Toxoplasma gondii Possesses a Superantigen Activity that Selectively Expands Murine T Cell Receptor Vβ5-bearing CD8+ Lymphocytes

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Summary

To investigate early immune responses to the intracellular parasite *Toxoplasma gondii*, we examined the capacity of nonimmune splenocytes to respond in vitro to intact tachyzoites and soluble tachyzoite antigen (Ag). Both types of stimuli induced high levels of proliferation as well as interferon γ (IFN-γ) secretion. Based on several key criteria, the response appeared to be driven by a superantigen present in the parasite. Thus, stimulation of C57BL/6 spleen cells with *T. gondii* resulted in a preferential threefold expansion of a T cell population expressing the Vβ5 chain of the T cell receptor, and a survey of different inbred mouse strains revealed an inverse correlation between Ag-induced proliferation and genetic deletion of Vβ5. Moreover, proliferation was induced using irradiated Ag-pulsed and infected splenic adherent cells, and was blocked by a major histocompatibility complex class II–specific monoclonal antibody. Furthermore, paraformaldehyde-fixed IA^b-, IA^k-, and IE^k-transfected fibroblast lines were able to specifically bind *T. gondii* Ag and drive proliferation of T lymphocytes, demonstrating that the response can be mediated by allogeneic class II molecules, and that it does not require cellular Ag processing. It is interesting to note that after 1 wk of culture with Ag, up to 70% of the expanded Vβ5-expressing cells were $CD8^+$. These results provide the first description of a superantigen activity in a protozoan pathogen. In the case of *T. gondii*, superantigen-driven expansion of IFN-γ–secreting $CD8^+$ lymphocytes may play a role in the development of the dominant IFN-γ–dependent, cell-mediated immunity characteristic of infection with this parasite.

The obligate intracellular protozoan *Toxoplasma gondii* infects a wide range of host species and cell types. Infection with this parasite is characterized by an acute phase during which tachyzoites rapidly proliferate, and a slow-growing chronic phase consisting of the formation of bradyzoite-containing cysts.

A remarkable feature of the parasite is that it induces long-lasting immunity that renders the immunocompetent host strongly resistant to Toxoplasma reinfection. This immunity is striking in that it is characterized by production of high levels of IFN-γ in both acute and chronic models of infection (1–3). Indeed, IFN-γ is critical for immunity as determined by in vivo depletion studies (2–4), and whereas Th-1 type $CD4^+$ cells play a role in this response (3), adoptive transfer experiments suggest that $CD8^+$ cells may be the major source of IFN-γ in vivo (5).

An issue of major importance in developing strategies of microbial vaccine development is to determine how immune responses becomes polarized towards a particular cytokine production phenotype such as a Th1 (in the case of intracellular pathogens) or Th2 (during helminth infection) pattern. Biasing of the response should, by definition, be driven by events occuring during early contact with pathogen, before the development of acquired immunity. For this reason, we have recently examined *T. gondii*–driven responses that occur independently of those mediated by primed cells of the immune system.

One potential means of inducing a vigorous early immune response which might influence the subsequent development of acquired immunity could be through stimulation by a superantigen. This class of Ags binds to Ia molecules outside of the conventional peptide-binding groove and activates T lymphocytes bearing specific TCR Vβ chains (6, 7). Thus, in contrast to conventional Ags, which activate only the low frequency of T cells bearing the appropriate clonotypic receptor, superantigens stimulate large proportions of resting T cells based on Vβ chain expression, and this results in proliferation and in some cases secretion of cytokines such as IFN-γ, IL-2, and TNF-α (8, 9).

Two classes of superantigen have been described: those that are encoded within the host genome by viruses (e.g., *Mtv* proviruses) and bacterial products such as the staphylococcal enterotoxins. In this paper, we show that live *T. gondii* tachyzoites as well as parasite extracts have stimulatory ac-

tivities consistent with the presence of a superantigen. Thus, exposure of resting mouse splenocytes to these stimuli results in selective proliferation of TCR Vβ5–bearing cells and release of IFN-γ. The effect is mediated through interaction with MHC class II molecules, and is distinguished from other Vβ-specific mitogens in that $CD8^+$ lymphocytes are preferentially expanded. Furthermore, inbred mice differ in their response based on expression of the Vβ5 chain. To our knowledge, this is the first example of a superantigen expressed by a protozoan pathogen. The ability of *T. gondii* to stimulate a strong IFN-γ response from unprimed splenocytes may provide a basis for understanding the mechanisms by which this parasite induces a dominant cell-mediated immune response after infection.

Materials and Methods

Mice and Parasites. Female mice of strains C57BL/6, C3H/HeN, A.BY, DBA/2, B10.D2, and BALB/c were obtained from The Jackson Laboratory (Bar Harbor, ME). Mouse strains BALB.B, B10.A, B10.A(2R), and B10.A(4R) were bred and maintained under specific pathogen-free conditions at Bioqual Inc. (Rockville, MD). Outbred, germ-free N:NIH-blk mice were bred and maintained by the National Institutes of Health (NIH) animal facility. Tachyzoites of the strains RH and ts-4 (a temperature-sensitive mutant that does not survive at 37°C) (10) were maintained by weekly passage on human foreskin fibroblasts at 37° and 34°C, respectively.

T. gondii Ag Preparation. Soluble tachyzoite Ag (STAg) was prepared as described (11) by sonicating RH parasites in the presence of protease inhibitors and centrifugation at 10,000 *g* followed by extensive dialysis of the supernatant fraction against PBS.

