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Stage-Specific Expression of Surface Antigens by *Toxoplasma gondii* as a Mechanism to Facilitate Parasite Persistence

Seon-Kyeong Kim and John C. Boothroyd

*Toxoplasma* persists in the face of a functional immune system. This success critically depends on the ability of parasites to activate a strong adaptive immune response during acute infection with tachyzoites that eliminates most of the parasites and to undergo stage conversion to bradyzoites that encyst and persist predominantly in the brain. A dramatic change in antigenic composition occurs during stage conversion, such that tachyzoites and bradyzoites express closely related but antigenically distinct sets of surface Ags belonging to the surface Ag 1 (SAG1)-related sequence (SRS) family. To test this contribution of this antigenic switch to parasite persistence, we engineered parasites to constitutively express the normally bradyzoite-specific SRS9 (SRS9*) mutants and tachyzoite-specific SAG1 (SAG1*) mutants. SRS9* but not wild-type parasites elicited a SRS9*-specific immune response marked by IFN-γ production, suggesting that stage-specificity of SRS Ags determines their immunogenicity in infection. The induction of a SRS9*-specific immune response correlated with a continual decrease in the number of SRS9* cysts persisting in the brain. In contrast, SAG1* mutants produced reduced brain cyst loads early in chronic infection, but these substantially increased over time accompanying a hyperproduction of IFN-γ, TNF-α, and IL-10, and severe encephalitis. We conclude that stage-specific expression of SRS Ags is among the key mechanisms by which optimal parasite persistence is established and maintained. The *Journal of Immunology*, 2005, 174: 8038–8048.

*Toxoplasma gondii* is an obligate intracellular, protozoan parasite highly prevalent in warm-blooded vertebrates (1). One route of infection is consumption of meat harboring cysts that contain bradyzoites (BZs).3 Once in the gut, BZs rapidly invade and convert to tachyzoites (TZs) that proliferate and disseminate throughout the body (2). This acute infection with TZs activates strong, long-lasting Ab and T cell responses that eliminate most of the parasites (3). Some parasites that survive undergo stage conversion to BZs, a process that may be induced by the immune response itself (4). BZs then encyst, establish a chronic infection primarily in the brain and harmlessly persist for the life of an immunocompetent host.

A key feature of *Toxoplasma* persistence is the requirement of a functional immune system to control the acute infection with TZs. In an immunodeficient host, uncontrolled TZ growth causes tissue destruction, and persistent infection is not established (5). When a chronically infected host later becomes immunodeficient, BZs re-activate to TZs causing severe neurological diseases (6). Another characteristic of *Toxoplasma* persistence is that the parasite persists in the face of a long-lasting anti-*Toxoplasma* immune response. *Toxoplasma* succeeds in this daunting task by using a variety of strategies applied by microbial agents, which allow them to persist until transmission to a new host (by carnivorism for *Toxoplasma*) can be accomplished. Such strategies include: modification of the intracellular environment within host cells to favor parasite survival (7); manipulation of the host immune response by producing immunomodulatory molecules (8, 9); establishment of chronic infection predominantly in the brain, where immune surveillance is controlled differently than in other peripheral tissues (10); and, the fact that BZs are metabolically quiescent and relatively nonproliferative during chronic infection (11).

Another immune-evasion mechanism commonly used in microbial persistence is the variation of antigenic composition that eliminates epitopes that would otherwise be targeted by protective immune responses (12–14). *Toxoplasma* stage conversion involves a differential expression of numerous genes in a stage-specific manner (4, 15), among which are the members of the surface Ag (SAG1)-related sequence (SRS) superfamily that encode GPI-anchored surface proteins (>160 putative genes) (16). The prototypic SAG1 is the most abundant TZ SRS Ag (17). SRS Ags are structurally related (18), yet antigenically distinct, sharing 25-35% overall amino acid sequence identity and up to 50–90% identity within a given subfamily (16). The structure of SRS Ags suggests their possible role as cell adhesion molecules (18). In addition, extensive polymorphisms found among the three canonical lines of *Toxoplasma* imply that SRS Ags are under selective pressure from the immune response (19). A distinctive feature of the SRS antigenic switch is that TZs and BZs express largely nonoverlapping sets of SRS Ags, but a single set of multiple SRS Ags is simultaneously expressed by most if not all of the parasite population in a given developmental stage (16).

Because stage conversion and a dramatic change of the parasite surface occur with the emergence of the adaptive immune response, it has been speculated that stage-specific expression of SRS Ags has evolved as an immune-evasion mechanism that allows *Toxoplasma* to persist. Surface proteins are excellent targets of neutralizing Abs and cell-mediated immune responses. In fact, SAG1 and SAG2A dominate the humoral response in the acute infection (20, 21), and SAG1 also activates T cells producing...
IFN-γ (22, 23), the major cytokine mediating resistance against Toxoplasma (24). However, an immune response to TZ-specific Ags such as SAG1 and SAG2A is expected to be ineffective against BZs that do not express these molecules. Although parasite Ags shared by both TZs and BZs can also activate an immune response (22, 25), this is clearly insufficient to eliminate BZs. Notably, immune responses to BZ-specific Ags, such as SAG2C/D (a member of the SRS family) and SAG4, LDH2, ENO1, and p-ATPase were undetectable in chronically infected humans (26). In this study (26), BAG1 and MAG1 were thought to be the only BZ-specific molecules immunogenic in infection. Recent data, however, demonstrated that MAG1 mRNA (15) and protein (27) are expressed in both the TZ and BZ stages, making it likely that a MAG1-specific response may have been elicited during acute infection with TZs. Because BAG1 shares a significant homology with other small heat shock proteins across species (28), cross-reactivity of an immune response to other microorganisms remains a distinct possibility. Thus, overall, BZ-specific Ags appear to be poorly or not at all immunogenic in infection and this condition may be one of the mechanisms by which BZs escape immune surveillance.

