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The Induction and Kinetics of Antigen-Specific CD8 T Cells Are Defined by the Stage Specificity and Compartmentalization of the Antigen in Murine Toxoplasmosis

Lai-Yu Kwok,* Sonja Lütjen,*‡ Sabine Solteck,* Dominique Soldati,‡ Dirk Busch,§ Martina Deckert,† and Dirk Schlüter*‡*

Toxoplasma gondii forms different life stages, fast-replicating tachyzoites and slow-growing bradyzoites, in mammalian hosts. CD8 T cells are of crucial importance in toxoplasmosis, but it is unknown which parasite stage is recognized by CD8 T cells. To analyze stage-specific CD8 T cell responses, we generated various recombinant Toxoplasma gondii expressing the heterologous Ag β-galactosidase (β-gal) and studied whether 1) secreted or cytoplasmic Ags and 2) tachyzoites or bradyzoites, which persist intracerebrally, induce CD8 T cells. We monitored the frequencies and kinetics of β-gal-specific CD8 T cells in infected mice by MHC class I tetramer staining. Upon oral infection of B6C (H-2^k^b^d^) mice, only β-gal-secretting tachyzoites induced β-gal-specific CD8 T cells. However, upon secondary infection of mice that had received a primary infection with tachyzoites secreting β-gal, β-gal-secretating tachyzoites and bradyzoites transiently increased the frequency of intracerebral β-gal-specific CD8 T cells. Frequencies of splenic and cerebral β-gal-specific CD8 T cells peaked at day 23 after infection, thereafter persisting at high levels in the brain but declining in the spleen. Splenic and cerebral β-gal-specific CD8 T cells produced IFN-γ and were cytolytic upon specific restimulation. Thus, compartmentalization and stage specificity of an Ag determine the induction of CD8 T cells in toxoplasmosis. *The Journal of Immunology, 2003, 170: 1949–1957.

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Abbreviations used in this paper: gal, galactosidase; HFF, human foreskin fibroblasts; p.i., postinfection; TE, Toxoplasma encephalitis; HSGRT, hypoxanthine-xanthine-guanine-phosphoribosyl-transferase; X-Gal, 5-bromo-4-chloro-3-indolyl b-D-galactoside.

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promoter of T. gondii. Analysis of mice infected with cysts of the various recombinant T. gondii parasites demonstrated that tachyzoites secreting β-gal induced a long-lasting dominant CD8 T cell response against the L3-restricted β-gal 76-84 epitope and a short-lived subdominant CD8 T cell response against the K1-restricted β-gal 175-183 epitope, but they did not induce a CD8 T cell response against the K1-restricted β-gal 96-103 epitope. Cerebral L3-restricted, β-gal 76-84-specific, IFN-γ-producing, and cytotxic CD8 T cells persisted at high frequency in chronic TE, indicating that control of persisting T. gondii is at least partially dependent on these CD8 T cells. These findings provide novel insights into the potential role of stage-specific expression and compartmentalization of Ags in the induction of a T. gondii-specific CD8 T cell response and may have important implications for the design of T. gondii-based vaccines.

Materials and Methods
Parasite strains and cell culture
Tachyzoites of T. gondii (Prugniaud strain) were propagated in human foreskin fibroblast (HFF) monolayers grown in DMEM (PAA Laboratories, Co, Germany) containing 3.7 g/L sodium bicarbonate (Sigma-Aldrich, Deisenhofen, Germany), 10 mM HEPES (Sigma-Aldrich), 1 mM l-glutamine (Sigma-Aldrich), 10% FCS (PAA Laboratories), and 10 µg/mL gentamicin (Sigma-Aldrich). The hypoxanthine-xanthine-guanine-phosphorosyl-transferase (HXGPRT) gene-deprived T. gondii PruHX was generated by double homologous recombination using the plasmid pRH containing HXGPRT and 6-thioxanthine selection (14, 15).

Parasite transfection and drug selection using HXGPRT as a marker of BAG1, a bradyzoite-specific Ag, (24) (kindly provided by Dr. W. Bohne, Universität Göttingen, Göttingen, Germany) followed by FITC- or Alexa Fluor 594 reactive dye-conjugated goat anti-mouse or goat anti-rabbit Ab, respectively (Molecular Probes, Leiden, The Netherlands).

Construction of expression vectors
To express cytoplasmic β-gal under the control of the sag1 promoter, the vector pSLACZ-HX was constructed by amplifying E. coli lacZ gene with PCR primers 5′-GCG ATG CAT ATG GAG AAG TTC CTA TTC-3′ (sense) and 5′-TGA TTA ATT AAG GAG ATC TTT TTT GAC ACC AGA CCA ACC GTT A-3′ (antisense) using the plasmid pSAG1-βGal (16) as template DNA. PCR was conducted using AmpliTaq DNA polymerase (PerkinElmer, Dreieich, Germany). The resulting PCR product was introduced with NsiI and PacI restriction enzyme sites at its 5′ and 3′ ends, respectively, and was subsequently cloned into the NsiI and PacI-digested plasmid, pHXGPRTloxSlacZ (17).