Abs. For cell cytometric analysis, the following mAbs were used (Pharmingen, San Diego, CA): B20.6 (anti-Vβ2), KJ-25 (anti-Vβ3), MR9-4 (anti-Vβ5), RR4-7 (anti-Vβ6), TR310 (anti-Vβ7), MR5-2 (anti-Vβ8), MR10-2 (anti-Vβ9), B21.5 (anti-Vβ10), RR3-15 (anti-Vβ11), MR11-1 (anti-Vβ12), MR12-3 (anti-Vβ13), 14-2 (anti-Vβ14), RM4-5 (anti-CD4), and 53-6.7 (anti-CD8). The Vβ chain–, and the CD4- and CD8-specific Abs were directly labeled with FITC (Vβ specific) and PE (CD4 and CD8 specific). Expression of Ia molecules on fibroblast lines was determined with Y-3P (anti-IA; mouse IgG2a) and 14-4-4 (anti-IE; mouse IgG2a) (American Type Culture Collection, Rockville, MD) and goat anti–mouse IgG-FITC (Jackson Immunoresearch Laboratories, West Grove, PA). For in vitro depletions, hybridoma supernatants containing mAb RL172.4 (anti-CD4), 3-155 (anti-CD8), RA3-3A1 (anti-B220), J11D (anti-B cell marker), and SW3A4 (anti-NK1.1) were used. In vitro blocking experiments used mAbs Y-3P, M5/114 (anti-IA plus IE; rat IgG2b), 14-4-4, KH9 (anti-H-$2D^b$; mouse IgG2b), GK1.5 (anti-CD4; rat IgG2b), and 2.43 (anti-CD8; rat IgG2b).

Fibroblast Transfectants. Fibroblast cell lines transfected with expression plasmids encoding IA^b (FT7.1C6), IA^k (RT7.3H3 B4.5), and IE^k (DCEK Hi7) Ia molecules (kindly provided by Dr. R. N. Germain, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, NIH) were produced as described (12).

Purification of T Lymphocytes. T cells were purified by passing splenocytes over an anti–mouse Ig column according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN). The resulting populations were typically >90% $CD3^+$ as determined by cytometric analysis. To purify $CD8^+$ lymphocytes, splenocytes (10^8) were incubated (45 min, 0°C) in 2.5 ml medium containing mAb specific for $CD4^+$, $NK1.1^+$, $B220^+$, and $J11D^+$ cells. After washing, cells were incubated (45 min, 37°C) in 5 ml of a 1:10 dilution of rabbit serum as a source of complement (Accurate Chemical & Scientific Corp., Westbury, NY). Cells were rewashed and the cycle repeated. The resulting cells were then applied to an anti–mouse Ig column (R&D Systems, Inc.) and those not binding were collected. Purity of the resulting $CD8^+$ cells, determined by flow cytometric analysis, was routinely ≥90%.

Cell Depletions. In vitro depletion of NK and T cells was carried out with mAb and complement as previously described (13).

Collection of Splenic Adherent Cells. Splenic adherent cells (SAC)[1] were obtained by incubating 10^8 spleen cells in DMEM (GIBCO BRL, Gaithersburg, MD) with 5% FCS (Hyclone Laboratories, Logan, UT) on a 100 × 20-mm tissue culture dish (Becton Dickinson Labware, Lincoln Park, NJ) for 1 h at 37°C. Nonadherent cells were removed by washing, then medium containing 50 μg/ml STAg or 2×10^7 15 kilorad (kR) irradiated ts-4 tachyzoites were added and cells incubated overnight at 37°C. After washing five times by low speed centrifugation (100 *g*) to remove soluble Ag and free parasites, cells were irradiated (3,300 rad) and used to stimulate T lymphocyte proliferation.

Cell Proliferation Assays. Splenocytes (4×10^5/well) were stimulated with parasite Ag diluted in culture medium consisting of DMEM (GIBCO BRL) with 10% FCS (Hyclone Laboratories), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), Hepes (30 mM), and 2-ME (5×10^{-5} M). After 96 h at 37°C, 1 μCi/well of [^{3}H]thymidine (Amersham Chemical Corp., Arlington Heights, IL) was added for 16 h. In the cases indicated, purified T cells (2×10^5/well) were added to SAC (2×10^5/well) and pulsed after 72 h. Alternatively, $CD8^+$ T cells (2×10^5/well) were added to SAC (4×10^5/well) and were pulsed after 72 h. Stimulation of cells with anti-CD3 mAb (145-2C11) was carried out as described (14). For experiments employing fibroblast lines as APC, the cells were grown to confluency on a 96-well tissue culture plate (Costar Corp., Cambridge, MA), fixed by incubation in 1% paraformaldehyde (10 min, 37°C) followed by quenching of the reaction with 0.15 M Tris, pH 7.2, and washed four times in PBS. Before addition of T lymphocytes (2×10^5 per well), the fibroblasts were incubated overnight at 4°C with STAg diluted in PBS, then washed three times to remove unbound Ag. Cells were pulsed with [^{3}H]TdR after 72 h. Radiolabeled cells were collected onto a glass fiber filter pad (LKB Wallac, Turku, Finland) using a 96-well harvestor (Tomtec, Orange, CT), scintillation cocktail (XSC/9200; LKB Wallac) added, and radioactivity determined on a liquid scintillation counter (Betaplate model 1205, LKB Wallac). Each sample was set up in triplicate, and mean ± standard deviation was calculated.

Extended In Vitro Culture. Spleen cells (4×10^5/well) were incubated in culture medium with 50 μg/ml STAg or a 1:1 ratio of 15 kR irradiated ts-4 tachyzoites on 96-well plates (model 3596, Costar Corp.). After 5 d, cells were collected, an equal volume of fresh medium and recombinant human IL-2 (Cetus Corp., Emeryville, CA) to a final concentration of 25 U/ml was added, and the cells were replated (1 ml/well) on 48-well plates (model 3548, Costar Corp.). In the case of cytokine assays, cell supernatants were assayed at day 7 without addition of exogenous IL-2.

Flow Cytometric Analysis. Cells (10^6/sample) were incubated with Ab (diluted in 50 μl HBSS supplemented with 1% FCS and

[1] *Abbreviations used in this paper:* kR, kilorad; SAC, splenic adherent cell; SEA, Staphylococcal enterotoxin A.

0.1% sodium azide) for 30 min at 0°C and then washed. Nonspecific Fc receptor binding was blocked by the addition of saturating levels of the Fc receptor–specific mAb 2.4G2. Cells were analyzed (10,000/sample) on an Epics 753 flow cytometer (Coulter Corp., Hialeah, FL) excluding dead cells by propidium iodide gating.