To explore the role of stage-specific expression of SRS Ags in Toxoplasma persistence, we chose to focus on SAG1 and SRS9, two of the most abundant SRS Ags specific to TZs and BZs (15), respectively. A key aspect of our work is the use of mutant parasites expressing SAG1 and SRS9 in a constitutive manner in both the TZ and BZ stages, which allowed us to study the interaction between the host immune response and SRS Ags in relation to their stage-specificity. We show that an immune response to SRS9, as well as to other BZ-specific SRS Ags, is lacking in natural infection and that stage-specific expression of SRS Ags has important consequences regarding their immunogenicity in infection and the ability of parasites to achieve optimal persistence in infected hosts.

Materials and Methods

Toxoplasma culture

Prugniaud (Pru) strain parasites were maintained as TZs by passage in human foreskin fibroblast (HFF) monolayers cultured in DMEM supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) (Invitrogen Life Technologies) in a humidified, 5% CO2 incubator. Switching to BZs was induced by culturing infected HFFs in HEPES-buffered RPMI 1640 (pH 8.1) supplemented with 2% FCS in an air incubator.

Recombinant SRS9 and polyclonal anti-SRS9 antisera

rSRS9 was produced in insect cells as a secreting protein using the pAcGP67/A baculovirus expression vector, as described for recombinant (rSAG1) production (18). SRS9 coding region lacking the N-terminal signal sequence and C-terminal GPI anchor signal was PCR cloned from Pru strain SAG1c (provided by Dati) (University of Geneva, Geneva, Switzerland) (29). Upstream (promoter) sequences from the TZ-specific GRA1 (pGRA1) (30) and from the BZ-specific SRS9 (pSRS9) were used to express transgenic SRS9 and SAG1 in the TZ and BZ stage, respectively. The SRS9 promoter region (~1.5 kb) was PCR cloned from the Pru strain genomic DNA using the following primers: 5′-GGGGAGACTTCGCTGCAGCTACG-3′ and 5′-GCCCATGCATTGTGTCGACCCGTGTGCACG-3′. Full-length SRS9 and SAG1 coding regions were cloned with the following primers: 5′-GGGGAGACTTCGCTGCAGCTACG-3′ and 5′-GCCCATGCATTGTGTCGACCCGTGTGCACG-3′. PCR products were digested with NotI and BglII, and cloned between the indicated promoter and 3′ GRA2 downstream sequence in a plasmid vector containing a copy of HPT gene driven by the dihydrofolate reductase promoter (pDHFR) (31) (see Fig. 3A). The plasmids were linearized with NotI and electroporated into Pru hpt as described (32). Clones were derived from over two independent populations after selecting for HPT activity using mycophenolic acid and xanthine (50 μg/ml each).

Mouse infection

Eight-week-old CBA/J female mice (The Jackson Laboratory) were infected i.p. with TZs. Infected HFFs were syringed-lysed using a 27-gauge needle to release TZs. TZs were washed in PBS by centrifugation at 250 × g for 10 min and counted with a hemacytometer. Mice were injected i.p. with 400 or 4000 TZs in 200 μl of PBS. We considered >3 wk postinfection as the chronic phase of infection. All animal studies have been reviewed and approved by Stanford University Administrative Panel for Laboratory Animal Care.

Recombinant SRS9 immunization and challenge infection

CBA/J females (8 wk of age) were immunized s.c. with rSRS9 (20 μg/ mouse in a 100 μl volume) prepared in Ribi adjuvant or adjuvant alone into one site at the back of the neck. Mice were boosted s.c. twice, 3 wk apart, with the same Ag preparations and challenged i.p. with 4000 wild-type (WT) TZs 7 days after the second boost.

Plaque assays to quantitate tissue parasite loads

Tissues were homogenized over 100-μm cell strainers. Aliquots of tissue homogenates were syringe-lysed using 27-gauge needles to prevent intact mouse cells from serving as host cells and thus interfering with plaque assays. Two-fold serial dilutions starting from 1% of the whole tissue were added to HFF monolayers in 12-well plates and cultured for 24 h in DMEM/5% FCS. Medium was replaced with DMEM/10% FCS the next day. HFFs were fixed in methanol at 4 days postinfection (dpi) and stained with rabbit anti-SAG1 antisera followed by Alexa Fluor 488. Plaques were transferred to a nitrocellulose membrane, which was blocked in PBS/5% nonfat dry milk and incubated with rabbit anti-SAG1 or –SRS9 antisera followed by peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories). Detection was by ECL chemiluminescence reagents (Amershams Biosciences).

