together, these data indicate that the Cre protein can escape from the parasitophorous vacuole and catalyze loxP-mediated deletion in the nucleus of infected host cells.

DISCUSSION

The class I MHC presentation pathway is designed primarily to detect pathogens that reside within the cytosol of host cells. It is therefore paradoxical that the parasite *T. gondii* is able to stimulate a class I restricted T-cell response, in spite of the fact that it is sequestered in a specialized vacuole that does not exchange with other host cell compartments (19, 27) and is impermeable to large molecules (40). One possible explana-

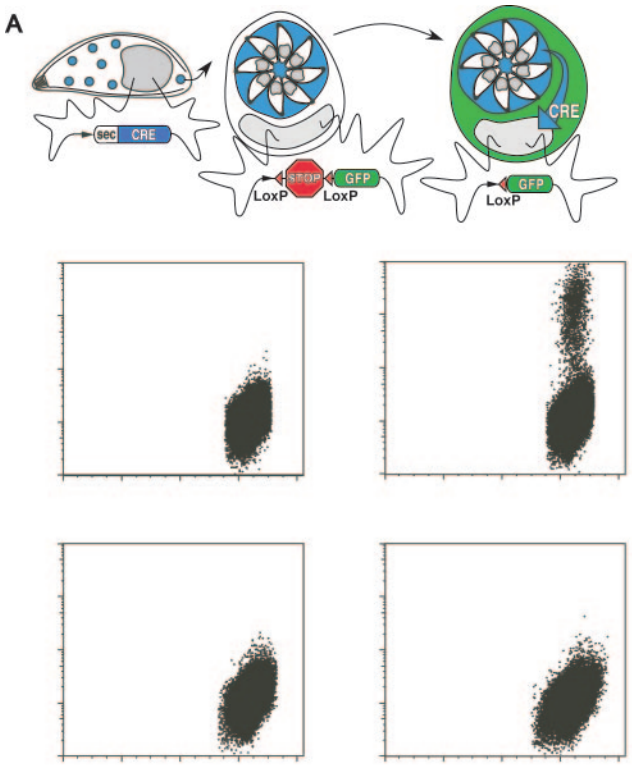

FIG. 4. Cre recombinase secreted by the parasite into the parasitophorous vacuole can escape into the cytoplasm of the host cell. (A) Schematic diagram of the Cre assay. Parasites are engineered to stably express a secreted version of Cre recombinase (secCre) (blue). These parasites are allowed to infect GFP-STOP cells. GFP-STOP cells carry a GFP gene (green), which is inactivated by a loxP site-flanked transcriptional terminator (STOP) (red). Cre escape from the parasitophorous vacuole into the host cell cytoplasm and nucleus excises the STOP sequence and results in GFP expression in the host cell. (B to E) A total of 5×10^6 cells GFP-STOP cells were infected in each experiment (MOI, 8) and analyzed by flow cytometry 24 h after infection. In all experiments GFP-STOP cells were gated on their forward and side scatter to exclude debris and free parasites from the analysis. (B) GFP-STOP cells infected with RH wild-type parasites. No significant GFP expression was detected (0.0054% of the cells were positive). (C) GFP-STOP cells infected with secCre parasites. GFP expression was detected in 0.35% of all GFP-STOP cells, corresponding to ~1% of the infected GFP-STOP cells. (D) GFP-STOP cells infected with cytoCre parasites expressing Cre in the cytoplasm. No GFP expression was detected (0.0085%). Note that we used a weaker promoter for the cytoCre construct (sagCre) as no stable transformants could be isolated with the tubCre plasmid. This was probably due to the toxic effects of Cre overexpression (45). While this could have contributed to the lack of GFP expression in this experiment, we were able to detect Cre activity from this construct by using a floxed reporter construct (data not shown). (E) GFP-STOP cells were incubated with culture supernatant of 10^9 lysed secCre parasites. No GFP expression was detected (0.0097%), indicating that secreted secCre was not taken up by a host cell from the exterior but needed to be released from the parasitophorous vacuole in an infected cell.

tion is that *T. gondii* antigens are presented not by actively infected host cells but by bystander cells via an alternative cross-presentation pathway. Another possible explanation is that parasite proteins can escape from the parasitophorous vacuole at sufficient levels to trigger a class I restricted T-cell response. Here we explored these possibilities using a secreted model antigen, ovalbumin. We found that ovalbumin presentation via class I MHC occurs primarily in actively infected cells rather than bystander cells and requires the TAP transporter protein. We also obtained evidence that the Cre protein encoded by the parasite in a form that is secreted into the parasitophorous vacuole can mediate loxP recombination in the host nucleus. Together, these data are consistent with the notion that class I presentation can occur by escape of *T. gondii* antigens from the parasitophorous vacuole into the host cell and subsequent entry into the endogenous class I presentation pathway.