To express secretory β-gal under the control of the sag4 promoter, the vector pS4spLACZ-HX was constructed by amplifying the lacZ gene fused to the signal peptide of sag4 from the plasmid pSAG-βgal with the PCR primers 5′-AAA CTG CAG TTT TCG GCA GTC GGA CCCG-3′ (sense) and 5′-TGA TTA ATT AAG GAG ATC TTT TTT GAC ACC AGA CCA ACC GTT A-3′ (antisense). The resulting PCR fragment was introduced with a PacI and a PacI site at the 5′ and 3′ ends, respectively, and was cloned into the NsiI and PacI-digested plasmid, psag4GFPsag4-HX (D. Soldati, unpublished data). All restriction enzymes were supplied by New England Biolabs (Frankfurt, Germany).

DNA sequences of all lacZ-containing plasmids used in this study were determined (MWG Biotech, Ebersberg, Germany) within the regions of the three known CDB T cell epitopes (18–20).

Parasite transfection and drug selection using HXGPRT as a selectable marker gene
Parasites PruHX were harvested from freshly lysed cultures of T. gondii-infected HFF cells and transfected by electroporation as described previously (21). T. gondii mutants PruS1LACZ and PruS4spLACZ were generated by transfecting the parental PruHX with the expression plasmids pS1LACZ-HX and pS4spLACZ-HX, respectively. The T. gondii mutant, PruS1SPLACZ, was obtained by cotransfected the parental PruHX with the plasmid pSAG1-βGal, which contains the lacZ gene fused to the signal peptide of sag1, and pminiHXGPRT. For each transfection, 50–100 µg of BamHI-linearized plasmid DNA were used. For cotransfection, parasites were transfected with the lacZ-containing plasmid together with the selection plasmid pminiHXGPRT (14) at a 1:10 ratio. Both plasmids were linearized with BamHI before the transfection. Cotransfection was performed using restriction enzyme-mediated insertion in the presence of BamHI (22). From 24 to 48 h after electroporation, parasites were cultivated in the presence of 25 µg/mL mycophenolic acid and 40 µg/mL xanthine and cloned 7–10 days later by limiting dilution in 96-well microtiter plates containing HFF cells.

Stable transformants were analyzed for the presence of the recombinant protein at the respective cellular compartment of the parasites by indirect immunofluorescence assay. In addition, parasites were subjected to 5-bromo-4-chloro-3-indolyl b-D-galactoside (X-Gal) staining for the detection of β-gal activity (16).

Direct immunofluorescence assay
All manipulations were conducted at room temperature as described previously (23). In brief, after fixation with 4% paraformaldehyde/0.05% glutaraldehyde in PBS, neutralization with 0.1 M glycine in PBS, permeabilization with 0.2% Triton X-100 in PBS, and blocking with 2% BSA/0.2% Triton X-100 in PBS, glass coverslips attached with the tachyzoite-infected HFF cells were incubated with rabbit polyclonal Abs directed against E. coli β-Gal (BioTrend, Cologne, Germany) and/or a mouse mAb against T. gondii BAG1, a bradyzoite-specific Ag, (24) (kindly provided by Dr. W. Bohne, Universität Göttingen, Göttingen, Germany), followed by FITC- or Alexa Fluor 594 reactive dye-conjugated goat anti-mouse or goat anti-rabbit Ab, respectively (Molecular Probes, Leiden, The Netherlands).

X-Gal staining
Tachyzoite-infected HFF cells with/without in vitro alkaline medium treatment and cyst-containing brain homogenates from chronically infected NMRI mice were fixed and subjected to X-Gal staining (16). In brief, brain tissue was isolated, minced through a cell strainer, and centrifuged. The pellet was resuspended in the fixative solution (2% formaldehyde/0.02% glutaraldehyde) and washed once with PBS before being resuspended in a substrate solution containing X-Gal (Sigma-Aldrich). After 2 h, the cells fixed in vitro were checked for blue color substrate formation. Brain homogenates were examined microscopically for blue-stained cysts after 12 h.

Screening for PruS4spLACZ T. gondii expressing β-gal after alkaline treatment
HFF cells attached on glass coverslips were infected with clones stably transfected with the expression vector pS4spLACZ-HX. After 6 h of incubation, T. gondii-infected cells were subjected to a pH shift by alkaline treatment as described before (25) for 3–5 days before testing for enzyme activity by X-Gal staining and the expression of both β-gal protein and bradyzoite-specific marker BAG1 by indirect immunofluorescence assay. Stable clones expressing β-gal selectively after alkaline treatment were selected for further in vivo analysis. The expression of β-gal in brain tissue cysts was confirmed by β-gal staining of cysts isolated from NMRI mice chronically infected with the selected clones.