Immunofluorescence microscopy

Infected HFFs grown on cover slips were fixed in 3.7% formaldehyde and permeabilized in 0.2% Triton X-100. Brain homogenates containing cysts were fixed in methanol. Samples were incubated in PBS/3% BSA with indicated anti-parasite Ag Abs followed by appropriate goat IgG coupled with Alexa Fluor 488, Alexa Fluor 594, or Cascade Blue (Molecular Probes). Coverslips were mounted on a glass slide with Vectashield (Vector Laboratories), and photographs were taken using the Image Pro Plus software and a 35 mm digital camera (model C4742-95; Hamamatsu) connected to an upright (model BX60; magnification, ×1000; Olympus) or an inverted fluorescence microscope (model TE300; magnification, ×200; Nikon).

Flow cytometry

TZs lysed out of HFFs were incubated in PBS/2% FCS (105 TZ in 100 μl volume) with indicated anti-parasite Ag Abs followed by appropriate goat IgG coupled with FITC or PE (BD Biosciences). Data acquisition and analysis were by FACScan and CellQuest software (BD Biosciences).

Generation of SRS9-constitutive (SRS9+) and SAG1-constitutive (SAG1+) mutants

SRS9+ and SAG1+ were derived from PruΔhpt (hypoxanthine-xanthine-guanine phosphoribosyltransferase-deficient Pru strain), a gift from D. Sol-dati (University of Geneva, Geneva, Switzerland) (29). Upstream (promoter) sequences from the TZ-specific GRA1 (pGRA1) (30) and from the BZ-specific SRS9 (pSRS9) were used to express transgenic SRS9 and SAG1 in the TZ and BZ stage, respectively. The SRS9 promoter region (~1.5 kb) was PCR cloned from the Pru strain genomic DNA using the following primers: 5′-GGGGAGACTTCGCTGCAGCTACG-3′ and 5′-GCCCATGCATTGTGTCGACCCGTGTGCACG-3′. Full-length SRS9 and SAG1 coding regions were cloned with the following primers: 5′-GGGGAGACTTCGCTGCAGCTACG-3′ and 5′-GCCCATGCATTGTGTCGACCCGTGTGCACG-3′. PCR products were digested with NotI and Polcl, and cloned between the indicated promoter and 3′ GRA2 downstream sequence in a plasmid vector containing a copy of HPT gene driven by the dihydrofolate reductase promoter (pDHFR) (31) (see Fig. 3A). The plasmids were linearized with NotI and electroporated into PruΔhpt as described (32). Clones were derived from over two independent populations after selecting for HPT activity using mycophenolic acid and xanthine (50 μg/ml each).

Mouse infection

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counted using an inverted fluorescence microscope (magnification, ×200). Parasite loads are expressed as PFU per entire organ.

**Enumeration of parasite cysts and T cells in the brain**

Brains were homogenized over 100-µm cell strainers. Cells were washed in 25 ml of PBS by centrifugation at 150 × g for 10 min and resuspended in 20 ml of PBS/2% FCS. The 5% of the entire brain sample was stained with fluorescein-conjugated Dolichos biflorus agglutinin (Vector Laboratories) to stain the cyst wall (33). After washing, samples were resuspended in 500 µl of PBS/2% FCS, and 50 µl of aliquots were seeded in a flat-bottom 96-well plate. Cysts were counted in all 10 wells using an inverted fluorescence microscope (magnification, ×200). To count T cells, 1% of the total brain homogenates preincubated with Fc block were stained with FITC-coupled anti-CD4 or anti-CD8 Abs (BD Biosciences). Serial dilutions of each sample were seeded in a flat-bottom 96-well plate and FITC+ cells were counted using an inverted fluorescence microscope (magnification, ×200).

**Histology and assessment of brain cyst size**

Brains were fixed for >24 h in 3.7% formaldehyde solution. Sections (10-µm thick) were cut 100 µm apart and stained with H&E (Histotec). Photographs of H&E-stained sections were taken with a camera attached to a microscope (magnification, ×200) and used to measure the diameter of each cyst as an arbitrary scale.

**ELISA with serum and splenocyte culture supernatant**

To quantitate Ag-specific IgG, serial dilutions of serum prepared from tail vein blood were added to ELISA plates coated with rSRS9 or rSAG1 (50 ng/well). After incubation with peroxidase-coupled goat anti-rabbit IgG and its substrate (Kirkegaard & Perry Laboratories), plates were read colorimetrically at 450 nm. To detect cytokines secreted by cultured splenocytes, spleens were homogenized over 100-µm cell strainers. After lysing RBC (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM Na2EDTA), splenocytes were stimulated with indicated Ags in a 12-well plate (8 × 106 cells/well in 2 ml of medium) using DMEM supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), l-glutamine (2 mM), and 2-ME (55 µM). At desired time points, 250 µl of supernatant were collected and analyzed for IFN-γ, TNF-α, and IL-10 levels using ELISA kits according to the manufacturer’s instructions (Pierce). Following incubation with peroxidase-coupled detection Ab and its substrate, plates were read colorimetrically at 450 nm. Serum cytokine levels were determined using the same ELISA kits.

**Statistics**

Student’s t test was performed to compare parasite loads in different experimental groups.

