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Identification of *T. gondii* epitopes, adjuvants, & host genetic factors that influence protection of mice & humans

Tze Guan Tan[1], **Ernest Mui**[1], **Hua Cong**[1,2], **William Witola**[1], **Alexandre Montpetit**[3], **Stephen P. Muench**[4], **John Sidney**[5], **Jeff Alexander**[6], **Alessandro Sette**[5], **Michael Grigg**[7], **Ajesh Maewal**[8], and **Rima McLeod**[1]

[1] Departments of Surgery and Pediatrics (Infectious Disease), Committees on Immunology, Molecular Medicine, and Genetics, Institute of Genomics and Systems Biology, and The College, The University of Chicago, Chicago, Illinois 60615, USA

[2] Department of Parasitology, School of Medicine, Shandong University, Jinan, Shandong 250012, China

[3] Centre d'Innovation, Génome Québec, Montréal, Québec H3A 1A4, Canada

[4] Institute of Membrane and Systems Biology, Faculty of Biological Sciences, University of Leeds, LS2 9JT, United Kingdom

[5] Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, La Jolla, California 92037, USA

[6] Pharmexa-Epimmune, San Diego, California 92121, USA

[7] National Institutes of Health, Bethesda, Maryland 20892, USA

[8] Synthetic Biomolecules, San Diego, California 92121, USA

Abstract

Toxoplasma gondii is an intracellular parasite that causes severe neurologic and ocular disease in immune-compromised and congenitally infected individuals. There is no vaccine protective against human toxoplasmosis. Herein, immunization of L^d mice with HF10 (HPGSVNEFDF) with palmitic acid moieties or a monophosphoryl lipid A derivative elicited potent IFN-γ production from L^d-restricted $CD8^+$ T cells *in vitro* and protected mice. $CD8^+$ T cell peptide epitopes from *T. gondii* dense granule proteins GRA 3, 6, 7, and Sag 1, immunogenic in humans for HLA-$A02^+$, HLA-$A03^+$, and HLA-$B07^+$ cells were identified. Since peptide repertoire presented by MHC class I molecules to $CD8^+$ T cells is shaped by endoplasmic reticulum-associated aminopeptidase (ERAAP), polymorphisms in the human ERAAP gene *ERAP1* were studied and associate with susceptibility to human congenital toxoplasmosis ($p<0.05$). These results have important implications for vaccine development.

Keywords

Toxoplasma gondii; vaccine; HLA Class 1 bound peptides

*Correspondence: MD; rmcleod@midway.uchicago.edu; Phone: 773-834-4133, Fax: 773-834-3577, Univeristy of Chicago, Department of Surgery, 5841 S. Maryland Ave, AMB S206, MC 2114, Chicago, IL 60637.

1. INTRODUCTION

Toxoplasma gondii is an obligate, intracellular, apicomplexan parasite that has a global distribution and a broad host range. The majority of *T. gondii* strains can be classified into 3 distinct archetypal clonal lineages, type I to III, or atypical, which differ in their patterns of virulence[1]. Infection in humans can cause severe ocular, neurologic, and sometimes systemic disease, especially in immunocompromised and congenitally infected individuals[2]. Proinflammatory cytokines produced by macrophages and lymphocytes, such as tumor necrosis factor-α (TNF-α), interleukin-12 (IL-12), and interferon-γ (IFN-γ) are critical for control of both acute and chronic phases of *T. gondii* infection [3,4]. In addition, $CD8^+$ T lymphocytes have been shown to be the major cell subset responsible for protective immunity against *T. gondii* by secreting IFN-γ and developing cytotoxic activity against infected cells [3,5]. Consistent with this, $CD8^+$ cytotoxic T lymphocyte (CTL)-mediated resistance to toxoplasmic encephalitis in H-2^d mice has been definitively mapped to the major histocompatibility complex (MHC) class I L^d allele[6]. Moreover, immune L^d-restricted CTLs are strain-specific, killing target cells that are infected with the type II Me49 parasite strain and its derivatives but not those infected with type I strains[7].

At present, there are no chemotherapeutic agents to definitively and completely prevent or cure *T. gondii* infection in humans[8,9]. A vaccine could solve these problems. The ideal vaccine against toxoplasmosis in humans would include antigens that can elicit a protective T helper cell type 1 (Th1) immune response, and characterized by the generation of long-lived IFN-γ-producing $CD8^+$ T cells. The administration of live attenuated tachyzoites such as the temperature-sensitive ts-4 strain has been found to induce a potent, protective cell-mediated immune response in animals [3,10] (Hutson et al., unpublished data), although live vaccines are generally considered insufficiently safe for use in humans. Studies have identified a number of *T. gondii* surface antigens (SAG) and secreted proteins such as the dense granule, rhoptry, and microneme proteins as antigens recognized by murine T lymphocytes [11]. Indeed, vaccine formulations based on native, recombinant proteins or DNA vaccine forms of these antigens have resulted in reduced mortality and cyst burden in infected animals [12–14]. Nonetheless, protection was incomplete because sterile immunity was not achieved.