Generation of MHC-tetramer reagents
MHC/peptide tetrameric complexes, L3′-β-gal 76-84, K1′-β-gal 175-183, and K1β-gal 175-183 were generated as described before (26). Briefly, recombinant K1′ and L3′ H chain and β2-microglobulin were expressed as insoluble inclusion bodies in E. coli and were further purified. The purified L3′ H chain was refolded in vitro in the presence of one of the peptides, DAPIYTVN (β-gal 175-183), and ICPMYARV (β-gal 175-183) to form stable and soluble MHC/peptide complexes that are specifically biotinylated in vitro by adding the enzyme BirA (AviDye, Denver, CO), d-biotin, and ATP. Complexes were purified by gel filtration over a Superdex 200 HR column (Amersham Pharmacia Biotech, Freiburg, Germany). Purified biotinylated MHC/peptide complexes were multimerized with streptavidin-PE (Molecular Probes). Tetrameric complexes were buffer exchanged with PBS containing 0.02% sodium azide, 1 µg/mL pepstatin, 1 µg/mL leupeptin, and 0.5 mM EDTA, and adjusted to a concentration of 2 mg/mL by ultrafiltration. Peptides were supplied by Jerini (Berlin, Germany).

Mice and infection
B6C (C57BL/6 x BALB/c) x NIMRI (outbred) mice (Janvier, Le Genest St Isle, France) were kept under specific pathogen-free conditions throughout the experiments. A total of 104 tachyzoites were injected i.p. into NMRI mice. Cysts were isolated from the brain of these animals 3–6 mo thereafter. Brain tissue was homogenized in HBSS (Life Technologies, Rockville, MD) before cyst counting. For all experiments, mice were infected orally with gavage.

Assessment of virulence of various T. gondii strains
The virulence of the various T. gondii transformants was analyzed by infecting B6C mice orally with cysts of the various clones (five cysts/mouse)
and assessing both the survival of mice and the number of intracerebral cysts at day 30 postinfection (p.i.). For cyst counting, brain tissue was isolated from five mice per group and homogenized in a final volume of 2 ml of HBSS/brain. A total of 300 µl of each brain homogenate was examined microscopically for the presence of cysts.

Isolation of splenocytes and cerebral leukocytes

At the indicated days p.i., animals were anesthetized and intracardially perfused with 0.9% NaCl to remove contaminating intravascular leukocytes from the brain. Splenic leukocytes were isolated by passing spleens through a cell strainer (BD Biosciences, Heidelberg, Germany), and erythrocytes were lysed with ammonium chloride. Cerebral leukocytes were isolated from the brains as described previously (27). In brief, brain tissue was minced through a cell strainer, and leukocytes were separated by Percoll gradient centrifugation (Amersham Pharmacia Biotech).

Flow cytometry

The kinetics of β-gal-specific CD8 T cells were determined by containing isolated splenic and cerebral leukocytes with CD8-FITC and one of the generated tetramer reagents, L^β/β-β-gal^876-884-, K^β/β-β-gal^876-901-, and K^β/β-β-gal^876-904-PE, respectively. To analyze the activation state of β-gal-specific CD8 T cells, splenic and cerebral T cells were costained with a rat anti-CD8α fluorochrome (clone 53-6.7), L^β/β-β-gal^876-904-PE, and either rat anti-mouse CD62L-FL-FITC (clone MEL-14) or rat anti-mouse CD44-FL-FITC (clone IM7). All Abs were obtained from BD Biosciences. Flow cytometric analysis was performed using a FACScan (BD Biosciences). Data were analyzed with the CellQuest 3.3 software (BD Biosciences).

ELISPOT assay

The frequency of IFN-γ-producing splenic and cerebral β-gal-specific CD8 T cells was determined by ELISPOT assay. Splenic and cerebral leukocytes at concentrations of 2 × 10^5, 2 × 10^6, and 2 × 10^3 cells/well, respectively, were placed in an ELISPOT plate coated with rat anti-mouse IFN-γ mAb (Biosource International, Camarillo, CA). Cerebral leukocytes were cocultured overnight with syngeneic B6C spleen cells from noninfected mice (4 × 10^5 cells/well) preloaded with 10^-7 M final concentration of the L^β/β-β-gal^876-884 peptide. ELISPOT plates were developed with biotin-labeled rat anti-mouse IFN-γ (BD Biosciences), peroxidase-conjugated streptavidin (Dianova, Hamburg, Germany), and amino-ethylcarbazole dye solution (Sigma-Aldrich). Spots were counted microscopically. The number of β-gal^876-884-specific T cells was expressed as the number of spots formed in each well per 10^6 leukocytes.