Our results raise the question of when and how parasitophorous vacuolar proteins are processed into antigenic peptides. The TAP dependence of antigen presentation that we observed implies that a cytosolic peptide intermediate is involved in presentation; however, it is not clear which cellular compartment is the site of proteolysis. Previous reports indicating that the parasitophorous vacuole membrane acts as a molecular sieve that excludes molecules larger than 1,300 Da (40) are most compatible with the notion that parasite proteins are processed into peptides within the parasitophorous vacuole prior to escape into the host cell. Numerous proteases that act on *T. gondii* secretory proteins during the invasion process have been described (for a review see reference 23), and some of these proteases might be active in the lumen of the parasitophorous vacuole to generate such peptides. However, our data demonstrating that active Cre protein expressed in a form that is secreted into the parasitophorous vacuole can gain access to the host cell nucleus suggest that intact ovalbumin may also escape from the parasitophorous vacuole and be processed in the cytosol along with the bulk of class I associated antigens. However, while we favor the view that the Cre and ovalbumin gain access to the cytosol by escape from the PV, we cannot exclude the possibility that this occurs via a different route (for example, by release into the cytosol during the initial invasion of the host cell by the parasite). Furthermore, a recent study has provided evidence which suggests that there is transport of a protein from the parasitophorous vacuole to the surface of the host cell (31).

It is important that the escape of parasite proteins from the parasitophorous vacuole that we describe here does not reflect a general breakdown of the parasitophorous vacuole membrane. Indeed, our data showing that secRFP is retained in the parasitophorous vacuole in the same host cells in which the GFP reporter is active argue that only a small fraction of proteins can escape from the parasitophorous vacuole. The level of protein escape from the parasitophorous vacuole is sufficient to mediate loxP recombination and class I MHC presentation, but it is too low to be detected by fluorescence microscopy. It is also noteworthy that only a low percentage of infected cells activated the GFP reporter. This may have been a reflection of the general inefficiency of the process, due to the low level of protein escape from the parasitophorous vacuole and the need to form Cre tetramers to generate an active recombinase (11). Alternatively, the low percentage of reporter-expressing cells may indicate that protein escape from the parasitophorous vacuole occurs only in rare host cells. We cannot currently distinguish between these possibilities. We also note that the class I presentation of ovalbumin may also occur in a subset of the infected antigen-presenting cells. A