Recently, Blanchard et al. demonstrated that a single decapeptide HF10 (HPGSVNEFDF), derived from the dense granule protein 6 (GRA6), was the protective, immunodominant L^d-restricted epitope in H-2^d mice infected with the Prugneaud, a type II, *T. gondii* strain [15]. Endogenous generation of this immunodominant peptide was critically dependent on the endoplasmic reticulum-associated aminopeptidase (ERAAP), a protease that trims precursor peptides delivered into the endoplasmic reticulum lumen to the requisite 8- to 10-amino acid length for loading onto MHC class I molecules[15]. L^d and human MHC molecules belonging to the HLA-B07 supertype exhibit a similar peptide binding motif as determined empirically from the specificity of their main anchor residues [16,17]. There is functional overlap in L^d and HLA-B07 in peptide binding shown in binding studies and crystal structures of various B7 supertype alleles are similar to L^d [18]. Consequently, it seemed highly likely that HF10 would also be the immunodominant epitope generated during the course of natural infection by type II *T. gondii* strains in HLA-$B07^+$ individuals. This provided a paradigm for evaluating the protective mechanisms of various HF10-containing vaccine formulations in H-2^d and HLA-$B07^+$ transgenic murine models. Interestingly, 3 other distinct L^d-restricted peptides were separately identified as immunogenic epitopes in H-2^d mice [19,20], thereby making the model of a single, immunodominant $CD8^+$ T cell epitope in *T. gondii* infections more complex. Thus, it remains important to define immunogenic epitopes restricted by HLA-B07 and other HLA supertypes for the development of an efficacious vaccine in humans.

The success of a vaccine also depends on other factors, including immuno-stimulatory properties of adjuvants employed and delivery system for both antigen and adjuvant. Conjugation of $CD8^+$ T cell determinants to lipid groups is known to enhance specific cell-mediated responses to target antigens in animals and humans, although mechanisms whereby immunity is achieved remains poorly understood. In particular, palmitoylated lipopeptide constructs have been demonstrated to elicit long-lived, protective cellular responses against a variety of pathogens, including Hepatitis B virus (HBV), influenza virus, and *Plasmodium falciparum* [21–24]. Lipopeptides hold several advantages over other conventional vaccine formulations; for instance, they are self-adjuvanting and display none of the toxicity-associated side effects of Th1-inducing adjuvant systems such as saponin or QS21 [25]. They are also convenient to store at ambient temperature and can be chemically synthesized, thus avoiding potential contamination problems associated with bacterial or other biosynthetic systems. There have also been a number of studies indicating that lipopeptides and lipid-associated peptide formulations are remarkably effective at inducing mucosal immunity, which represents a major goal for vaccines against *T. gondii* infection [23,26]. The inclusion of PADRE, a synthetic peptide that binds promiscuously to variants of the human MHC class II molecule DR and is effective in mice, in lipopeptides and DNA vaccine vectors containing CpG motifs, also can augment $CD8^+$ T cell effector functions by inducing $CD4^+$ T helper cells [21,27]. Detoxified derivatives of lipopolysaccharide (LPS), such as monophosphoryl lipid A (MLA), also are potent activators of Th1 responses via the Toll-like receptor 4 (TLR4) signaling pathway in macrophages and dendritic cells [28].

Herein, we designed 3 HF10-containing vaccine formulations comprising 2 lipopeptides that incorporate PADRE, as well as an oil-in-water emulsion that includes a synthetic MLA derivative. The efficacy of these vaccines in eliciting L^d-restricted, $CD8^+$ T cell-mediated IFN-γ production *in vitro* from H-2^d BALB/c mice was examined. To determine if HF10 could be an immunodominant epitope generated for HLA-$B07^+$, we tested HF10 immunized HLA-$B07^+$ transgenic mice and naturally infected HLA-$B07^+$ individuals for their reactivity to HF10 as determined by the levels of IFN-γ secretion. We also utilized bioinformatic algorithms to identify novel, *T. gondii*-derived, $CD8^+$ T cell epitopes restricted by the HLA-A02, -A03, and -B07 supertypes, which collectively provide broad coverage for over 80 to 90% of the human population worldwide, regardless of ethnicity [29]. Since ERAAP shapes the final repertoire of peptide antigens presented by MHC class I molecules to $CD8^+$ T cells and ERAAP-deficient mice are much more susceptible to *T. gondii* than control mice [30], we hypothesized that polymorphisms in the human ERAAP gene that is orthologous to mouse ERAAP, *ERAP1*, might influence the outcome of congenital parasite infection in people. Indeed, our analysis of a cohort of congenitally infected children and their parents using the transmission disequilibrium test (TDT) indicates that congenital toxoplasmosis associates with 2 single nucleotide polymorphisms (SNPs) in *ERAP1*.

2. METHODS

2.1 Peptides, lipopeptides, and formulated MLA mimetic

All peptides and both HF10-containing lipopeptides were synthesized by Synthetic Biomolecules (San Diego, CA) at >90% purity in lyophilized form. Both lipopeptides have structures similar to previously described lipidated constructs [21,22]. Murine cytomegalovirus (MCMV) pp89-derived peptide YL9 (YPHFMPTNL) and Hepatitis C virus (HCV) core 169 (LF9 (LPGCSFSIF)) were used as irrelevant L^d-restricted and HLA*B0702-restricted peptides respectively. The TLR4 agonist was a MLA mimetic that was synthesized by the Infectious Diseases Research Institute (IDRI, Seattle, WA) as a stable oil-in-water emulsion.

2.2 Mice

BALB/c mice were obtained from Taconic Farms. HLA-B*0702 transgenic mice[31] were produced at Pharmexa-Epimmune (San Diego, CA) and bred at the University of Chicago. All studies were conducted with the approval of the Institutional Animal Care and Use Committee at the University of Chicago.