Measurement of CTL activity

At day 23 p.i. with the strains PruHX^−, PruS1SLACZ, and PruS4SLACZ, leukocytes were isolated from the brains and the spleens. P815 (H-2d) were used as target cells and were coated with 10^-7 M β-gal^876-884 peptide in MEM-α supplemented with 10% FCS at 37°C, 5% CO_2. During the last hour of peptide incubation, P815 cells were labeled with ^51Cr (100 µCi/1 × 10^5 cells) (Amersham Pharmacia Biotech). Thereafter, target cells were washed three times with MEM-α supplemented with 10% FCS to remove unbound peptide and extracellular ^51Cr. Isolated leukocytes and target cells were incubated at E:T ratios of 200:1, 100:1, 30:1, 10:1, 3:1, 1:1, and 0.3:1 in triplicate. After incubation at 37°C with 5% CO_2 for 5 h, 100 µl of the supernatant from each well were collected, and the released ^51Cr was counted in a gamma counter (Beckman Coulter, Munich, Germany). The specific release was calculated according to the following formula: 100 × ([test release − spontaneous release]/(maximal release − spontaneous release]) where test release was in the presence of effector cells, spontaneous release was in the presence of medium alone, and maximal release was in the presence of detergent.

Results

Generation and characterization of transgenic Pruniaud T. gondii

Expression vectors pS1LACZ-HX and pS4SLACZ-HX were generated from the plasmid pSAG1-βGal as illustrated in Fig. 1A. Three transgenic clones of T. gondii, namely PruS1LACZ, PruS1SLACZ, and PruS4SLACZ, were generated by transfecting the parental PruHX^− T. gondii with the respective expression vectors. PruS1LACZ expressed β-gal in the tachyzoite cytoplasm under the control of the tachyzoite-specific sag1 promoter (Fig. 1B); tachyzoites of PruS1SLACZ clone secreted β-gal under the control of the tachyzoite-specific sag1 promoter (Fig. 1B); and bradyzoites of the PruS4SLACZ secreted β-gal under the control of the bradyzoite-specific sag4 promoter both in vivo and in vitro (Fig. 1, B and C). The predicted expression of β-gal in the various parasitic clones was controlled by 1) location of β-gal expression by an indirect immunofluorescence assay in vitro (Fig. 1, B and C) and 2) the stage-specific expression of β-gal in vitro in tachyzoites and bradyzoites as well as in tissue cysts by β-gal staining (Fig. 1, B and C).

In accordance with Suzuki et al. (9) oral infection of B6C mice with T. gondii cysts induced a chronic persisting nonlethal cerebral toxoplasmosis, which was characterized by the presence of T. gondii cysts associated with inflammatory leukocytes including CD8 T cells. After infection with PruHX^−, PruS1LACZ, and PruS4SLACZ, the number of inflammatory leukocytes as well as CD8 T cells in the T. gondii-infected brain increased from day 15 to 23 p.i. and declined thereafter with development of chronic latent TE (data not shown). At each stage of infection, 40–50% of all inflammatory cerebral leukocytes were CD8 T cells. Furthermore, upon infection with the various T. gondii clones all animals developed a nonlethal chronic TE with identical numbers of intracerebral CD8 T cells and cysts (data not shown). Thus, the transfection procedure did not alter the virulence of the parasites and the course of TE.

Impact of the cellular compartment of the expressed Ag for the induction of a CD8 T cell response

To analyze the impact of the compartment in which the respective T. gondii Ags are expressed, i.e., the parasite cytoplasm or the parasitophorous vacuole, on the CD8 T cell response, mice were infected orally with the PruHX^− control PruS1SLACZ (vacuolar/secretory β-gal expression) and PruS4SLACZ (cytoplasmic β-gal expression) clones, respectively. Infection with the PruS1SLACZ clone induced a strong β-gal^876-884-specific CD8 T cell response (Fig. 2). At day 9 p.i., 0.5% of all CD8 T cells were specific for β-gal^876-884 peptide. At day 21 p.i., the highest frequency of β-gal^876-884-specific CD8 T cells (2.6%) was observed with a decline to 0.9% of the total number of CD8 T cells until day 49 p.i. Infection with PruS1LACZ or PruHX^− T. gondii did not induce a β-gal^876-884-specific CD8 T cell response. These results suggest that the secretion of a T. gondii protein into the parasitophorous vacuole is a prerequisite for the induction of an Ag-specific CD8 T cell response.