**Results**

**SAG1 induces an IFN-γ- and IL-10-producing immune response, whereas SRS9 is not immunogenic in natural infection**

SRS9 was first identified as an abundant, BZ-specific mRNA (15). To confirm that SRS9 is expressed as a BZ-specific surface protein, we performed immunofluorescence assays using polyclonal antisera raised against rSRS9 produced by the baculovirus system. In vitro BZs were obtained by culturing in a high pH medium (34) HFFs infected with the PruΔhpt parasite engineered to express GFP from the TZ-specific GRA1 promoter (pGRA1), a gift from G. Arrizabalaga (University of Idaho, Moscow, ID). We show that SRS9 is expressed by GFPhigh BZs but not by GFPbright TZs (Fig. 1A). SAG1, as expected, is expressed by TZs but not by BZs (Fig. 1B). BZs within brain cysts isolated from a PruΔhpt-infected CBA/J mouse (3 wk postinfection) were costained with fluorescein-conjugated Dolichos that binds to the cyst wall and rabbit anti-SRS9 or anti-SAG1 followed by Alexa Fluor 594-coupled goat anti-rabbit IgG. Ten-micron scale bars are shown.

In contrast, SRS9-specific serum IgG was conspicuously absent in the same infected mice (Fig. 2A), as well as in 12 different chronically infected people who have most likely acquired infection by ingesting BZ cysts (data not shown). The lack of detectable IgG in infection was not unique to SRS9 but was also true for other BZ-specific SRS Ags, such as SAG2C, SAG2X, and SAG2Y, despite the fact that all these molecules were highly immunogenic in animals when given along with an appropriate adjuvant (data not shown). A T cell response to SRS9 was also undetectable: splenocytes from the infected mice did not produce IFN-γ or IL-10 when stimulated with rSRS9 in vitro (Fig. 2B).

**Generation of SAG1C and SRS9C mutants**

The stage-specific expression of SRS Ags may be one of the reasons why BZs are not cleared by the immune system but persist. Because SRS9 given with an adjuvant is highly immunogenic in animals, we predicted that it should be immunogenic during an infection if it could be expressed in the TZ stage and that the resulting immune response could eliminate BZs that subsequently appeared. Likewise, SAG1, if still expressed after TZ-to-BZ conversion, might continue to attract the robust SAG1-specific response leading to the elimination of BZs. To test these hypotheses, we engineered parasites to express SAG1 or SRS9 in a developmentally inappropriate, “constitutive” manner. SRS9 and SAG1C mutants were derived from PruΔhpt by using the TZ-specific pGRA1 and BZ-specific pSRS9 promoters to drive SRS9 and SAG1 expression, respectively (Fig. 3A). To control for the presence of the HPT gene in the expression vectors, WT parasites were...
engineered by transforming PruΔhpt with the same vectors from which the SAG1 or SRS9 coding region was removed (Fig. 3A). Clones were obtained from at least two independent populations per construct after selecting for HPT activity.

Because WT TZs lack SRS9 expression, SRS9c mutants were readily identified by Western blotting with TZ lysates (Fig. 3B), as well as by flow cytometry with live TZs (Fig. 3C). Transgenic SRS9 reaches the parasite surface (Fig. 3C) and can be cleaved with phosphatidylinositol phospholipase C indicating that it is GPI-anchored as strongly predicted from its sequence (data not shown). The relative abundance of SAG1 vs SRS9 in SRS9c mutants was estimated to be ~5-fold (~178,000 SAG1 and ~36,000 SRS9 molecules per parasite) by comparing band intensities in a Western blot performed with rabbit anti-SAG1, a gift from M. Grigg (University of British Columbia, Vancouver, BC, Canada) or anti-SRS9 antisera and serial dilutions of denatured recombinant proteins and TZ lysates (data not shown). Because of the TZ-specific nature of pGRA1 (Fig. 1), it is highly unlikely that SRS9 is over-expressed in the BZ stage of SRS9c mutants compared with WT parasites.

SAG1c mutants were identified after infecting mice with individual clones and examining SAG1 expression in brain cysts by immunofluorescence microscopy. Different mutant clones showed similar SAG1 expression levels in BZs (Fig. 3D), which appeared lower than in TZs (data not shown). Due to the difficulty in obtaining a sufficient number of pure cysts from infected mice, the exact levels of SAG1 expression by SAG1c BZs vs TZs could not be determined. In the TZ stage, SAG1 expression levels between WT and SAG1c mutants were indistinguishable (data not shown), as expected from the endogenous pSRS9 being active exclusively in the BZ stage (Fig. 1). Also, even in the context of the plasmid construct we used, pSRS9 is BZ-specific because the same construct containing the GFP gene instead of the SAG1 gene results in GFP expression only in the BZ stage (data not shown).
two independent populations were used in all studies. WT parasites were maintained as either nonclonal or clonal populations, both of which showed similar growth and dissemination rates in acute infection (data not shown), as well as similar brain cyst loads in chronic infection (Fig. 4, B and D). WT and SRS9c mutants showed similar growth rates during coinfection of HFFs in vitro (data not shown). Brain cyst loads continually decrease during chronic infection with SRS9c mutants

In CBA/J mice infected i.p. with 4000 TZs of WT and SRS9c mutants, mortality usually began to occur 12–14 dpi, following the peak of acute infection (Fig. 4A) and around the time most parasites are found in the brain (35). With all infecting strains, mortality often occurred throughout the chronic phase (>3 wk postinfection) with SRS9c-infected mice showing similar or slightly better (but not statistically significant) survival than WT-infected mice (Fig. 4A). The number of cysts persisting in the brains of WT-infected mice remained relatively constant during the chronic infection (Fig. 4B). Brain cyst loads in SRS9c-infected mice were similar to or sometimes lower than those in WT infection in the early phase of chronic infection (3 wk postinfection), but they significantly declined over time and were 17–50% of those in WT-infected brains by the late phase of chronic infection (6 wk postinfection) (Fig. 4B).