Cytokine Assays. IFN-γ and IL-5 were quantitated in cell supernatants by means of two-site ELISA as previously described (3). The cytokines IL-2 and IL-4 were measured using CTLL proliferation assays (3). Results are expressed as means ± standard deviations of triplicate points.

Statistical Analysis. The statistical significance of the association of cell proliferation and expression of Vβ5 was assessed using Spearman's rank correlation test.

Results

Live Tachyzoites and Soluble Tachyzoite Extract Induce Proliferation of Normal Murine Splenocytes. Resting spleen cells from normal, uninfected C57BL/6 mice proliferated strongly when cultured with irradiated ts-4 strain tachyzoites and this response was abolished by heat inactivating the parasites (56°C, 30 min) before addition to the cultures (Fig. 1 *A*). Moreover, a high level of cell proliferation was induced using a soluble fraction (STAg) obtained by sonication and centrifugation of tachyzoites (Fig. 1 *B*). Unlike the response to intact parasites, the response to STAg was resistant to identical heat treatment. The above observations suggest that metabolically active tachyzoites are required to deliver the stimulatory parasite molecules to the cell, either through infection or active secretion into the culture supernatant, but that the active molecules themselves are resistant to mild heat treatment.

The proliferation obtained after stimulation with tachyzoites and soluble Ag is not attributable to endotoxin contamination because the response could not be inhibited by polymyxin (5 μg/ml). Furthermore, the biological activity was lost after incubation at 100°C for 10 min, as well as with proteinase K incubation, evidence that the active molecules themselves are protein rather than LPS (data not shown). In addition, tachyzoites passed three times through C57BL/6 mice continued to stimulate proliferation of C57BL/6 cells demonstrating that the response is triggered by *T. gondii* rather than contaminating host allogeneic tissue (data not shown). Finally, highly significant parasite-driven proliferation was observed using spleen cells from germ-free (N:NIH-blk) mice (see Table 3) arguing that the response is not due to preinfection with a cross-reacting microbial agent.

To further characterize the mechanism by which *T. gondii* stimulates proliferation of normal splenocytes, we examined whether T lymphocytes respond to SAC bearing parasite Ags. When SAC were cultured overnight with tachyzoites, washed to remove free parasites, irradiated, and added to purified T cells, the latter proliferated strongly (Fig. 1, *C*). As expected, uninfected SAC failed to evoke a response. Similarly, SAC preincubated with STAg also stimulated T cells to proliferate (Fig. 1 *D*).

$CD8^+$ Lymphocytes Expressing Vβ5 TCR Are the Major Cell Population Expanded in Response to Parasite Stimulation. By 7 d of culture, up to 70% of the spleen cells stimulated with either live tachyzoites or STAg were $CD8^+$ in contrast to cultures stimulated with Staphyloccal enterotoxin A (SEA) where $CD4^+$ and $CD8^+$ cells were equally stimulated (Fig. 2). However, prior removal of $CD4^+$ lymphocytes by treatment with mAb and complement rendered the remaining cells nonresponsive to parasite Ag (data not shown). This observation suggested that $CD8^+$ lymphocyte proliferation was, at least initially, dependent upon $CD4^+$ helper activity, most probably provided by IL-2. In support of the latter hypothesis, it was shown that $CD8^+$ cells (purified by negative selection with mAB and complement) proliferate in response to STAg-pulsed irradiated SAC, but only in the presence of exogenous IL-2 (see Fig. 5 *A*).

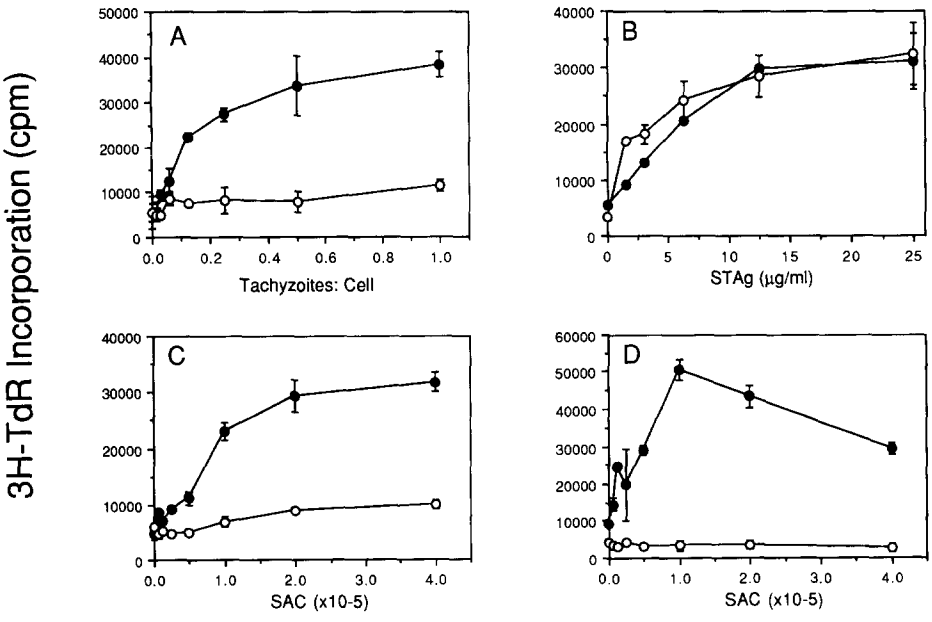

Figure 1. *T. gondii* tachyzoites and soluble Ag induce proliferation of nonimmune C57BL/6 splenocytes. Cells (4 × 10^5/well) were cultured with (*A*) irradiated ts-4 tachyzoites or (*B*) STAg. (●) Nontreated parasites and Ag; (○) the same samples treated 30 min at 56°C. In addition, purified T cells (4 × 10^5/well) were incubated with irradiated SAC that were (*C*) preinfected or (*D*) preloaded with STAg. (*C* and *D*, ●) Infected and Ag-pulsed SAC; (○) normal SAC. Cultures that were directly stimulated with Ag and parasites and those that were stimulated with preloaded and preinfected SAC were pulsed with [^{3}H]TdR at 96 and 72 h respectively, times predetermined to be optimal for low background and maximum proliferation. Indistinguishable results were obtained using tachyzoites of the RH strain from which the ts-4 mutant was derived (not shown).