Kinetics and hierarchy of Ag-specific CD8 T cells after primary infection with PruS1SLACZ T. gondii

In additional experiments, the kinetics and hierarchy of β-gal-specific CD8 T cells specific for all three identified MHC class I restricted peptides, namely the L^β/β-β-gal^876-884, the K^β/β-β-gal^996-1013, and the K^β/β-β-gal^97-504, were monitored by MHC class I tetramer staining. At day 7 p.i. with PruS1SLACZ T. gondii, CD8 T cells specific for the L^β/β-β-gal^876-884 peptide were detectable in the spleen (Fig. 3). Thereafter, their frequency increased sharply with a peak of 4% of the total CD8 T cell population up to day 23 p.i. The frequency of β-gal^876-884-specific CD8 T cells declined rapidly from day 25 to 28 p.i. and thereafter returned gradually to baseline levels. However, low numbers of specific CD8 T cells corresponding to 0.3% of all CD8 T cells persisted until day 180 p.i. On the contrary, CD8 T cells specific for the K^β/β-β-gal^996-1013 peptide remained undetectable throughout the entire course of infection, and only a small peak of 0.5% CD8 T cells specific for the K^β/β-β-gal^97-504 was detected at day 14 p.i. (data not shown). Thereafter, the frequency of CD8 T...
cells specific for the β-gal<sub>497-504</sub> epitope declined to background levels. To confirm the frequencies of CD8 T cells specific for β-gal, an IFN-γ ELISPOT assay was performed at days 14 and 25 p.i. These experiments confirmed the data obtained by tetramer staining. Splenic leukocytes produced IFN-γ upon restimulation with β-gal<sub>876-884</sub> peptide at days 14 and 25 p.i. Some splenic leukocytes produced IFN-γ after restimulation with β-gal<sub>497-504</sub> peptide at day 14 p.i., and no IFN-γ production was observed upon restimulation with β-gal<sub>96-103</sub> peptide (data not shown).

Thus, oral infection with T. gondii-secreting β-gal under the control of the tachyzoite-specific SAG1 promoter induced a dominant L<sup>d</sup>-restricted β-gal<sub>876-884</sub>-specific CD8 T cell response.

**FIGURE 1.** Generation of lacZ expressing T. gondii parasites. A, Transgenic parasites were generated by transfecting PruHX<sup>T. gondii</sup> with corresponding lacZ-containing expression vectors. To obtain a T. gondii clone secreting β-gal under control of the sag1 promoter, the T. gondii strain PruHX<sup>T. gondii</sup> was cotransfected with the plasmids pSAG-βGal and pminiHXGPRT. Primers 1 and 3 were used to generate the pS4spLACZ-HX expression vector, and primers 2 and 3 were used to obtain the PruSILACZ expression vector from pSAG-βGal. Amino acid positions of β-gal CD8 T cell epitopes are indicated. B, The subcellular localization of β-gal expression in tachyzoites grown in HFF cells was characterized by indirect immunofluorescence assay with FITC-labeled Abs. PruSILACZ tachyzoites expressed β-gal only in the cytoplasm of the parasite but not in the parasitophorous vacuole. The PruSISPLACZ mutant secreted β-gal in the parasitophorous vacuole, which stained completely positive. The phase contrast micrographs show the same parasites as the indirect immunofluorescence staining. The β-gal expression of tachyzoites grown in HFF cells and brain tissue cysts isolated from infected NMRI mice was also characterized by X-Gal staining. PruHX- parasites were consistently negative; PruSILACZ and PruSISPLACZ mutants were only positive as tachyzoites; and the PruS4SPACZ mutant was only positive as bradyzoites after X-Gal staining. C, Three days after differentiation of tachyzoites into bradyzoites by alkaline medium treatment, the mutant PruS4SPACZ was characterized by X-Gal staining and indirect immunofluorescence assay for expression of β-gal (Alexa Fluor 594 reactive dye labeled) and coexpression of bradyzoite-specific marker BAG1 (FITC-labeled). In the immunofluorescence assay, the parasitophorous vacuole is completely positive for β-gal, and all parasites express BAG1.
reaching a maximum at day 23 p.i. and a subdominant K\textsuperscript{b}-restricted H9252-gal\textsubscript{497–504}-specific CD8 T cell response, which peaked as early as day 14 p.i.

**Impact of stage-specific Ag expression for the induction of a CD8 T response**

To analyze whether a parasite-specific CD8 T cell response is exclusively directed against a tachyzoite-specific Ag or whether a bradyzoite-specific Ag can also be a target for recognition by CD8 T cells, mice were infected orally with *T. gondii* clones secreting H9252-gal either as tachyzoites (PruS1SPLACZ) or bradyzoites (PruS4SPLACZ) as well as the parental PruHX\textsuperscript{−} control strain.