When the inoculum size was reduced by 10-fold to 400 TZ per mouse, the onset of death was generally delayed by more than a week and the occurrence of mortality was still observed throughout the chronic phase (Fig. 4C). SRS9c-infected mice showed a similar or marginally better survival during chronic infection than WT-infected mice, which was not statistically significant but consistently observed in all five independent experiments performed (data not shown). SRS9c-infected mice showed a better overall health status than WT-infected mice as they weighed more (27.6 ± 3.4 g; n = 7) than WT-infected mice (20.1 ± 2.5 g; n = 6) at 9 wk postinfection (age-matched, naive mice weighed 30.5 ± 0.8 g; n = 3) (data not shown). As with the higher inoculum and after initial parity, the brain cyst loads continually decreased in SRS9c-infected mice and were 20–30% of those in WT infection by 9 wk postinfection (Fig. 4D). Notably, the 10-fold difference in inoculum size, which causes a clear difference in acute phase parasite loads in the whole body (data not shown), yielded similar brain cyst loads in chronic infection (Fig. 4, B and D). This implies that the number of persisting parasites is not simply determined by the inoculum size but may be controlled by complex factors including, e.g., the host response.

Because brain cyst loads in the early phase of chronic infection were similar between WT and SRS9c mutants, the subsequent reduction in SRS9c cyst loads is unlikely due to a growth defect. Indeed, up to 15 dpi, both WT and SRS9c mutants showed similar parasite loads in the spleen (the major site of parasite replication after i.p. infection) (Fig. 5A) and in the brain (Fig. 5B), indicating that dissemination from the site of infection and subsequent growth in target tissues during the acute phase is comparable. Brain cyst counts at 15 dpi were also similar between WT (323 ± 140; n = 3) and SRS9c-K2 (300 ± 46; n = 3) (data not shown). Moreover, there was no measurable difference in brain cyst size between WT and SRS9c mutants measured in the H&E-stained brain sections (data not shown), suggesting that a defect in BZ growth is not responsible for the decrease in SRS9c cyst loads.

**FIGURE 4.** Continual decrease in brain cyst loads in SRS9c infection. A, Survival curves of CBA/J mice infected i.p. with 4000 TZs of indicated parasite strains (n = 12 per group). WT used is not a clone but a stable population. B, Brain cyst loads in representative mice from A determined at 3 and 6 wk postinfection (wpi). C, Survival curves of mice infected i.p. with 400 TZs of indicated parasite strains (n = 13 per group). WT c3 and c28 are clonal populations. D, Brain cyst loads in representative mice from C determined at 4 and 9 wk postinfection (wpi). Each diamond represents a single mouse. Bars represent average cyst counts. *, Significant difference (p < 0.05) in cyst loads between WT and SRS9c at the given week postinfection; #, significant difference (p < 0.05) in SRS9c cyst loads between the two time points.
Another possible reason for observing decreased brain cyst loads in SRS9 infection would be if the transgenic SRS9 expression in TZs altered tissue tropism of the parasite such that the parasite preferentially located to and encysted in other tissues than in the brain. This possibility is unlikely because parasite loads were similar between WT- and SRS9-infected brains up to 4 wk postinfection (Figs. 5B and A, B and D). Also, regardless of the infecting strain, cysts detectable after dolichos-fluorescein staining were present only in the brains and were absent in the lungs and livers that are targets of the acute infection (35) (zero cysts in 5% of the total tissue homogenates, both at 4 and 9 wk postinfection; data not shown).

**SRS9** mutants induce a SRS9-specific immune response in infection

A SRS9-specific immune response that was absent in WT infection was readily detectable in SRS9 infection. Sera from SRS9-infected mice (6 wk postinfection) had a high titer SRS9-specific IgG unlike WT-infected mice (Fig. 6A). As expected, SAG1-specific IgG titers were high in all infected mice (data not shown). SRS9 infection also activated a SRS9-specific T cell response marked by IFN-γ but not IL-10 production by cultured splenocytes (Fig. 6B). Although SAG1 clearly dominates the humoral response in *Toxoplasma* infection, it is not known whether it also dominates the T cell response. In SRS9 infection, the amount of rSAG1-stimulated IFN-γ production was substantially higher than that induced by rSR9. Because SAG1 is the most abundant SRS Ag in TZs and expressed in ∼5-fold excess over SRS9 in SRS9 TZs, the size of the T cell response to SAG1 is expected to be greater than that to SRS9 (and most other TZ-specific Ags for that matter). Neither T cell population produced detectable levels of TNF-α regardless of the infecting strain (data not shown). Generally, cytokine secretion in the absence of an antigenic stimulus (Fig. 6B) or in the presence of PMA and ionomycin (Fig. 6C) was higher with splenocytes from infected mice than with cells from naive mice, indicating an infection-driven accumulation of immune cells still detectable in the spleen at 6 wk postinfection.