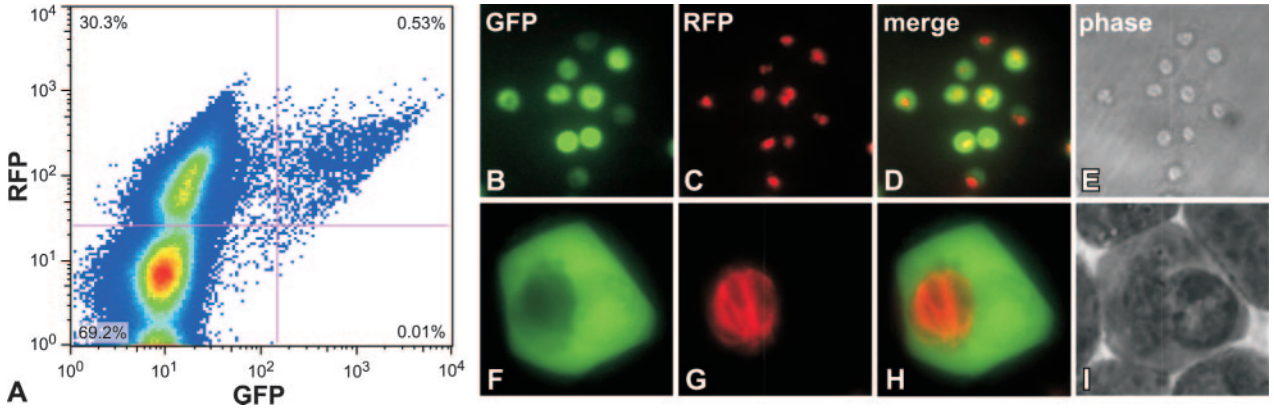

FIG. 5. GFP-expressing GFP-STOP cells are infected and harbor parasites within an intact parasitophorous vacuole. A parasite line coexpressing secreted versions of RFP and Cre (secRFP and secCre were both secreted into the vacuolar space) was constructed and used to infect GFP-STOP cells. (A) At 24 h after infection cells were subjected to sorting. All GFP-expressing cells (0.53% of all cells and 1.7% of infected cells) were also RFP positive (30.83% of all cells). (B to E) GFP-positive cells were sorted into a well of an optical bottom 96-well plate for microscopy. Fluorescence microscopy showed that all green cells (B) indeed harbored red parasitophorous vacuoles (C). For higher-resolution microscopy GFP-STOP cells infected with secCre/secRFP parasites were imaged on glass coverslips (F to I). Note that secRFP was always restricted to the lumen of the parasitophorous vacuole (G and C) ($n > 100$) and that cytoplasmic host cell GFP was excluded from this vacuole (F and H), strongly suggesting that the vacuolar membrane was intact.

sensitive assay to measure antigen presentation on individual cells is required to address this question.

Our results extend the results of a previous study in which presentation of the model antigen LacZ by *T. gondii* was examined. In that study a secreted form of LacZ, but not a cytoplasmic form, induced a CD8 T-cell response in infected mice. Our results provide an explanation for this observation. In particular, the implication that the major source of class I presentation is actively infected cells rather than bystander cells, together with evidence that secreted parasite proteins but not cytoplasmic parasite proteins can escape into the host cell, suggests that proteins in the parasite cytoplasm do not have access to the class I presentation pathway. On the other hand, if cross-presentation contributed significantly to these CD8 responses, we might expect proteins in the parasite cytoplasm to be presented as well as secreted proteins (43). However, although cross-presentation did not appear to make a substantial contribution in our studies, cross-presentation of *T. gondii* antigens in vitro has been reported previously (7). Cross-presentation may also make an important contribution to immune responses to *T. gondii* in vivo, depending on factors such as the nature and stability of the antigen (33, 52), as well as the type of immune responses studied, the parasite strain, and the type of host. Importantly, Kwok et al. (25) also found that expression of LacZ in the tachyzoite stage, but not the bradyzoite stage, induced a CD8 response. Thus, the acute infection model used here is appropriate for studies of antigen presentation during the initiation of CD8 T-cell responses to *T. gondii*.

Previous work implicated dendritic cells in the sensing of *T. gondii* during infection and in shaping the immune response through the production of cytokines. such as interleukin-12 (for reviews see references 5 and 41). Our data emphasize the importance of dendritic cells in presenting *T. gondii* antigens to T cells. In particular, we found that dendritic cells are a major target of *Toxoplasma* infection in vivo. We also found that dendritic cells accounted for much of the ovalbumin presentation activity in splenocytes of mice infected with *T. gondii* secOVA. Together, these results suggest that dendritic cells are likely to be important antigen-presenting cells for inducing CD8 T-cell responses during *Toxoplasma* infection.

T. gondii is perhaps the most successful parasite known, infecting up to 30% of humans worldwide (12). Part of the key to its success is the balance that the parasite achieves with the host immune system, which allows it to infect and establish a chronic infection without causing overt pathology in the vast majority of cases. CD8 T cells are an important part of the protective response of the host to the parasite (6, 26, 54). Perhaps the limited ability to present *T. gondii* antigens to CD8 T cells helps to ensure that the host immune response is sufficient to prevent pathology while the parasite can still establish a chronic infection and ultimately spread to other hosts.