In noninfected mice, H9252-gal\textsubscript{876–884}-specific CD8 T cells were undetectable in the spleen and in the brain (data not shown). At day 15 p.i. with PruS1SPLACZ, when a significant number of leukocytes including CD8 T cells had already been recruited to the brain (Ref. 7 and present study, data not shown), 2.0 and 4.2% of total CD8 T cells in the spleen and the brain stained positively with L\textsuperscript{d}/H9252-gal\textsubscript{876–884} tetramer, respectively (Fig. 4, A and B). In both the spleen and the brain, the percentage of tetramer H9252-gal\textsubscript{876–884}-positive CD8 T cells reached a peak at day 23 p.i. when 2.1 and 5.2% of the total CD8 T cells were tetramer positive, respectively (Fig. 4). The peak of intracerebral tetramer-positive CD8 T cells...
coincided with the peak of CD8 T cell infiltration into the brain (D. Schlüter, unpublished results). Thereafter, the percentage of β-gal-876–884–specific CD8 T cells declined in the spleen from 1.3 (day 38 p.i.) to 0.7% of all CD8 T cells at day 60 p.i. (Fig. 4A). In the brain, the frequency of β-gal-876–884–specific CD8 T cells declined slightly to 3.5% of all CD8 T cells at day 38 p.i. and remained at this frequency (3.7%) until day 60 p.i. (Fig. 4B). The majority of the splenic and cerebral L^2/β-gal-876–884 tetramer-positive CD8 T cells expressed high levels of CD44 and no CD62L, indicating that they were activated (data not shown).

However, in both the spleen and the brain of mice infected with PruHX^− and PruS4SPLACZ T. gondii, β-gal-876–884–specific CD8 T cells were not detectable throughout the course of infection (Fig. 4, A and B). Thus, only tachyzoites, but not bradyzoites secreting β-gal, induced a primary CD8 T cell response, which declined in the spleen during the infection but remained at a high and stable level in the brain of chronically infected mice.

β-gal-secreting bradyzoites increase the number of β-gal-876–884–specific CD8 T cells in mice primed with tachyzoites secreting β-gal

To analyze whether bradyzoites induce an expansion of Ag-specific CD8 T cells, which had already been primed by a primary infection with tachyzoites, mice were orally infected with cysts of the clone PruS1SPLACZ to develop a primary β-gal-876–884–specific CD8 T cell response. Thereafter, mice were orally reinfected with either the PruS4SPLACZ, the PruS1SPLACZ, or the PruHX^−.

At day 41 after primary infection, i.e., the day 0 of reinfection, 3.2% of all cerebral CD8 T cells were specific for β-gal-876–884 (Fig. 5A). At day 7 after secondary infection, the frequency of β-gal-876–884–specific CD8 T cells had increased to 5.1% of all CD8 T cells in mice infected with the PruS1SPLACZ T. gondii and to 6.8% of all CD8 T cells in mice infected with the PruS4SPLACZ T. gondii, respectively (Fig. 5B). In contrast, without reinfection and upon reinfection with the PruHX^− strain, the frequency of cerebral β-gal-876–884–specific CD8 T cells decreased to 2.8 and 2.3%, respectively, at day 7 after secondary infection (Fig. 5B). The increase of β-gal-specific cerebral CD8 T cells at day 7 after secondary infection was a transient phenomenon, because at day 35 after secondary infection with PruS1SPLACZ or PruS4SPLACZ T. gondii, the frequency of β-gal-876–884–specific CD8 T cells declined to 2.7 and 3.1%, respectively. These frequencies corresponded to control animals without secondary infection (2.9% of all CD8 T cells) or to mice that were reinfected with the PruHX^− strain (1.9% of all CD8 T cells) (Fig. 5B). In contrast to the brain, a reinfection with PruS4SPLACZ T. gondii did not result in an increase of the frequency of β-gal-876–884–specific CD8 T cells in the spleen (data not shown).

These results illustrate that bradyzoites secreting β-gal stimulate the expansion of the β-gal-specific CD8 T cell population in the brain upon secondary infection only after priming with tachyzoites secreting β-gal.