2.3 Immunizations

Age-matched female BALB/c mice were inoculated subcutaneously (s.c.) at the base of the tail using a 27-gauge needle with 50 μg HF10, 50 μg HF10 and 20 μg EM005, or 50 nmol of each lipopeptide in 100 μl of phosphate-buffered saline (PBS). As controls, mice were injected with PBS or a combination of PBS and 20 μg EM005. Mice that received PBS/EM005, HF10, and HF10/EM005 were boosted twice at 2-week intervals, while mice that received PBS and either of the 2 lipopeptides were boosted once at 21 days after the first immunization. Sex- and age-matched HLA-B*0702 mice were inoculated s.c. at the scruff of the neck with the same peptide immunogens at the same time intervals.

2.4 Peripheral blood mononuclear cells

Cryopreserved peripheral blood mononuclear cells (PBMC) from both *T. gondii*-seropositive and seronegative individuals were used in the ELISPOT assay. PBMCs were previously isolated from heparinized blood samples by density gradient centrifugation using Histopaque (Sigma-Aldrich), washed twice with PBS, and cryopreserved in AIM-V medium (Gibco) containing 20% FCS and 10% DMSO. All blood samples were obtained with written informed consent from the donors and in accordance with institutional and NIH guidelines.

2.5 *T. gondii* serotyping and HLA typing of chronically infected individuals

Serotyping of the *T. gondii* strain in infected individuals was performed by Michael Grigg (National Institutes of Health) as previously described [32]. HLA haplotype analysis of seropositive individuals was determined using serologic typing by Paul Terasaki (UCLA, Tissue Typing Laboratory, Los Angeles, CA) as previously described [33].

2.6 *Ex vivo* preparation of murine splenocytes

Mice were euthanized 7 to 14 days after the last immunization. Spleens were harvested, pressed through a 70 μm screener to form a single-cell suspension, and depleted of erythrocytes with AKC lysis buffer (160 mM NH_4Cl, 10 mM $KHCO_3$, 100 μM EDTA). The remaining splenocytes were washed twice with Hank's Balanced Salt Solution (HBSS) and resuspended in complete RPMI medium (RPMI-1640 supplemented with 2 mM L-GlutaMax [Invitrogen], 100U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, and 10% FCS) before they were used in subsequent *in vitro* assays.

2.7 Enzyme-linked immunospot (ELISPOT) assay

For human ELISPOT assays, MSIPS4W10 Multiscreen HTS-IP 96-well plates (Millipore, Bedford, MA) were coated with 50 μl of 15 μg/ml anti-human IFN-γ (1-D1K) monoclonal antibody (mAb) in sterile PBS overnight at 4°C. Wells were washed with sterile PBS and blocked with RPMI-1640 medium containing 10% FCS at room temperature for 2–3 hr. PBMCs were then plated in complete RPMI-1640 medium at 2×10^5 cells per well. Peptide or peptide pools were added to each well at 10 μg/ml and plates were incubated at 37°C and 5% CO_2 for 20–24 hr. Media containing an equivalent concentration of DMSO was used to measure background response levels, and 5 μg/ml of *T. gondii* lysate antigen (TLA), 2.5 μg/ml of Concavalin A, or 10 μg/ml of a relevant HLA-B07-restricted Epstein-Barr virus (EBV)-derived peptide[31] was used as a positive control. Plates were successively incubated with 100 μl of

1 μg/ml biotinylated anti-human IFN-γ mAb (7B6-1) for 2 hr and streptavidin-conjugated alkaline phosphatase at a 1:1000 dilution for 1 hr at room temperature, before spots were developed using 5-bromo-4-chloro-3-indolyl-phosphate/*p*-nitro blue tetrazolium chloride (BCIP/NBT). Wells were washed with PBS in between incubation stages. Spot formation was quenched by extensive washing with distilled water. Plates were air-dried overnight at 4°C, and spots counted the next day using an automated ELISPOT reader (CTL ImmunoSpot).

Murine ELISPOT assays were performed similarly, except that anti-mouse IFN-γ mAb (AN18) and the biotinylated anti-mouse IFN-γ mAb (R4-6A2) were used as the cytokine-specific capture antibodies instead. In addition, 5×10^5 splenocytes were plated per well. All antibodies and reagents used for the ELISPOT assay were obtained from Mabtech (Cincinnati, OH). Cells were plated in at least 3 replicate wells for each condition. Results were expressed as the number of spot forming cells (SFCs) per 10^6 PBMCs or per 10^6 murine splenocytes.

2.8 Antibody blocking in ELISPOT

Murine splenocytes were incubated with the relevant blocking antibody for 1.5 to 2 hr at 37° C and 5% CO_2 before they were seeded at 2.5 to 5×10^5 cells per well. Anti-CD4 (RM4-5, BioLegend), Anti-CD8α (53-6.7, BD Biosciences) mAb, and their relevant isotype control (Rat IgG_{2a}, BD Biosciences) were added to each sample at a final concentration of 10 μg/ml. To assay for L^d-restricted IFN-γ production, splenocytes were incubated with 0.5 μg of FcBlock™ (BD Biosciences) per 10^6 cells for 10–15 min at 4°C and washed before anti-L^d (BioLegend) mAb or its isotype control (mouse IgG_{2a}, BioLegend) was added at a final concentration of 20 μg/ml. Results were expressed as the percentage of SFCs generated in the presence of antibody relative to that in the absence of antibody as follows: percent SFC production = (number of SFCs in presence of antibody)/(number of SFCs in absence of antibody) × 100% ± s.e.m.