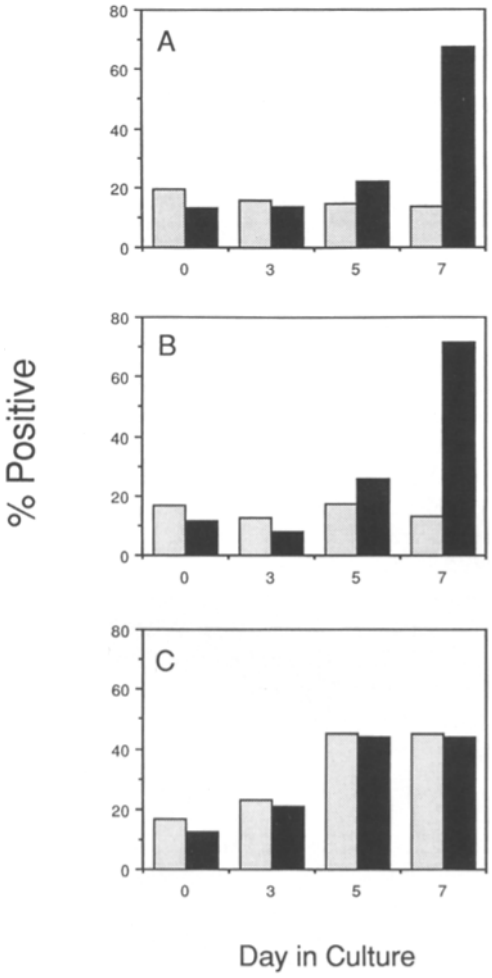

Figure 2. *T. gondii* induces preferential expansion of CD8+ lymphocytes. Spleen cells (4×10^5) were stimulated with a 1:1 ratio of (*A*) irradiated tachyzoites, (*B*) 50 μg/ml STAg, or (*C*) 3 ng/ml SEA. At the indicated days, the percentage of total cells expressing (*shaded bars*) CD4 and (*solid bars*) CD8 was examined by flow cytometric analysis. This experiment is representative of three performed.

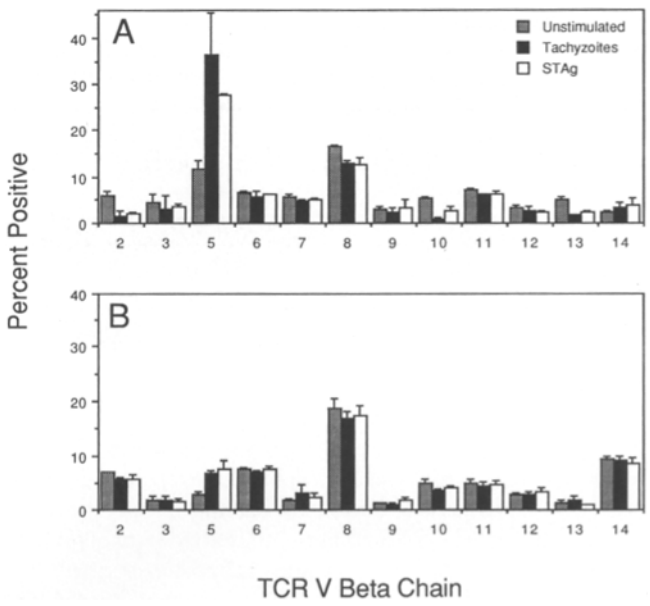

Figure 3. T cell receptor Vβ chain usage in *T. gondii*–stimulated splenocyte cultures. Two-color FACS® analysis was used to examine Vβ expression by (*A*) CD8+ and (*B*) CD4+ cells from freshly isolated splenocytes and day 7 splenocyte cultures stimulated with a 1:1 ratio of ts-4 tachyzoites or 50 μg/ml STAg. The data show mean ± SEM from three independent experiments.

When TCR Vβ chain usage by parasite-stimulated lymphocytes was examined, we found a striking threefold expansion in CD8+ lymphocytes expressing the Vβ5.1/5.2 chain after stimulation with live tachyzoites and soluble Ag as determined by two-color flow cytometric analysis (Fig. 3 *A*). When recalculated for absolute number of cells, culture with parasite Ag induced a 16-fold increase in CD8+Vβ5+ cells but only a minor increase in CD8+ lymphocytes bearing other Vβ chains (for example, 3.5-fold for CD8+Vβ6+ cells). We do not presently know the reason for the small increase in CD8+ cells bearing Vβs other than Vβ5, but possibilities include presence of a conventional mitogen or nonspecific cytokine-induced proliferation. The small number of CD4+ cells in these cultures were also enriched for Vβ5 expression, although less dramatically so than the CD8+ lymphocytes (Fig. 3 *B*). The observed preferential Vβ5+ expansion in these cultures was not an in vitro artifact since SEA stimulation of spleen cells under the same conditions induced the expected increases in Vβ11-bearing CD4+ and CD8+ lymphocytes with no evidence of Vβ5 expansion (data not shown). In addition to the Vβ5 response, *T. gondii* also induced a variable and less dramatic increase in CD8+ Vβ14-bearing cells (Fig. 3 *A*).

Proliferative Response Is Dependent on MHC Class II IA Molecules. To determine if the proliferative response was mediated through MHC class II molecules, purified T lymphocytes were added to parasite-exposed SAC in the presence of class II–specific mAb. The response to both infected (Fig. 4 *A*) and Ag-pulsed (Fig. 4 *B*) SAC was reduced to levels found in the presence of normal SAC by culture with 1 μg/ml anti-IA mAb. The same concentration of an isotype-matched anti-IE mAb failed to effect the response, consistent with the fact that C57BL/6 strain mice do not express functional EαEβ molecules. In addition, a mAb to the MHC class I D^b molecule failed to block the response.