Functional capacity of β-gal-867–884–specific CD8 T cells induced by infection with tachyzoites secreting β-gal

To analyze the functional capacity of both splenic and cerebral β-gal-867–884–specific CD8 T cells, their IFN-γ production and cytotoxic activity were determined. At day 25 after infection with the clone PruS1SPLACZ, 1.1% of the splenic and 3.0% of the cerebral leukocytes were L^2/β-gal-867–884 tetramer-positive CD8 T cells (data not shown), and 64/10^4 (0.64%) of all splenic and 267/10^4 (2.67%) of all cerebral leukocytes produced IFN-γ upon restimulation with the β-gal-867–884 peptide (Fig. 6A). Both splenic and cerebral leukocytes isolated from mice infected with PruS1SPLACZ T. gondii were able to lyse target cells loaded with β-gal-867–884 peptide (Fig. 6, B and C), and the cytotoxic activity paralleled the percentage of tetramer L^2/β-gal-867–884-positive cells in both the spleen and the brain (Fig. 6, B and C). In control mice infected with PruHX^− or PruS4SPLACZ T. gondii, β-gal-867–884–specific CD8 T cells were not detectable by tetramer staining. In addition, splenic and cerebral CD8 T cells isolated from these mice did not produce IFN-γ upon restimulation with the β-gal-867–884 peptide and did not lyse β-gal-867–884–loaded target cells (Fig. 6, B and C).

These findings indicate that tachyzoites secreting β-gal induce IFN-γ-secreting, cytotoxic β-gal-876–884–specific CD8 T cells in the brain of mice primarily infected with tachyzoites secreting β-gal. B6C mice were orally infected with cysts of the PruS1SPLACZ strain, which secretes β-gal tachyzoite specifically. At day 41 p.i., three mice were sacrificed, and cerebral leukocytes were isolated and stained for CD8 and L^2–specific TCR with PE-conjugated L^2 tetramer (A). On the same day, i.e., day 41 p.i., groups of mice were either orally reinfected 1) with cysts of the PruS1SPLACZ strain, which secretes β-gal tachyzoite specifically; 2) with cysts of the PruS4SPLACZ strain, which secretes β-gal bradyzoite-secretively; 3) with cysts of the parental PruHX^− strain; or 4) were left not reinfected. On days 48 and 76 after primary infection, i.e., on days 7 and 35, respectively, following secondary infection, cerebral leukocytes were isolated and stained for CD8 and L^2–specific TCR with PE-conjugated L^2 tetramer (B). The dot plots (A and B) represent CD8^+–gated T cells stained with rat anti-mouse CD8 FITC (x-axis) and β-gal-876–884 PE-conjugated tetramer (y-axis).
both the spleen and the brain of infected animals. The frequency of β-gal<sub>867-884</sub>-tetramer-positive cells was directly correlated to the number of β-gal<sub>867-884</sub>-specific IFN-γ-producing cells as well as the level of β-gal<sub>867-884</sub>-specific cytotoxicity.

Discussion

In the present study, we analyzed the impact of the stage-specific expression of a parasitic Ag on the induction of a CD8 T cell response. Because the location of a pathogen-derived Ag also determines the induction of a pathogen-specific CD8 T cell response, we first identified whether secreted or cytoplasmic Ags of <i>T. gondii</i> induce a CD8 T cell response. To address this question, we have expressed β-gal, a model Ag that contains one L<sup>a</sup> and two K<sup>b</sup> epitopes (18–20), under specific conditions in a low-virulent cyst-forming type II strain of <i>T. gondii</i>. The combined use of β-gal as a model Ag and the infection of B6C (H<sup>2b</sup>) mice with β-gal<sup>eu</sup>,<sup>eu</sup> was shown at different E:T ratios. A control is included, which shows the percentage of specific lysis of Pr15 (H<sup>2d</sup>) cells pulsed with β-gal<sub>876-884</sub> peptide are shown. The data represent the mean ± SD from three mice per group. B and C, CTL activity of splenic (B) and cerebral (C) leukocytes. The percentage of specific lysis of Pr15 (H<sup>2d</sup>) cells pulsed with β-gal<sup>eu</sup>,<sup>eu</sup> is shown at different E:T ratios. A control is included, which shows the percentage of specific lysis of unloaded P815 cells in the presence of isolated leukocytes from mice infected with the PruS1PLACZ strain at the highest E:T ratio.

The experiments illustrated in this figure were repeated in a separate experiment, and similar data were obtained.

β-gal, induced a CD8 T cell response. These data clearly illustrate that the stage-specific expression of an Ag is a major factor determining the induction of a CD8 T cell response in toxoplasmosis. Thus, intestinal and intracerebral bradyzoite-containing cysts were insufficient to induce an Ag-specific CD8 T cell response. Several mutually nonexclusive factors, including the kinetics of bradyzoite/tachyzoite conversion, the organ distribution of these parasitic stages, the amount of β-gal produced by either bradyzoites or tachyzoites, as well as the transport or efflux of secreted Ags across the cyst wall or parasitophorous vacuolar membrane may account for this result. After oral infection, bradyzoites switch to tachyzoites within 12–18 h (1), and this rapid stage conversion may result in the production of too low amounts of Ag, which is insufficient for the induction of a bradyzoite-specific CD8 T cell response. Thereafter, predominantly tachyzoites, but not bradyzoites, multiply in the gastrointestinal tract, disseminate in the host, and infect multiple parenchymatous and lymphatic organs (1, 2). The conversion of some tachyzoites into bradyzoites, which eventually form intracellular tissue cysts in parenchymatous but not in lymphatic organs, is not initiated before day 6 p.i. Moreover, the cyst wall may prevent the efflux or transport of secreted Ag into the host cell cytoplasm where the MHC class I processing pathway is located, whereas the parasitophorous membrane allows the efflux of small molecules between 1.3 and 1.9 kDa from the vacuolar space into the host cell cytoplasm (28, 29).