2.9 Bioinformatic prediction of $CD8^+$ T cell epitopes

A total of 9 surface and secreted proteins (SAG1, SUSA1, GRA2, GRA3, GRA6, GRA7, ROP2, ROP16, ROP18) from the type II *T. gondii* strain, Me49, were selected for bioinformatic analysis. Initially, protein sequences were screened for nonameric $CD8^+$ T cell epitopes on the basis of their predicted binding affinity to HLA-A02, -A03, or -B07 supertype molecules using the ARB, SMM, and ANN algorithms, as well as predicted proteasomal cleavage and processing by TAP as previously described [34,35]. A total of 118 unique peptides representing the top 1% of all ranked nonameric peptides were identified (Supplementary Table 1–3). All protein sequences were obtained from ToxoDB 5.1 (http://toxodb.org/toxo/). Subsequently, a new algothythm was utilized (http://www.immuneepitope.org). Peptides from the proteins listed above that were present in both Type I and II parasite with predicted avidities <100 nM were tested in the IFN-γ Elispot assays described below.

2.10 Modeling of peptide/MHC structures

The previously determined crystal structures of L^d (Protein Data Bank accession code 1LDP) and HLA-B*3501 (Protein Data Bank accession code 2CIK), a HLA-B07 supertype molecule, were used as templates to model the respective HF10/MHC complexes. The molecular graphics program PyMol was used to generate the structure figures.

2.11 Parasites

The transgenic *T. gondii* strain used for *in vivo* challenges in this work, Pru-FLUC, was derived from the type II Prugneaud (Pru) strain and expresses the firefly luciferase (FLUC) gene constitutively by tachyzoites and bradyzoites. It was created, maintained and utilized as previously described [36].

2.12 Patient cohort and genotyping of *ERAP1* and *ERAP2*

Case-parent trios were from the National Collaborative Chicago-based Congenital Toxoplasmosis Study (NCCCTS) as previously described [37]. Briefly, the NCCCTS consisted of 176 North American children clinically diagnosed with congenital toxoplasmosis and their parents. DNA was successfully extracted from 149 case-parent samples and genotyped at 18 SNPs in *ERAP1* and 7 SNPs in *ERAP2*. Of these 149 infected children, 124 (83%) had clinically confirmed brain calcifications with/without hydrocephalus and/or retinal lesions at birth or time of diagnosis.

2.13 *In vivo* bioluminescence imaging

Mice infected with Pru-FLUC tachyzoites[36] were imaged at 5 to 7 days post-challenge using the *in vivo* imaging system (IVIS; Xenogen, Alamedia, CA) as previously described [38]. Briefly, mice were injected i.p. with 200 μl of D-luciferin and immediately anesthetized in an O_2-rich induction chamber with 2% isofluorane. After 12 min, mice were imaged in ventral positions and photonic emissions were assessed using Living image® 2.20.1 software (Xenogen). Data were presented as pseudocolor representations of light intensity and mean photons/s/region of interest (ROI).

2.14 MHC-peptide binding assays

Quantitative assays to measure the binding of peptides to HLA class I molecules are based on the inhibition of binding of a radiolabeled standard peptide. MHC molecules were purified by affinity chromatography from EBV transformed or single allele transfected 721.221 cell lines, and assays performed, as described previously [39–41]. Peptides used in this assay included: HLA-A2: VVFVVFMGV (GRA6); FMGVLVNSL (GRA6); FLVPFVVF (GRA3); HLA-A3 KSFKDILPK (SAG1); AMLTAFFLR (GRA6); RSFKDLLKK (GRA7); HLA-B7: LPQFATAAT (GRA7); VPFVVFLVA (GRA3); HPGSVNEFDF (GRA6). Peptides were tested at six different concentrations covering a 100,000-fold dose range in three or more independent assays, and the concentration of peptide yielding 50% inhibition of the binding of the radiolabeled probe peptide (IC_{50}) was calculated. Under the conditions used, where [radiolabeled probe] < [MHC] and $IC_{50} \geq$ [MHC], the measured IC_{50} values are reasonable approximations of the true K_d values [42,43].

2.15 Statistical analyses

Statistical analyses for all *in vitro* assays were performed using a 2-tailed student's *t* test. Peptides were considered immunogenic in mice if they induced IFN-γ spot formation from immunized mice that was significant ($p<0.05$) relative to: (i) spot formation from control mice, and (ii) spot formation from immunized mice incubated with an irrelevant peptide. Peptides and peptide pools were considered immunogenic in humans if they induced IFN-γ spot formation from PBMCs that was significant ($p<0.05$) relative to spot formation from the same cells incubated with media containing an equivalent concentration of DMSO. All Elispot experiments were replicated a minimum of twice. The experiment with luciferase expressing parasites used to challenge immunized BALB/c mice was performed with a total of 5–9 mice per treatment group. Different mice were imaged on each of the three days (5–7 days after challenge). For the genetic study, tag SNPs were selected from the HapMap project in *ERAP1* and *ERAP2* using 10 kb flanking sequence on each side, a MAF cutoff of 5% in CEU and r^2 threshold of 0.8. Allelic association analysis was performed for the 124 infected children in the cohort with confirmed clinical findings in the eye and/or brain using a conventional TDT and *p* values were calculated using Haploview (http://www.broadinstitute.org/haploview). *P* values of 0.05 or less were considered significant for association with disease.