Mice immunized with rSR9 show improved survival and reduced brain cyst loads following a challenge infection with WT parasite

To test whether the SRS9-specific immune response contributed to the decrease in brain cyst loads in SRS9 infection, CBA/J mice were immunized s.c. with rSR9 prepared in Ribi adjuvant or with adjuvant alone and then challenged i.p. with 4000 WT TZs. Following infection, rSR9-immune mice showed better survival (Fig. 7A) and lower brain cyst loads (Fig. 7B) than the control group. This result suggests that the induction of a SRS9-specific immune response in SRS9 infection may have been a key mechanism that led to the clearance of BZ cysts during chronic infection.

**Brain cyst loads continually increase during chronic infection with SAG1−/− mutants**

Because SAG1 dominates the immune response during the acute infection with TZs, one would anticipate that BZs still expressing
SAG1 will be effectively cleared by the immune response. Indeed, the average brain cyst count in SAG1\(^c\) infection was often lower than that in WT infection at 3–4 wk postinfection (Fig. 8, A and B). However, in contrast to SRS9\(^c\) infection, the cyst count substantially increased over time in SAG1\(^c\)-infected brains and, by 6–7 wk postinfection, far exceeded that in WT-infected brains by up to 3.5-fold (Fig. 8, A and B). This increase in SAG1\(^c\) cyst loads was associated with an increase in mortality during the late phase of chronic infection (Fig. 8C), which was not statistically significant but consistently observed in all five independent experiments we performed (data not shown). At 6 wk postinfection, SAG1\(^c\)-infected mice weighed 17.5 ± 1.8 g (n = 3), less than WT-infected (20.5 ± 1.8 g; n = 3) or SRS9\(^c\)-K2-infected mice (21.2 ± 0.7 g; n = 3) (age-matched, naive mice weighed 25.2 ± 0.8 g; n = 3), reflecting the poor overall health status (data not shown).

The increase in brain cyst loads in SAG1\(^c\) infection is unlikely due to an enhanced growth of SAG1\(^c\) mutants. Following i.p. infection, parasite loads expanded almost equally from 3 to 7 dpi in the spleens of all infected mice (Fig. 9A). At 11 dpi, although statistically not significant, the mean parasite loads in the spleen tend to be somewhat lower in SAG1\(^c\) infection than in WT infection (Fig. 9A), possibly indicating an improved parasite clearance due to SAG1 expression by BZs. Parasite loads in the brain at 11 dpi were similar in WT and SAG1\(^c\) infection (Fig. 9A). The brain cyst size measured in the H&E-stained brain sections was also similar between WT and SAG1\(^c\) mutants (data not shown), indicating no significant difference in the rate of BZ growth.

As with SRS9\(^c\) mutants, cysts formed by SAG1\(^c\) mutants were only detectable in the brain and not in the lung and liver (data not shown), indicating that constitutive SAG1 (SAG1\(^c\)) expression probably did not result in altered tissue tropism.

**SAG1\(^c\) infection leads to cytokine hyperproduction in the spleen**

In immunocompetent mice infected with WT parasites, brain cysts occasionally rupture during chronic infection but this does not lead to active parasite replication because of an immune response resulting in the killing of the parasites (36). The increase in brain cyst loads in SAG1\(^c\) infection suggests that the immune response may not be properly regulated in infected brains and, therefore, it may not effectively control parasite replication or it could even promote differentiation to BZs.

To test this possibility, we compared immune responses elicited by WT, SRS9\(^c\) and SAG1\(^c\) mutants and found that splenocytes from SAG1\(^c\)-infected mice (6 wk postinfection) produced much higher levels of IFN-\(\gamma\) when stimulated with rSAG1 in vitro (>50 ng/ml at 48 h culture) (Fig. 9B) than did the cells from WT- and SRS9\(^c\)-infected mice (<20 ng/ml at 48 h culture) (Fig. 6B). Moreover, unlike in WT and SRS9\(^c\) infection, significant levels of TNF-\(\alpha\) were produced by splenocytes from SAG1\(^c\)-infected mice when stimulated with rSAG1 (Fig. 9B). SAG1-specific IL-10 production was also enhanced in SAG1\(^c\) infection (Fig. 9B) compared with WT and SRS9\(^c\) infection (Fig. 6B).

This result suggests that SAG1\(^c\) infection causes cytokine dysregulation due to an excessive expansion and/or activation of SAG1-specific T cells in the spleen despite the comparable in vivo growth rates of WT and SAG1\(^c\) parasites. However, serum IFN-\(\gamma\)
levels were similar among mice infected with WT, SRS9c, or SAG1c mutants: ~2000 and ~700 pg/ml at 4 and 6 wk postinfection, respectively, compared with ~300 pg/ml in naive mice (data not shown). Serum IL-10 and TNF-α were undetectable in all mice (data not shown). This result is perhaps not surprising given that the major site of parasite replication during chronic infection is the brain. Cytokines locally produced in the brain may be more relevant to the contrasting phenotypes of SRS9c and SAG1c mutants.