However, upon oral infection bradyzoites secreting β-gal increased the frequency of β-gal-specific CD8 T cells in animals that were already infected with a <i>T. gondii</i> strain secreting β-gal tachyzoite specifically. This finding illustrates that the conditions for the induction and restimulation of <i>T. gondii</i>-specific CD8 T cells differ with respect to the stage specificity of the Ag. Because the infection with β-gal-secreting tachyzoites induced β-gal-specific CD8 T cells persisting in a lymphatic organ, i.e., the spleen, as well as in a parenchymatous organ, i.e., the brain, β-gal produced by bradyzoites after reinfec­tion may be rapidly recognized...
by these CD8 T cells and foster an expansion of this cell population.

Both in brain and spleen, β-gal-specific CD8 T cells peaked at day 23 p.i. Compared with many bacterial and viral infections, this peak in the spleen is rather late (26, 30) and occurs at a time point when the splenic parasitic load, which peaks around day 10 p.i., has already declined (1). This unusual kinetics may be explained by the fact that proliferation of splenic T cells is actively inhibited by macrophage-derived nitric oxide and IL-10 between days 7 and 14 p.i. (31, 32). Beyond day 23 p.i., the frequency of β-gal-specific CD8 T cells dropped rapidly, which may be caused by a fast contraction of the Ag-specific CD8 T cell population in the spleen as well as a recruitment of β-gal-specific CD8 T cells to the brain (7).

In fact, the formation of intracerebral T cell infiltrates in TE strictly depends on the recruitment of peripheral T cells to the brain. In the brain, the frequency of β-gal-specific CD8 T cells also peaked at day 23 p.i. and persisted at high levels thereafter. Thus, intracerebral CD8 T cells specific for a secreted Ag of tachyzoite were not eliminated from the brain in chronic TE, i.e., when the parasite persists as bradyzoites within cysts. The persistence of these CD8 T cells is consistent with the observation that intracerebral T cells form a stable cell pool in chronic TE, which is only slowly downsized by a low level of apoptosis (7). Because β-gal-specific CD8 T cells were functionally active and rapidly produced IFN-γ (the major cytokine in resistance against T. gondii (33)) upon peptide restimulation and also killed peptide-pulsed APCs, they appear to play an important role in the control of intracerebral parasites in acute and chronic TE. Because the intracerebral conversion of bradyzoites to tachyzoites is a major risk for chronically infected hosts—in AIDS patients the loss of T cell-mediated immunity against T. gondii is considered to be the major factor leading to a lethal necrotizing TE by reactivated T. gondii (34)—the intracerebral persistence of tachyzoite-specific CD8 T cells may be important for the rapid elimination of reactivated T. gondii. This assumption is experimentally supported by the crucial protective role of CD8 T cells in the TE of retrovirus-infected immunocompromised mice (35, 36). The failure of CD8 T cells to completely eliminate T. gondii from the brain may be caused by an immune evasion of tachyzoites in which CD8 T cells switch into bradyzoites, which do not induce a primary β-gal-specific CD8 T cell response, as well as the formation of cysts in neurons (37, 38), which in general lack expression of MHC class I and II Ag (39).

The observation that a heterologous Ag of T. gondii is successfully targeted to the parasitophorous vacuole and subsequently induces a strong Ag-specific CD8 T cell response indicates that T. gondii may serve as a vaccine vector against diseases in which CD8 T cells are protective. In fact, T. gondii has recently been proved for its potential to serve as a vaccine vector in murine malaria (40). However, the application of T. gondii as a vaccine vector might potentially be limited by the fact that resistance to T. gondii as well as strong and persisting Ag-specific CD8 T cell responses are genetically restricted to L1, as observed in the present and previous studies (8, 41).

Collectively, the finding that a heterologous Ag secreted under control of the tachyzoite-specific SAG1 promoter induces Ag-specific functionally active CD8 T cells, which persist in the brain in chronic TE, has substantial implications for both the development of T. gondii-based vaccines and the interaction of T. gondii and the host CD8 T cell response. Although, it has to be kept in mind that endogenous tachyzoite- and bradyzoite-specific proteins differ from each other and also from β-gal, which may also have an impact on the ensuing CD8 T cell response.

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References