3. RESULTS

3.1 Lipopeptides and HF-10 with MLA elicit potent IFN-γ-production from L^d-restricted $CD8^+$ T cells *in vitro* and partially protect mice

IFN-γ was defined as a key mediator of protective immunity during both acute and chronic phases of disease in mice [4]. To evaluate adjuvancy of lipid groups and MLA in vaccines against *T. gondii* infection, we designed and studied ability of 2 lipopeptides (Figure 1A,B) and a MLA/HF10 mixture to induce antigen-specific IFN-γ production in H-2^d mice. BALB/c (H-2^d) mice were immunized with the HF10-adjuvant formulations and spleens harvested 7 to 14 days after the last inoculation. Frequency of IFN-γ-secreting splenic cells generated in immunized mice and control mice was measured and compared using an *ex vivo* ELISPOT assay. Both lipopeptides and MLA/HF10 elicited significantly higher HF10-specific IFN-γ production relative to control mice (Figure 1C, $p<0.01$). In addition, there was no significant difference in IFN-γ spot formation among mice that received the 3 different vaccine formulations ($p>0.05$). To assess the relative contribution of $CD8^+$ T cells to overall IFN-γ response, we blocked activity of splenic $CD4^+$ and $CD8^+$ T cells using antibodies (Ab) specific to those 2 surface co-receptors. IFN-γ spot formation was significantly reduced in the presence of anti-CD8 Ab ($p<0.01$) but not anti-CD4 Ab when compared to spot formation induced by isotype control Ab (Figure 1D). L^d restriction was also demonstrated by abrogation of IFN-γ responses with Ab to L^d but not with its isotype control Ab (Figure 1E, $p<0.05$). Thus, all 3 adjuvant systems elicited significant frequencies of IFN-γ-producing L^d-restricted $CD8^+$ T cells in immunized mice. Mice were partially protected, i.e. reduced luciferase expessing parasites, by these immunizations (Figure 2). Protection was present for each of the immunized groups of mice, each of which had spleen cells that produced IFN-γ in ELISPOT assays when cultured with HF10 in parallel experiments. All adjuvanted immunizations protected mice on each of the 3 days tested. Different mice were imaged on each of the 3 days, 5–7 days after challenge. There were a total of 5–9 mice in each control or immunized group.

3.2 Absence of Immunogenicity of HF10 in HLA-B*0702 transgenic mice

Studies have shown that many polymorphic MHC class I molecules bind overlapping peptide repertoires, which are to a large extent determined by anchor residues at the second and C-terminal positions of presented peptides. We noted that L^d shares the same peptide binding motif as the HLA-B07 supertype of human MHC molecules; both present peptides containing proline at the second position and aliphatic or hydrophobic residues at the C-terminal end [16,17]. Consequently, we hypothesized that immunogenic *T. gondii*-derived epitopes that are bound by L^d in mice (such as HF10) might be similarly presented by HLA-B07 supertype molecules in humans. Bioinformatic analysis also predicted a relatively high binding affinity of HLA-B07 supertype molecules to HF10. We therefore immunized HLA-B*0702 mice with the same HF10-containing vaccine formulations that were previously administered to BALB/c mice and demonstrated to elicit significant IFN-γ-producing $CD8^+$ T cells *in vitro*. Splenocytes were similarly harvested from immunized HLA-B*0702 mice and stimulated with HF10 or an irrelevant HLA-B07-restricted peptide, LF9 in an ELISPOT assay. Contrary to our prediction, neither lipopeptide nor the MLA/HF10 combination generated significant numbers of IFN-γ spots relative to the control mice, although very modest but not significant levels of IFN-γ were produced to HF10 compared to those for LF9 in the immunized mice (Figure 3A). The functionality of our experimental system was confirmed using a positive control group of mice that were inoculated with an immunogenic, HLA-B07-restricted, EBV-derived peptide and which produced significant frequencies of IFN-γ spots in response to the relevant EBV antigen (data not shown). Thus, HF10 was not immunogenic in HLA-B*0702 mice when administered as lipopeptide or with MLA.

3.3 Absense of Immunogenicity of HF10 and 3 other L^d-restricted *T. gondii* epitopes in HLA-B07$^+$ humans

Although relevance of HLA-B*0702 mice as a model system for identifying human HLA-B07-restricted epitopes has been previously described [31], it was still possible that H-2^b molecules which are also expressed in the transgenic mice to interfere with presentation of HF10 by HLA-B*0702. To directly determine immunogenicity of HF10 in humans, we tested PBMCs from *T. gondii*-infected donors for their reactivity to HF10 using an ELISPOT assay. All donors were seropositive for the type II strain of *T. gondii* (data not shown) and expressed HLA-B07 supertype alleles (referred to as HLA-B07$^+$). None of the 4 seropositive individuals tested at times from acquisition of their infection ranging from months to 35 years exhibited significant IFN-γ responses to HF10 (Figure 3B), even when peptide concentration was titrated upwards to 50 μg/ml and stimulation period was extended to 48 hrs (data not shown). We therefore concluded that HF10 was not the type II *T. gondii* epitope that was naturally processed and presented by HLA-B07 supertype molecules to CD8$^+$ T cells in humans.

It was notable that HF10 did not represent the only L^d-restricted epitope that has been identified to be immunogenic in *T. gondii*-infected mice. Recent work by Frickel et al. suggest that 2 unique nonameric epitopes (ROP7-derived IF9 [IPAAAGRFF] and GRA4-derived SM9 [SPMNGGYYM]) were generated in a stage-specific manner during natural infections in BALB/c mice [20]. In addition, a SAG1-derived peptide TL9 (TPTENHFTL) was found to be immunogenic and partially protective in H-2^d mice [19]. We hypothesized that 1 or more of these L^d-bound epitopes might be recognized in HLA-B07$^+$ individuals instead. However, none of the peptides was found to elicit IFN-γ production by HLA-B07$^+$ human cells (Figure 3B).