We next performed mAb blocking experiments on purified CD8+ cells in the presence of infected SAC. Since in this system exogenous IL-2 is required (Fig. 5 *A*), the studies were performed in the presence of recombinant cytokine (40 U/ml). Responder cell proliferation was blocked by mAb specific for the CD8 or MHC class II molecules, but not by an isotype-matched anti-CD4 mAb nor by a mAb specific for H-$2D^b$ (Fig. 5 *B*). These results confirm that CD8+ lymphocytes, and not a contaminating cell type, proliferate in response to parasite Ag presented by SAC. Furthermore, they show that the MHC class II IA protein is directly involved in the CD8+ cell response.

Lack of a Requirement for MHC Class II Haplotype Matching Between Responder and Stimulator Cells. We used MHC class

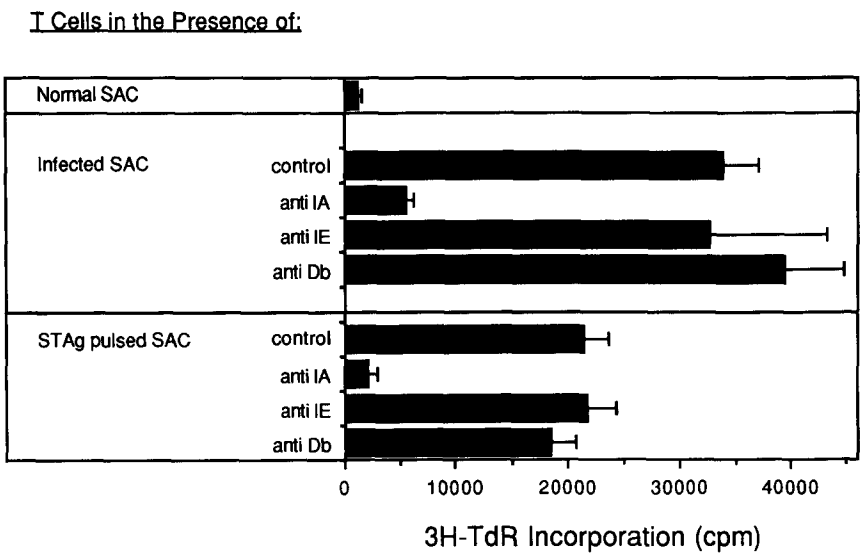

Figure 4. Proliferation of T cells is blocked by anti-IA mAb. SAC were preinfected or pre-pulsed with STAg, irradiated, and cultured (2×10^5/well) with purified T lymphocytes (2×10^5/well) in the presence or absence of 1 μg/ml of the indicated mAb. This experiment is representative of two performed.

II-transfected fibroblast lines to examine if the response was mediated solely by IA^b, or whether IE as well as IA of different haplotypes could present *T. gondii* Ag to T lymphocytes. Fig. 6 shows the IA and IE binding profiles of the fibroblasts used in these experiments. Whereas DAP.3, the parent line, expresses neither IA or IE (Fig. 6, *A* and *B*), RT7.1C6 staining demonstrates the presence of IA^b but not IE (Fig. 6, *C* and *D*). Similarly, RT7.3H3 B4 expresses IA^k and no IE (Fig. 6, *E* and *F*). Lastly, the fibroblast line DCEK. Hi7 was transfected with the IE^k and not the IA molecule (Fig. 6, *G* and *H*). When the cell lines were fixed with 1% parafor-

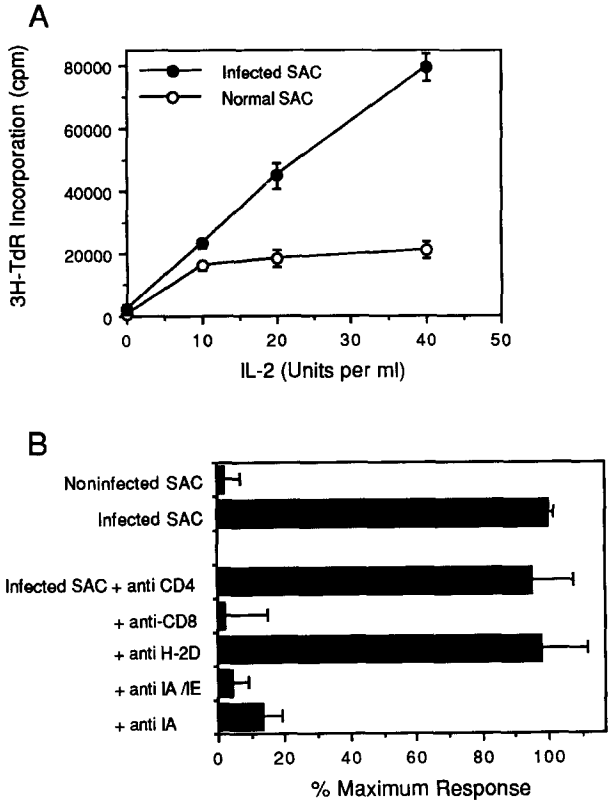

Figure 5. Il-2- and IA-dependent response of purified $CD8^+$ lymphocytes to *T. gondii*. $CD8^+$ cells (2×10^5), purified by Ab and complement-mediated depletion of non-$CD8^+$ cells, were incubated with 2×10^5 irradiated normal and STAg-pulsed SAC and increasing amounts of IL-2 (*A*). (*B*) $CD8^+$ lymphocytes were added to SAC in the presence of 40 U/ml IL-2 and 0.5 μg/ml of the indicated mAb. These experiments were performed three times with essentially identical results.