Distinct immunological events occur in the brains infected with WT, SRS9c, and SAG1c mutants

In histologic examination of H&E-stained brain sections, meningitis and encephalitis were apparent in all infected mice (6 wk postinfection) regardless of the infecting strain (Fig. 10, A–F). Mononuclear infiltrates were mostly found in the cerebral cortex where the great majority of cysts were localized. Compared with WT (Fig. 10B) and SRS9c infection (Fig. 10, C and D), SAG1c-infected brains showed much severe inflammation with prominent areas of necrosis (Fig. 10, E and F).

Both CD4+ and CD8+ T cells are known to significantly contribute to Toxoplasma encephalitis (37), and were recruited to and accumulated in the brains in WT, SRS9c, and SAG1c infection and gradually decreased in numbers during the chronic infection (Fig. 10G). Given that T cells, especially IFN-γ-secreting CD8+ T cells, play a critical role in resistance to Toxoplasma (38, 39), it is notable that more CD8+ T cells were recruited to the brain in SAG1c infection than in WT or SRS9c infection (Fig. 10G).

In chronically infected brains, the majority of cysts were not associated with mononuclear infiltrates (Fig. 10Ha). However, many sites of focal inflammation were found throughout the cerebral cortex, some of which were seen to be intimately surrounding cysts (Fig. 10Hb) whereas others were not (Fig. 10Hc). The latter (Fig. 10Hc) might have been seen to associate with a cyst if sections had been cut at different levels or may represent the situation after a cyst had been cleared by the infiltrates. Counting these sites of focal inflammation revealed that a significantly higher proportion of SAG1c cysts was closely associated with infiltrates than WT cysts (Fig. 10H). This is not simply due to the increased numbers of cysts and infiltrates present in SAG1c infection because SRS9c infection that caused moderate inflammation and produced fewer cysts than WT also had a higher proportion of cysts associated with infiltrates (Fig. 10H).

Discussion

Toxoplasma surface proteins belonging to the SRS superfamily are stage-specifically expressed during TZ-to-BZ conversion concomitant with the emergence of an adaptive immune response in acute infection. The stage conversion is critical in the establishment and maintenance of a chronic infection, but it has not been previously tested whether SRS antigenic switch indeed serves as an immune-evasion mechanism that allows parasites to persist. Using genetically manipulated parasites expressing the normally stage-specific SAG1 and SRS9 Ags in a constitutive manner, we have demonstrated that altering parasite stage-specificity substantially affects the immune response they elicit in infection and interferes with the ability of parasites to achieve optimal persistency.

BZ cysts persist despite the long-lasting immune response to TZ-specific SRS Ags, such as immunodominant (at least in terms of humoral response) SAG1 and SAG2A, as well as the response to Ags shared by both TZs and BZs, suggesting that BZ-specific immune responses are required to clear BZs. Somehow, Toxoplasma avoids eliciting such a response because BZ-specific SRS Ags, such as SRS9, SAG2C, SAG2X, and SAG2Y, are not immunogenic in infection. This result is true not only in mice infected i.p. with TZs but also in humans who have most likely acquired infection through oral ingestion of BZ cysts. Therefore, the lack of immunogenicity of BZ SRS Ags appears to hold regardless of the infection route (perhaps because BZs convert to TZs quickly upon reaching the gut before BZ Ags can be seen by the immune system). However, we found that constitutive SRS9 expression elicited a readily detectable SRS9-specific immune response and this response correlated with a significant decrease in cyst loads persisting in the brain. This finding supports the hypothesis that the BZ-specific nature of SRS9 expression is one of the key factors contributing to parasite persistence. The timing of SRS expression likely determines parasite immunogenicity in infection by regulating the way they interact with the immune system: the amount of SRS Ags available for presentation to the immune system is far greater during acute infection with rapidly multiplying TZs than in chronic infection with metabolically quiescent BZs. Also, systemically disseminating TZs may more readily encounter and activate
The contrasting phenotypes of SRS9c and SAG1c mutants may explain why evolution has led to the immunodominant SAG1 molecule being expressed only in the TZ stage. Although SAG1c expression effectively targets BZ cysts for clearance in the early phase of chronic infection, an over-stimulation of SAG1-specific T cells seems to occur leading to severe encephalitis, and somehow, an increase in brain cyst loads over time. A similar phenomenon has been seen in an attempt to obtain a protective immunity by immunizing mice with SAG1: an i.p. immunization with purified SAG1 protein along with CFA resulted in an increase in mortality and brain cyst burdens in mice subsequently challenged with avirulent *Toxoplasma* strain (40). This observation and the phenotype of SAG1c in infected mice indicate that an excessive immune response to SAG1 can somehow lead to increased parasite growth and exacerbated disease in the chronic phase of infection. The studies with SRS9c and SAG1c mutants have demonstrated one of many purposes that may be served by stage-specific SRS expression: BZ-specific expression avoids the induction of a potentially protective immune response, whereas TZ-specific expression avoids BZ clearance by an immune response and minimizes immunopathology to the host.

The brain has been viewed as an immunoprivileged site due to such characteristics as the blood-brain barrier, lack of conventional lymphatics, better allograft acceptance, and low T cell trafficking (10). These features will undoubtedly contribute to the lack of immunogenicity of BZ Ags in infection and facilitate BZ persistence in the brain. Recently, however, many studies have shown that resident cells of the brain do have the ability to serve as potent APCs that are quite capable of supporting the induction of immune responses (41). Moreover, Ags exclusively localized in the brain can be transported to the cervical lymph nodes and activate T cells, which are then recruited to the brain (42–44), and selectively act on target cells (45). Thus, it seems reasonable to speculate that BZ Ags should be able to activate an immune response during chronic infection of the brain and that the lack of immunogenicity of SRS Ags may be the result of complex mechanisms including their localization in the brain.