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REFERENCES

1. **Bousso, P., and E. Robey.** 2003. Dynamics of CD8 T cell priming by dendritic cells in intact lymph nodes. Nat. Immunol. **4:**579–586.
2. **Brown, C. R., and R. McLeod.** 1990. Class I MHC genes and CD8$^+$ T cells determine cyst number in *Toxoplasma gondii* infection. J. Immunol. **145:** 3438–3441.
3. **Channon, J. Y., R. M. Seguin, and L. H. Kasper.** 2000. Differential infectivity and division of *Toxoplasma gondii* in human peripheral blood leukocytes. Infect. Immun. **68:**4822–4826.
4. **Coligan, J., A. Kruisbeek, D. Margulies, E. Shevach, and W. Strober.** 1995. Current protocols in immunology. John Wiley & Sons, Inc, New York, N.Y.
5. **Denkers, E. Y., B. A. Butcher, L. Del Rio, and S. Bennouna.** 2004. Neutrophils, dendritic cells and *Toxoplasma*. Int. J. Parasitol. **34:**411–421.
6. **Denkers, E. Y., and R. T. Gazzinelli.** 1998. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. Clin. Microbiol. Rev. **11:**569–588.

7. **Denkers, E. Y., R. T. Gazzinelli, S. Hieny, P. Caspar, and A. Sher.** 1993. Bone marrow macrophages process exogenous *Toxoplasma gondii* polypeptides for recognition by parasite-specific cytolytic T lymphocytes. J. Immunol. **150:**517–526.
8. **Garg, N., M. P. Nunes, and R. L. Tarleton.** 1997. Delivery by *Trypanosoma cruzi* of proteins into the MHC class I antigen processing and presentation pathway. J. Immunol. **158:**3293–3302.
9. **Gazzinelli, R., Y. Xu, S. Hieny, A. Cheever, and A. Sher.** 1992. Simultaneous depletion of $CD4^+$ and $CD8^+$ T lymphocytes is required to reactivate chronic infection with *Toxoplasma gondii*. J. Immunol. **149:**175–180.
10. **Gazzinelli, R. T., F. T. Hakim, S. Hieny, G. M. Shearer, and A. Sher.** 1991. Synergistic role of $CD4^+$ and $CD8^+$ T lymphocytes in IFN-gamma production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine. J. Immunol. **146:**286–292.
11. **Gopaul, D. N., F. Guo, and G. D. Van Duyne.** 1998. Structure of the Holliday junction intermediate in Cre-loxP site-specific recombination. EMBO J. **17:**4175–4187.
12. **Greig, J. R., and R. Holliman.** 1999. Neonatal screening for toxoplasma infection. Lancet **354:**1030–1031.
13. **Gu, H., Y. R. Zou, and K. Rajewsky.** 1993. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell **73:**1155–1164.
14. **Gubbels, M. J., C. Li, and B. Striepen.** 2003. High-throughput growth assay for *Toxoplasma gondii* using yellow fluorescent protein. Antimicrob. Agents Chemother **47:**309–316.
15. **Gubbels, M. J., M. Wieffer, and B. Striepen.** 2004. Fluorescent protein tagging in *Toxoplasma gondii*: identification of a novel inner membrane complex component conserved among *Apicomplexa*. Mol Biochem Parasitol. **137:**99–110.
16. **Guermonprez, P., L. Saveanu, M. Kleijmeer, J. Davoust, P. Van Endert, and S. Amigorena.** 2003. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature **425:**397–402.
17. **Heath, W. R., and F. R. Carbone.** 2001. Cross-presentation, dendritic cells, tolerance and immunity. Annu. Rev. Immunol. **19:**47–64.
18. **Houde, M., S. Bertholet, E. Gagnon, S. Brunet, G. Goyette, A. Laplante, M. F. Princiotta, P. Thibault, D. Sacks, and M. Desjardins.** 2003. Phagosomes are competent organelles for antigen cross-presentation. Nature **425:**402–406.
19. **Joiner, K. A., S. A. Fuhrman, H. M. Miettinen, L. H. Kasper, and I. Mellman.** 1990. *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. Science **249:**641–646.
20. **Joshi, S. K., K. Hashimoto, and P. A. Koni.** 2002. Induced DNA recombination by Cre recombinase protein transduction. Genesis **33:**48–54.