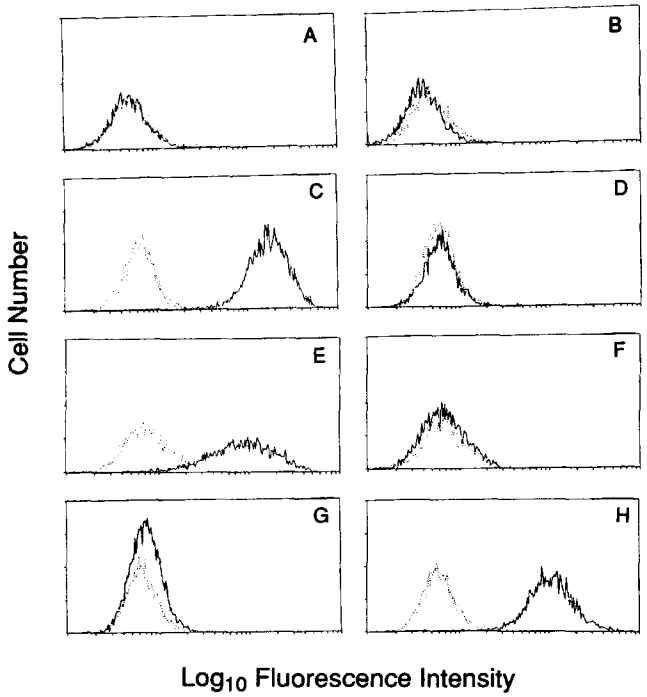

Figure 6. Expression of MHC class II IA and IE molecules by fibroblast cell lines. Cells were stained with (*A*, *C*, *E*, and *G*) mAb to IA and (*B*, *D*, *F*, and *H*) IE followed by FITC-labeled goat anti-mouse IgG. The parent line (*A* and *B*) DAP.3 was transfected with genes encoding the (FT7.1C6; *C* and *D*) IA^b, (RT7.3H3 B4; *E* and *F*) IA^k, and (DCEK Hi7; *G* and *H*) IE^k heterodimer.

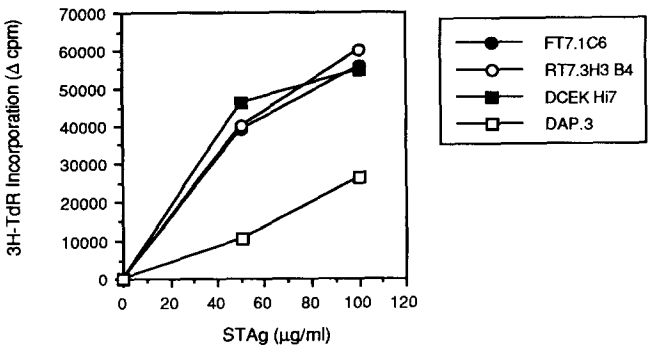

Figure 7. Ability of fixed fibroblasts expressing syngeneic and allogeneic Ia molecules (see legend to Fig. 6) to stimulate C57BL/6 T lymphocytes. Fibroblasts were treated with 1% paraformaldehyde, incubated with STAg, then added to purified T lymphocytes. See Materials and Methods for details. This experiment is representative of three performed.

maldehyde, incubated with STAg, then washed and presented to T cells, the transfectants stimulated strong proliferation of the latter cells in contrast to the parent line, DAP.3 (Fig. 7). The low level of proliferation induced by DAP.3 may reflect nonspecific Ag carryover and subsequent cross-feeding to APC remaining in the responder population.

These results demonstrate that APC presentation of the active parasite molecules to T lymphocytes, while requiring IA molecules, does not require haplotype matching between stimulator and responder populations, and furthermore that IE, which is not expressed in C57BL/6 animals, is able to act as a presentation molecule to responder cells of the latter strain when provided exogenously. Finally, the ability of fixed fibroblasts to present Ag suggests that the response occurs independently of conventional Ag processing. It is unlikely that presentation is due to preexisting peptides in STAg, since these Ag preparations are extensively dialyzed and have previously been shown to lack biologically detectable MHC binding low molecular weight molecules (11).

Table 1. *Cytokine Production in 7-d Cultures of Splenocytes*

Cytokine	Media	Tachyzoites*	STAg‡	Con A§
IFN-γ‖	<1.0	39.5 ± 0.5	24.5 ± 2.5	41.5 ± 3.0
IL-2¶	<1.0	<1.0	<1.0	100.0 ± 6.0
IL-4¶	<0.5	<0.5	<0.5	90.0 ± 1.0
IL-5‖	<0.1	<0.1	<1.0	1.8 ± 0.3

* 10^7 15 kR irradiated tachyzoites plus 10^7 splenocytes.
‡ 50 μg/ml added to 10^7 splenocytes.
§ 1.5 μg/ml.
‖ ng/ml.
¶ U/ml.

Parasite-stimulated T Lymphocytes Selectively Produce IFN-γ. When cytokines present in the supernatants of 7-d cultures of *T. gondii*–stimulated splenocytes were assayed, high levels of IFN-γ were observed after either tachyzoite or soluble Ag stimulation (Table 1). In contrast, neither IL-2, IL-4, nor IL-5 were detected although Con A stimulated production of these cytokines in control cultures.

Table 2. *$CD8^+$ Lymphocytes Are the Major Cell Population Producing IFN-γ in Response to T. gondii*

		Splenic Adherent Cells*	
	Responders‡	Uninfected	Infected§
Experiment 1	Nondepleted	1.86 ± 0.02‖	9.76 ± 0.70
	NK cell depleted	1.10 ± 0.02	7.78 ± 0.66
	T cell depleted	0.82 ± 0.02	1.75 ± 0.40
	None	0.56 ± 0.04	1.06 ± 0.45
Experiment 2	$CD8^+$	0.62 ± 0.31	1.40 ± 0.08
	$CD8^+$ + IL-2¶	1.46 ± 0.22	8.52 ± 0.37
	$CD8^+$ plus IL-2 plus anti-CD8	0.91 ± 0.24	1.77 ± 0.18
	None	1.16 ± 0.08	1.98 ± 0.11

* 2.5×10^5 irradiated (3,000 rad) cells.
‡ Responder cells (2.5×10^5) were obtained by treatment with mAb plus complement. $CD8^+$ cells (90% purity) were similarly obtained followed by passage over anti-mouse Ig-Sepharose. See Materials and Methods for details.
§ Cells were infected with 2×10^7 15-kR irradiated ts-4 tachyzoites.
‖ ng/ml, measured at day 6 of culture.
¶ 40 U/ml.