Thus, there appeared to be a disparity between peptide repertoires naturally presented by L^d and HLA-B07 supertype molecules to CD8$^+$ T cells in mice and humans despite their similar binding motifs. To test this hypothesis, we modeled L^d/HF10 and HLA-B07/HF10 peptide/MHC (pMHC) structures based on previously determined crystal structures of pMHC complexes involving L^d and HLA-B*3501 [44,45], a HLA-B07 supertype molecule [17]. The peptide-binding pocket of both murine and human MHC molecules displayed good surface complementarity to bound peptide, with no obvious predicted steric occlusion between peptide and MHC molecule (Figure 3C,D). A moderate electrostatic complementarity was also observed, with basic patches of peptide-binding pocket proximal to acidic residues (Glu and Asp) in HF10 and hydrophobic regions of peptide-binding pocket surrounding remaining nonpolar residues of HF10 (Figure 3C,D). Hence there did not appear to be significant differences in modeled structures of both pMHC complexes that might indicate a bias for binding of HF10 to L^d instead of HLA-B*3501, or that L^d and HLA-B07 supertype molecules would bind significantly different peptide repertoires. Our preliminary structural analysis (Figure 3C,D) thus was consistent with bioinformatic predictions of overlapping binding motifs of L^d and HLA-B07 supertype molecules. Although there were no differences in configuration of L^d or individual HLA-B07 supertype members there were differences both in size (L^d larger) and charge that we thus hypothesized must be responsible for the lack of immunogenicity of HF10 and other L^d binding peptides for HLAB7 humans and transgenic mice.

3.4 Identification of candidate CD8$^+$ T cell epitopes restricted by HLA-A02, -A03, and -B07 supertypes

Since the GRA6 peptide HF10, and the ROP7, GRA4, and SAG1 peptides that bound L^d did not elicit a CD8$^+$ T cell response in the HLA B7 persons tested or the HLA B7 mice, to attempt to directly identify CD8$^+$ T cell epitopes in humans, we utilized several bioinformatic algorithms to screen a panel of 9 candidate proteins for potential epitopes that might be endogenously processed and presented by the HLA-A02, -A03, and -B07 supertypes.

Candidate proteins include 2 surface antigens (SAG1, and SUSA1, a surface marker specific to the slow-growing, bradyzoite form of *T. gondii*) and 7 secreted proteins (GRA2, GRA3, GRA6, GRA7, ROP2, ROP16, ROP18) that have previously been reported to be highly immunogenic in mice and humans or to reach host cell cytoplasm, as is necessary for entry into a MHC class I processing pathway [46]. A total of 118 unique nonameric peptides were identified (42 for HLA-A02, 40 for HLA-A03, and 36 for HLA-B07) and screened in pools (Tables A1–A3) for reactivity in MHC-matched individuals who were seropositive for the type II strain of *T. gondii*. We observed that 1 HLA-A02-restricted pool, A2.P4, induced significant IFN-γ production from all 3 HLA-A02 tested individuals ($p<0.01$, Table 1 and Figure 4A). Intriguingly, only 1 out of 3 donors responded significantly to the HLA-A03-restricted pool, A3.P2, or the HLA-B07-restricted pool, B7.P1 (Table 1 and Figure 4B,C). Moreover, the B7.P1 pool also elicited modest IFN-γ spot formation from a second HLA-B07$^+$ individual (Figure 4C, $p=0.087$). This pool might therefore contain peptides that are only moderately immunogenic in HLA-B07$^+$ humans, possibly due to an intermediate peptide binding affinity for HLA-B07 supertype molecules. Given the considerable genetic and environmental variability across the human population, it is not entirely surprising that individuals might respond differently to the same peptides. Nevertheless, the 3 aforesaid peptide pools, A2.P4, A3.P2, and B7.P1, represented interesting targets for further deconvolution studies so that individual immunogenic epitopes might be defined.

Subsequent studies were performed following the studies with deconvolution of peptide pools found with the initial algorhytms decribed above. Selection of additional predicted best binding peptides was based on peptides identified using a new algorithm at http://www.immuneepitope.org. We selected peptides with predicted binding avidities <100 nM that were present in both Type I and II parasites. Those peptides that elicited IFN-γ from T cells from all of the seropositive persons of the specific haplotype are: HLA-A02 (GRA6 [VVFVVFMGV], GRA6[FMGVLVNSL], GRA3[FLVPFVVFL]), HLA-B07 (GRA7 [LPQFATAAT], GRA3[VPFVVFLVA]), and HLA-A03 (SAG1[KSFKDILPK], GRA7 [RSFKDLLKK], GRA6[AMLTAFFLR]). Figures 4D,E,F show data from one seropositive person of each haplotype. The data in Figures 4D, E and F show responses that are representative of data from each of the seropositive persons. Specifically, four of 4 seropositive persons of each haplotype (4 with A2, 4 with A3-11, and 4 with B7) responded to each of the peptides identified and listed above, for their haplotype. Three of these seropositive persons were then tested with these 8 peptides pooled and all three also responded to the pooled peptides. For seronegative persons, 0 of 3 A2 persons, 0 of 4 A3-11 persons and 0 of 2 B7 persons responded to the peptides pooled. These data for seronegative persons are not shown, as there was no response to these peptides

These are the first *T. gondii* octamer/nonamer peptides predicted to bind to HLA Class I molecules that have been identified. These are the first *T. gondii* octamer/nonamer peptides that have been found to elicit IFN-γ production by human T cells from seropositive and not seronegative persons.