The type of the immune response induced by TZ-specific SRS Ags may be one of the factors contributing to parasite persistence by down-modulating the induction of an immune response to BZ Ags. T cells are critical for controlling *Toxoplasma* replication and their protective effect is largely mediated by cytokines, especially IFN-γ (24). We found that, in addition to IFN-γ as previously shown (22, 23), SAG1-specific T cells produce IL-10. As a potent antagonist of IFN-γ, IL-10 is a key regulator in preventing excessive inflammation in the brain and other tissues infected with *Toxoplasma* (46–48). T cells simultaneously producing IFN-γ and IL-10 have been frequently observed in persistent infections, including tuberculosis (49), malaria (50), leishmaniasis (51), and *Borrelia burgdorferi* infection (52). IL-10 produced by these T cells limits the IFN-γ-driven inflammation, and in doing so, hampers complete clearance of infectious agents. Previously, an elevated level

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**FIGURE 10.** Distinct immunological events occurring in the brains in WT, SRS9c, and SAG1c infection. H&E stained brain sections from CBA/J mice uninfected (A) and infected (B–F) (6 wk postinfection) with indicated parasite strains. Arrowheads indicate cysts. G, Number of T cells per brain at indicated week postinfection (wpi). Shown are mean ± SD (n = 3 per group). When examined, all CD4+ and CD8+ T cells were costained with anti-CD3 Ab (data not shown). H, Proportion of brain cysts associated with immune response (6 wk postinfection) was calculated based on the numbers of lone cysts (a), cysts surrounded by infiltrates (b), and inflammation foci apparently lacking cysts (c). Infiltrates around blood vessels or those broadly distributed over a large area of the cortex were excluded from the analysis.
of IL-10 mediating immunosuppression was noted in the spleen of a mouse acutely infected with Toxoplasma (53). Conceivably, a T cell response to the dominant SAG1 Ag may greatly contribute to parasite clearance via IFN-γ production and also be the major source of IL-10 that serves as a self-regulatory mechanism to turn off the IFN-γ-driven Th1 type response. While preventing immunopathology in favor of host survival, IL-10 may also create an immunosuppressive environment that is not ideal for eliciting primary responses to newly emerging BZ-specific Ags.

The pattern of changes in brain cyst loads and cellular infiltration during chronic infections with SRS9c and SAG1c mutants is clearly different from that seen in WT infection. This finding indicates that mutant BZs are attracting an active immune response to the brain that is greater than what is seen in WT infection resulting in BZ clearance in SRS9c infection and the emergence of new cysts in SAG1c infection. The fact that more brain cysts are intimately surrounded by immune infiltrates in the SAG1c and even SRS9c infection than in WT infection argues for the possibility that some of these infiltrates may be T cells recruited to the brain due to their specificity for SAG1 and SRS9. Alternatively, or in addition, the T cell response may be targeting an extracerebral parasite reservoir, thus changing the number of parasites colonizing the brain during chronic infection. Understanding how SAG1- and SRS9-specific T cells are activated and maintained in the periphery, how they are recruited to the brain and what their exact effector functions are in dealing with persisting parasites will require the ability to track Ag-specific T cells and parasites in vivo in a quantitative manner. Data available at the present time indicate that the cytokine response induced by SRS9c and SAG1c mutants in the periphery is qualitatively and quantitatively different: while SRS9c-specific T cells produce moderate levels of IFN-γ in SRS9c infection, IFN-γ, TNF-α, and IL-10 are hyperproduced by SAG1c-specific T cells in SAG1c infection. Dysregulated cytokine production has been linked to many immunologic diseases and shown to be a critical event in some cases (54, 55). Although it is difficult to fully appreciate exact mechanisms, a similar phenomenon may be responsible for regulating parasite growth/differentiation and encephalitis seen in this study in the brains of mice infected with SRS9c and SAG1c mutants.

Another challenging issue in understanding the biology of the SRS superfamily and its role in parasite persistence is the possible immune-interference by presentation of altered peptide ligands (56, 57). Some SRS Ags may contain Ab and T cell epitopes that can interfere with immune responses to closely related SRS Ags. Such interference unlikely exists between SRS9 and SAG1, as immune responses to both Ags were readily detectable in SRS9c infection. However, SRS9 and SAG1 belong to different subgroups within the SRS superfamily sharing only ~25% sequence identity. An altered peptide ligand phenomenon is more likely to be operative among the SRS Ags of the same subgroup (in which sequence identity can be up to 90%) and could be one of the mechanisms contributing to the lack of immunogenicity of some of the SRS Ags in infection.

Overall, the results presented broaden our understanding of the immunologic roles played by the developmentally regulated SRS Ags of Toxoplasma and clearly argue that the SRS antigenic switch accompanying TZ-to-BZ stage conversion has evolved as one of the critical mechanisms that allow optimal parasite persistence in its intermediate host until such time as transmission to a new host becomes feasible.