21. **Kasper, L. H., I. A. Khan, K. H. Ely, R. Buelow, and J. C. Boothroyd.** 1992. Antigen-specific (p30) mouse $CD8^+$ T cells are cytotoxic against *Toxoplasma gondii*-infected peritoneal macrophages. J. Immunol. **148:**1493–1498.
22. **Khan, I. A., K. H. Ely, and L. H. Kasper.** 1991. A purified parasite antigen (p30) mediates $CD8^+$ T cell immunity against fatal *Toxoplasma gondii* infection in mice. J. Immunol. **147:**3501–3506.
23. **Kim, K.** 2004. Role of proteases in host cell invasion by *Toxoplasma gondii* and other *Apicomplexa*. Acta Trop. **91:**69–81.
24. **Kumar, S., and R. L. Tarleton.** 2001. Antigen-specific Th1 but not Th2 cells provide protection from lethal *Trypanosoma cruzi* infection in mice. J. Immunol. **166:**4596–4603.
25. **Kwok, L. Y., S. Lutjen, S. Soltek, D. Soldati, D. Busch, M. Deckert, and D. Schluter.** 2003. The induction and kinetics of antigen-specific CD8 T cells are defined by the stage specificity and compartmentalization of the antigen in murine toxoplasmosis. J. Immunol. **170:**1949–1957.
26. **Lieberman, L. A., and C. A. Hunter.** 2002. The role of cytokines and their signaling pathways in the regulation of immunity to *Toxoplasma gondii*. Int. Rev. Immunol. **21:**373–403.
27. **Lingelbach, K., and K. A. Joiner.** 1998. The parasitophorous vacuole membrane surrounding *Plasmodium* and *Toxoplasma*: an unusual compartment in infected cells. J. Cell Sci. **111:**1467–1475.
28. **Luder, C. G., and F. Seeber.** 2001. *Toxoplasma gondii* and MHC-restricted antigen presentation: on degradation, transport and modulation. Int. J. Parasitol. **31:**1355–1369.
29. **Luft, B. J., and J. S. Remington.** 1992. Toxoplasmic encephalitis in AIDS. Clin. Infect. Dis. **15:**211–222.
30. **Mordue, D. G., S. Hakansson, I. Niesman, and L. D. Sibley.** 1999. *Toxoplasma gondii* resides in a vacuole that avoids fusion with host cell endocytic and exocytic vesicular trafficking pathways. Exp. Parasitol. **92:**87–99.
31. **Neudeck, A., S. Stachelhaus, N. Nischik, B. Striepen, G. Reichmann, and H. G. Fischer.** 2002. Expression variance, biochemical and immunological properties of *Toxoplasma gondii* dense granule protein GRA7. Microbes Infect. **4:**581–590.
32. **Nielsen, H. V., S. L. Lauemoller, L. Christiansen, S. Buus, A. Fomsgaard, and E. Petersen.** 1999. Complete protection against lethal *Toxoplasma gondii* infection in mice immunized with a plasmid encoding the SAG1 gene. Infect. Immun. **67:**6358–6363.
33. **Norbury, C. C., S. Basta, K. B. Donohue, D. C. Tscharke, M. F. Princiotta, P. Berglund, J. Gibbs, J. R. Bennink, and J. W. Yewdell.** 2004. $CD8^+$ T cell cross-priming via transfer of proteasome substrates. Science **304:**1318–1321.
34. **Prigione, I., P. Facchetti, L. Lecordier, D. Deslee, S. Chiesa, M. F. Cesbron-Delauw, and V. Pistoia.** 2000. T cell clones raised from chronically infected healthy humans by stimulation with *Toxoplasma gondii* excretory-secretory antigens cross-react with live tachyzoites: characterization of the fine antigenic specificity of the clones and implications for vaccine development. J. Immunol. **164:**3741–3748.
35. **Reichmann, G., H. Dlugonska, and H. G. Fischer.** 2002. Characterization of TgROP9 (p36), a novel rhoptry protein of *Toxoplasma gondii* tachyzoites identified by T cell clone. Mol. Biochem. Parasitol. **119:**43–54.
36. **Reichmann, G., H. Dlugonska, E. Hiszczynska-Sawicka, and H. Fischer.** 2001. Tachyzoite-specific isoform of *Toxoplasma gondii* lactate dehydrogenase is the target antigen of a murine CD4(+) T-cell clone. Microbes Infect. **3:**779–787.