3.5 Binding Assays

Binding assays identified physiologically relevant differences in binding avidity for the peptides noted to stimulate T cell production of IFN-γ in each of the seropositive persons of each supermotif haplotype and none of the seronegative controls of each supermotif hypothesized. (Table 2). HF10 (HPGSVNEFDF) also was tested. With the exception of HPGSVNEFDF all of the peptides identified were good binders, binding 3 or more alleles within the respective supertype with an affinity of 500 nM, or better. The HF10 peptide, HPGSVNEFDF bound several HLA B7 alleles with only weak affinity (500–5000 nM), but

none with high affinity This finding explains the lack of response to HF10 in people and mice HLA-B07.

3.6 Congenital toxoplasmosis associates with *ERAP1* in humans

Previous studies have identified ERAAP as a 'susceptibility gene' for *T. gondii* infection in mice because ERAAP is required for proteolytic generation of the immunodominant HF10 epitope. We hypothesized that ERAAP might also play a critical immunomodulatory role in humans by shaping the peptide repertoire presented by MHC class I molecules to protective $CD8^+$ T cells. To test this hypothesis, we genotyped a total of 32 tag SNPs that were selected in the human ERAAP genes, *ERAP1* (also *ARTS-1*) which is the homologue of the mouse ERAAP gene and *ERAP2* (also *L-RAP*) in a North American cohort of case-parent trios comprising 124 congenitally infected children, many with ocular and/or brain disease, using a r^2 threshold of 0.8. Overall, 25 SNPs passed all the genotyping QCs. We found that 2 SNPs in *ERAP1*, rs149173 and rs17481856, associated with congenital toxoplasmosis (Table 3, $p<0.05$), while none of the 7 SNPs in *ERAP2* was observed to associate with disease (Table 3, $p>0.05$). The 2 SNPs that showed significant association with congenital disease, rs149173 and rs17481856, were in high LD with each other ($r^2 = 0.4$) and are therefore likely part of the same associated haplotype (Figure 5). This suggests that one of those SNPs or an etiological variant in strong LD with these 2 markers accounts for the observed association with susceptibility to this congenital disease. Although neither rs149173 nor rs17481856 result in nonsynonymous amino acid changes in *ERAP1* other variants in strong LD with these likely are responsible for the observed association through a change in the protein sequence or through regulation of ERAP1 expression *in vivo* (for example rs27044 causes Glu to Gln change and has r^2 of 0.4 as does rs17481856, although slightly less associated with the trait). This work provides the first evidence for *ERAP1* as a 'susceptibility gene' in *T. gondii* infection in humans and confirms the importance of MHC Class I antigen processing in humans in response to this infection.

4. DISCUSSION

In this study, we showed that palmitoylated lipopeptides elicit potent IFN-γ production *in vitro*, comparable to that induced by the synthetic TLR4 agonist, MLA. Furthermore, IFN-γ responses were primarily dependent on antigen-specific, L^d-restricted $CD8^+$ T cells, which are critical mediators of protective immunity in *T. gondii*-infected mice. However, HF1O was not immunogenic in HLA-B*0702 mice, and neither HF10 nor 3 other L^d-restricted *T. gonii* candidate peptides were found to be immunogenic in infected $HLA\text{-}B07^+$ humans, even though bioinformatic and structural analyses suggest that both L^d and HLA-B07 supertype molecules bind extensively overlapping peptide repertoires. We then identified additional epitopes from *T. gondii* dense granule protein GRA6, GRA7, GRA3, and surface antigen 1, SAG1, that are immunogenic in people expressing HLA-A02, -A03, and -B07 supertype alleles. In addition, we showed for the first time that 2 SNPs in *ERAP1* that were in strong LD with each other associated with susceptibility to congenital toxoplasmosis in humans.

Administration of HF10 with adjuvants protects mice. In Figure 1C we show that HF10 alone does not induce IFN-γ but HF10 included in lipopeptide constructs does. Immunization with lipopeptides protected mice against challenge with *T. gondii*. We selected lipopeptides to test because this formulation has successfully induced other $CD8^+$ T cell reponses in humans as well as mice [21–24]. The lipopeptides we used were selected because of prior demonstrations by others that they were immunogenic and stable. Specifically, Livingston et al (21) used a 2 PAM Cys lipopeptide formulation in studies in humans, finding this to be effective in eliciting an immune response against hepatitis B. Deliyannis et al (22) used a 4 PAM Cys lipopeptide formulation in studies in mice, finding this to be effective in producing prolonged memory

CD8 + T cells directed against influenza virus. Thus, these constructs were selected for our proof of principal studies to attempt to induce a protective immune response against *T. gondii*. In our laboratory for these studies, lipopeptides have been active in eliciting IFN-γ, in experiments performed during a period of 8 months after they were synthesized. They were stored at −20 degrees C during this time prior to care.