Table 3. *Association of T. gondii–induced Proliferation with Vβ5 Expression*

Strain	*Mtv-6, 9*	IE	Vβ5*	[³H]-TdR incorporation (cpm)		
				Infected SAC‡		Anti-CD3§
				2 × 10⁵	1 × 10⁵	1 × 10⁵
N:NIH-blk	Unknown	–	9.7 ± 0.7	4.23 ± 0.43	1.21 ± 0.32	1.94 ± 0.21
C57BL/6	–, +	–	6.8 ± 0.6	4.14 ± 0.17	1.01 ± 0.28	2.68 ± 0.15
A.BY	+, –	–	6.1 ± 0.1	5.20 ± 0.69	1.65 ± 0.39	3.17 ± 0.14
BALB.B	+, +	–	4.0 ± 0.3	1.57 ± 0.67	0.87 ± 0.08	2.26 ± 0.12
B10.A(4R)	–, +	–	3.5 ± 0.4	2.02 ± 0.44	0.93 ± 0.06	2.79 ± 0.08
DBA/2	+, –	+	2.7 ± 0.4	4.54 ± 0.20	2.76 ± 1.10	3.32 ± 0.16
B10.D2	–, +	+	1.9 ± 0.4	0.83 ± 0.03	0.68 ± 0.01	ND
C3H/HeN	+, –	+	1.8 ± 0.1	0.22 ± 0.03	0.48 ± 0.02	2.69 ± 0.07
BALB/c	+, +	+	1.6 ± 0.1	0.73 ± 0.57	0.20 ± 0.01	ND
B10.A	–, +	+	0.9 ± 0.2	0.88 ± 0.19	0.13 ± 0.06	3.43 ± 0.24
B10.A(2R)	–, +	+	0.6 ± 0.1	0.95 ± 0.21	0.25 ± 0.02	2.98 ± 0.21

* Percentage of splenic CD3⁺ cells expressing Vβ5 determined by two-color flow cytometric analysis.
‡ Indicated number of splenic T cells were added to infected and noninfected SAC. Data show parasite-induced proliferation (× 10^{-4}) after background subtraction. See Materials and Methods for details.
§ Incorporation of [³H]TdR (× 10^{-5}) induced by plate-bound mAb 145-2C11.

The observed IFN-γ response stimulated by *T. gondii* was almost completely abolished by T cell depletion (92% reduction) but was only minimally affected by anti-NK1.1 treatment (20% reduction) (Table 2, *Experiment 1*) arguing against NK cells (15) as the major source of the cytokine in these cultures. Direct evidence that CD8⁺ cells produce IFN-γ in response to parasite stimulation is shown in experiment 2 (Table 2). As was the case for proliferation (Fig. 5 *A*), purified CD8⁺ lymphocytes produced high levels of the cytokine in response to infected SAC, but only when IL-2 was added. As expected, lymphokine secretion was abrogated with the inclusion of anti-CD8 mAb (1 μg/ml), demonstrating that the IFN-γ originates from the CD8⁺ lymphocytes which constitute the majority of the cell population. The similar level of cytokine produced by purified T cells (Table 2, *Experiment 1*) and CD8⁺ lymphocytes (*Experiment 2*) suggests that the latter cells are the major IFN-γ producers in the Ag-stimulated cultures. Nonetheless, the results do not formally exclude the possibility that residual CD4⁺ lymphocytes contribute to a portion of the IFN-γ produced.

Parasite-induced Proliferative Response Correlates with the Level of Vβ5 Expression in Inbred Mouse Strains. Inbred mice delete to varying degrees Vβ5-bearing cells during lymphopoiesis, giving rise in many cases to extremely low levels of peripheral T lymphocytes bearing this particular TCR chain. This phenomenon is now know to codepend upon expression of MHC class II IE molecules and the endogenous retroviruses *Mtv-9* and *Mtv-6* (16–19) (Table 3). Based on these observations and the data presented above, we predicted that parasite-driven T cell proliferation would be directly related to the degree of expression of peripheral Vβ5⁺ lymphocytes in a given mouse strain. Indeed, we found a highly significant association between Vβ5 expression and proliferation using infected SAC and 2 × 10^5 ($p < 0.015$) or 10^5 ($p < 0.002$) T lymphocytes of different mouse strains (Table 3). In contrast, there was no significant correlation between proliferation and Vβ5 expression in control cultures polyclonally stimulated with anti-CD3 mAb, indicating that the low proliferation of some strains after *T. gondii* stimulation cannot be attributed to a generally diminished T cell responsiveness.

Discussion

The results presented in this paper demonstrate a superantigen activity associated with live *T. gondii* tachyzoites as well as soluble molecules derived from these parasites. To our knowledge these data provide the first evidence of a superantigen expressed by a protozoan pathogen. The proliferation induced by tachyzoites and STAg does not appear to reflect a conventional primary immune response, as has recently been reported for the interaction of *Trypanosoma cruzi* with human PBL (20) and *Leishmania major* with mouse T cells (21), for the following reasons. First, a class II molecule is involved in presentation of Ag to CD8⁺ cells in this system, and whereas conventional Ag is presented to CD8⁺ lymphocytes in association with MHC class I molecules, superantigens such as staphylococcal enterotoxins are able to stimulate CD8⁺ cells through class II glycoproteins (22–24). Second, T cells bearing Vβ5 TCR are selectively expanded after culture with Toxoplasma, and mouse strains expressing low levels of this particular Vβ chain display correspondingly low proliferation after stimulation with *T. gondii*. Third, fibro-

blasts transfected with allogeneic MHC class II molecules can present parasite Ag to T cells. Finally, the response does not require conventional Ag processing since paraformaldehyde-fixed APC are able to present soluble Ag to responder lymphocytes.