37. **Remington, J. S., R. McLeod, P. Thulliez, and G. Desmonts.** 2001. Toxoplasmosis, p. 205–346. *In* J. S. Remington and J. Klein (ed.), Infectious diseases of the fetus and newborn infant, 5th ed. W. B. Saunders, Philadelphia, Pa.
38. **Saavedra, R., M. A. Becerril, C. Dubeaux, R. Lippens, M. J. De Vos, P. Herion, and A. Bollen.** 1996. Epitopes recognized by human T lymphocytes in the ROP2 protein antigen of *Toxoplasma gondii*. Infect. Immun. **64:**3858–3862.
39. **Sacks, D., and A. Sher.** 2002. Evasion of innate immunity by parasitic protozoa. Nat. Immunol. **3:**1041–1047.
40. **Schwab, J. C., C. J. Beckers, and K. A. Joiner.** 1994. The parasitophorous vacuole membrane surrounding intracellular *Toxoplasma gondii* functions as a molecular sieve. Proc. Natl. Acad. Sci. USA **91:**509–513.
41. **Scott, P., and C. A. Hunter.** 2002. Dendritic cells and immunity to leishmaniasis and toxoplasmosis. Curr. Opin. Immunol. **14:**466–470.
42. **Shastri, N., and F. Gonzalez.** 1993. Endogenous generation and presentation of the ovalbumin peptide/Kb complex to T cells. J. Immunol. **150:**2724–2736.
43. **Shen, H., J. F. Miller, X. Fan, D. Kolwyck, R. Ahmed, and J. T. Harty.** 1998. Compartmentalization of bacterial antigens: differential effects on priming of CD8 T cells and protective immunity. Cell **92:**535–545.
44. **Sibley, L. D.** 2004. Intracellular parasite invasion strategies. Science **304:**248–253.
45. **Silver, D. P., and D. M. Livingston.** 2001. Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. Mol. Cell **8:**233–243.
46. **Soldati, D., and J. C. Boothroyd.** 1993. Transient transfection and expression in the obligate intracellular parasite *Toxoplasma gondii*. Science **260:**349–352.
47. **Striepen, B., M. J. Crawford, M. K. Shaw, L. G. Tilney, F. Seeber, and D. S. Roos.** 2000. The plastid of *Toxoplasma gondii* is divided by association with the centrosomes. J. Cell Biol. **151:**1423–1434.
48. **Striepen, B., C. Y. He, M. Matrajt, D. Soldati, and D. S. Roos.** 1998. Expression, selection, and organellar targeting of the green fluorescent protein in *Toxoplasma gondii*. Mol. Biochem. Parasitol. **92:**325–338.
49. **Subauste, C. S., A. H. Koniaris, and J. S. Remington.** 1991. Murine $CD8^+$ cytotoxic T lymphocytes lyse *Toxoplasma gondii*-infected cells. J. Immunol. **147:**3955–3959.
50. **Suzuki, Y., M. A. Orellana, R. D. Schreiber, and J. S. Remington.** 1988. Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. Science **240:**516–518.
51. **Suzuki, Y., and J. S. Remington.** 1990. The effect of anti-IFN-gamma antibody on the protective effect of Lyt-2^+ immune T cells against toxoplasmosis in mice. J. Immunol. **144:**1954–1956.
52. **Wolkers, M. C., N. Brouwenstijn, A. H. Bakker, M. Toebes, and T. N. Schumacher.** 2004. Antigen bias in T cell cross-priming. Science **304:**1314–1317.
53. **Yano, A., F. Aosai, M. Ohta, H. Hasekura, K. Sugane, and S. Hayashi.** 1989. Antigen presentation by *Toxoplasma gondii*-infected cells to $CD4^+$ proliferative T cells and $CD8^+$ cytotoxic cells. J. Parasitol. **75:**411–416.
54. **Yap, G. S., and A. Sher.** 1999. Cell-mediated immunity to *Toxoplasma gondii*: initiation, regulation and effector function. Immunobiology **201:**240–247.
55. **Yewdell, J. W., E. Reits, and J. Neefjes.** 2003. Making sense of mass destruction: quantitating MHC class I antigen presentation. Nat. Rev. Immunol. **3:**952–961.

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