Whereas previously described microbial and viral superantigens stimulate $CD4^+$, or $CD4^+$ in conjunction with $CD8^+$ cells (7), the superantigen activity in *T. gondii* is unusual in that $CD8^+$ lymphocytes are selectively expanded. Although the mechanism underlying this phenomenon is unclear, it is possible that whereas both subsets initially respond, $CD8^+$ lymphocytes either multiply faster or produce a factor that inhibits growth of $CD4^+$ cells. Whatever the explanation, it is noteworthy that several investigators (25–28) have reported an increase in $CD8^+$ over $CD4^+$ cells during toxoplasmosis in both mice and humans, possibly providing an in vivo correlate of the observations reported here. Our in vitro system may thus provide a means of dissecting the mechanisms underlying the preferential induction of $CD8^+$ lymphocytes in these cases.

Whereas $CD4^+$ lymphocytes do not persist in culture, their role in the superantigen-driven response is not superfluous. Thus, removal of these cells before Ag stimulation renders the remaining $CD8^+$ cells nonresponsive unless exogenous IL-2 is supplied (Fig. 5). It would seem likely, therefore, that $CD4^+$ lymphocytes provide $CD8^+$ cells with an IL-2–mediated helper function in this in vitro system. Indeed, preliminary evidence (not shown) indicates that Ab-mediated neutralization of IL-2 with mAb blocks the proliferative response. The $CD4^+$ dependency of $CD8^+$ proliferation mimics responses seen in ts-4–vaccinated mice (3, 5) and animals chronically infected with ME49 (2), in that $CD4^+$ cells appear to augment, or synergize with, $CD8^+$ effector function. The experiments reported here suggest a mechanistic basis for the requirement of $CD4^+$ cells in $CD8^+$ lymphocyte function, and demonstrate that the $CD4^+$ dependency can be traced back to the earliest stages of contact between *T. gondii* and cells of the immune system.

The immune response that develops after *T. gondii* infection is characterized by rapid development of strong protective cell-mediated immunity that is highly dependent upon IFN-γ during both acute and chronic phases of infection (2–4, 29). In immunologically intact mice, most of this cytokine appears to derive from $CD8^+$ and Th1-type $CD4^+$ cells (3, 30). Indeed, the effectiveness of Toxoplasma in inducing protective immunity most probably lies in its ability to selectively stimulate this arm of the immune system, and determining how this polarized state is achieved is an issue of considerable importance within the context of vaccine development.

One way of driving the immune system to this response phenotype is by the early *T. gondii*–induced production of IFN-γ, since this cytokine promotes development of both Th1 and $CD8^+$ cells (31, 32). Indeed, during murine *L. major* infection, development of the resistant Th1 phenotype is dependent upon early NK cell production of IFN-γ (33, 34), and recent results from our laboratory demonstrate that *T. gondii* also stimulates NK cell IFN-γ production through an IL-12–dependent mechanism (13, 15, 35). The results of this paper suggest that superantigen-driven stimulation of $CD8^+$ cells could provide an additional early source of IFN-γ, and it is perhaps the combination of these two factors that leads to the remarkably strong cell-mediated response elicited by Toxoplasma.

Our results (Table 3) show a highly significant correlation between Vβ5 expression by different inbred mouse strains and the ability of nonimmune T cells to respond to parasite superantigen. In addition to confirming the association of proliferation with Vβ5 expression, these data argue against Vβ5 expansion being driven by *Mtv-6* or *Mtv-9* induced as a result of *T. gondii* infection for the following reasons. First, *Mtv-6* and *Mtv-9* require IE to mediate their effects (16–19), and this is not the case for the activity described here. In addition, *Mtv-9* reacts with Vβ5 and Vβ11-bearing lymphocytes (36), and we see no evidence for expansion of the latter subset in our experiments. Finally, several of the mouse strains examined lack *Mtv-6* yet still respond. Nevertheless, we cannot formally exclude the possibility that the effects observed here result from parasite-induced expression of a previously undescribed endogenous superantigen.

It is interesting to note that by comparing our findings with inbred strain surveys performed by others (1, 37, 38), it appears that the mouse strains most susceptible to *T. gondii*, as defined by increased mortality, tend to be those expressing high levels of Vβ5. Thus, for example, C57BL/6 mice have high Vβ5 expression and are susceptible to infection, whereas BALB/c mice, having low levels of Vβ5, are resistant. These differences in susceptibility have been ascribed in the past to classical MHC gene effects (1, 37, 38). Our data, however, suggest an alternative mechanism of MHC influence on infection in which the class II IE gene acting in concert with the appropriate endogenous retroviruses could exert an indirect effect by mediating deletion of Vβ5-bearing cells. As a result of this deletion the mouse would be protected against the potentially harmful consequences of a superantigen-driven response. In this model, the response induced by the *T. gondii* superantigen activity, in analogy with those driven by bacterial enterotoxins, would be detrimental as opposed to beneficial to the host (39). Further studies examining the relationship between superantigen responsiveness and parasite-induced pathology are necessary to formally test the latter hypothesis.

In addition to the potential effects of the *T. gondii* superantigen activity in determining the response phenotype of parasite-specific immunity, it is possible that congenitally acquired or chronic toxoplasmosis could lead to elimination or anergy of V$\beta5^+$-bearing lymphocytes, as has been described for other superantigens (40–43). Indeed, preliminary data from our laboratory in which T cells from infected mice appear to lose their responsiveness to anti-Vβ5 stimulation support this hypothesis. Such an occurrence in human populations, where the Toxoplasma infection rate is 30–80% (44), could have important clinical consequences. Indeed, recent evidence suggests that *T. gondii* has superantigen-like effects

on human lymphocytes (Drs. McLeod, R. and D. Mack, personal communication), and consistent with superantigen-induced deletions in the T cell repertoire, congenitally infected infants display lowered T cell responses to *T. gondii* in vitro (45). Thus, individuals may undergo altered immune responses as a result of *T. gondii*–induced changes in the T cell repertoire, a phenomenon that might profoundly affect the ability of individuals to control Toxoplasma infections as well respond to other diseases. Such unresponsiveness should be genetically linked to the alleles determining the TCR specificities responsible for superantigen recognition thereby providing a possible explanation for the variable manifestation of congenital toxoplasmosis in exposed individuals.

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