The new MLA-like adjuvant was provided by The Infectious Disease Research Institute Seattle (IDRI) and it has been very effective as an adjuvant providing $CD4^+$ T cell help for immunizations against other protozoan infections [25] [S. Reed, IDRI, Seattle, personal communication to RMc, 2009]. Our studies provide initial evidence *in vitro* and *in vivo* for efficacy of lipopeptides as inducing protective immune responses against *T. gondii*. MLA also was an effective adjuvant. While MLA promotes a Th1 response by activating TLR4, mechanisms by which lipopeptides enhance cell-mediated immune responses remain to be defined, with speculation that they may be TLR2 ligands. Earlier studies have suggested that lipopeptides upregulate antigen presentation by dendritic cells and macrophages to T cells, possibly by associating with hydrophobic cell membranes, or otherwise inducing maturation of professional antigen-presenting cells and consequent release of pro-inflammatory cytokines [23,47,48]. Lipopeptides represent attractive candidates as vaccines, because they are safe, easy to manufacture and store, and do not require additional adjuvants [21–24]. Future studies will investigate relative immunogenicity of other lipid-based vaccines such as NISV or bilosome enclosed peptides or DNA vaccines, as well as efficacy of different routes of vaccine delivery and use of MLA with lipopeptides. Both lipopeptides and liposomes have also been shown to induce mucosal immune responses, which are crucial for limiting toxoplasmic pathology and transmission since initial infection in humans occurs via the gastrointestinal tract through ingestion of oocysts or encysted bradyzoites in undercooked meat [11], except in cases of congenital infection. We will also determine whether sterile, long-term immunity can be attained in immunized mice when challenged with a type II strain of *T. gondii* and whether co-delivery of cytokines such as interleukin-15 (IL-15), IL-12, or inhibition of TOR with rapamycin enhance antigen-specific recall responses.

Initially, quite, surprisingly, our results in HLA-B*0702 mice and $HLA\text{-}B07^+$ individuals suggested that HF10 was not the immunogenic epitope presented by HLA-B07 supertype molecules to $CD8^+$ T cells in mice and humans. We confirmed that the assay was satisfactory and robust with both EBV and later with specific *T. gondii* peptides. This lack of immunogeneticity of HF10 was confirmed in our studies with HLA-B07 transgenic mice, where other peptides were immunogenic. This finding of lack of immunogeneticity of *T. gondii's* HF10, GRA4, ROP7, and SAG1 peptides was unexpected since both HLA-B07 supertype molecules and murine L^d exhibit the 'X-P' peptide binding motif. We predicted that one of several factors might contribute to this observation. HLA-B07 supertype molecules might bind to HF10 with a lower functional avidity relative to L^d due to differences in size and charge in structures of their peptide-binding pockets. Length of the HF10 decapeptide might fit better in the larger L^d binding pocket or prevent productive interactions between the HLA-B07/HF10 complex and the human $CD8^+$ T cell receptor. In this scenario, presentation of HF10 by L^d might be the exception rather than the rule. A lack of appropriate precursors in human T cell repertoires that recognize the HLA-B07/HF10 complex would also prevent induction of a significant, detectable human cytokine response. It is also possible that GRA6 is not endogenously processed to yield HF10 in humans the same way it is in mice due to differences in MHC class I antigen processing pathways in humans and mice. Binding studies provided the empirical evidence to resolve these questions. These data confirmed that the peptides we identified, using our bioinformatics approaches followed by testing responses of T cells from humans, avidly bind HLA A2, A3-11 or B7, and are consistent with those that elicit IFNγ producing T cells. However, HF10 was not an avidly binding peptide for HLA B07 explaining

our empirical results in which HF10 did not stimulate human HLA B7 lymphocytes from infected persons to produce IFNγ nor was HF10 immunogeneic in HLA B7 mice.

Specifically, this work identifies, for the first time, the importance of nine specific *T. gondii* peptides present in both Type I and II parasites in eliciting production of interferon γ by human CD8+T cells for persons with the three major HLA supermotifs. These supermotifs are present in ~90% of humans in the world. It also identifies the parasite proteins from which these peptides are derived. Thereby, we demonstrate that these proteins have peptides that traffic into an MHC Class 1 pathway during this infection in humans. These are proteins which the parasite secretes or that are on the parasite surface. In addition this work defines the critical role of ERAP1, an aminopeptidase in the MHC Class I antigen processing pathway, in susceptibility and resistance to the parasite in human congenital toxoplasmosis. This emphasizes the importance of MHC Class I antigen processing in defense of humans against this obligate intracellular parasite. This is of special interest because this parasite is sequestered in a vacuole in the host cell away from host cell cytoplasm. This work proves that in human cells these peptides must be trafficked from secreted or surface proteins of the parasite into an MHC Class I pathway. Thus, this work provides the foundation and the molecular tools for studying mechanisms of cell-mediated immune responses to *T. gondii* in humans or transgenic mice with human MHC Class I molecules. It also provides a paradigm for defining peptides important in MHC Class 1 responses for obligate intracellular organisms in humans. This approach will be useful for defining protective immune responses against other pathogens, and in fact potentially for a variety of other types of pathologic processes and diseases as well.

Although this supermotif binding algorhythm approach ultimately proved to be exceptionally useful to predict the *T. gondii* peptides that human T cells do recognize, it first provided new insights into potential confounding aspects of extrapolating information from Class I MHC antigen processing and presentation in murine models to peptides recognized by CD8+T cells following human MHC Class I antigen presentation. The importance of other features for peptide binding to murine and human MHC molecules was demonstrated. Specifically, initial algorhythms and work predicted that the shared amino acids at the second and 8–9th position of the peptides that binds in the antigen presenting molecule groove of HLA B7 and a critical murine MHC molecule L^d are the same. This indicated that HLA B7 and L^d should bind the same peptides and present them to T cells. However, the experiments performed to test these predictions did not result in presentation of the same peptides with the same efficiency for L^d mice as for B7 mice or B7 humans. In fact, we first predicted those peptides defined recently to bind L^d would be the needed clue for the work with human HLA B7 cells and HLA B7 transgenic mice. However, HLA B7 human's T cells, from infected persons, that recognize other peptides with this shared binding motif did not recognize those peptides defined recently as critical for presentation by murine L^d.

Blanchard et al utilized ERAAP knock out mice to prove that MHC Class I processing was essential for L^d presentation of HF10 and resistance of mice to *T. gondii*. It was therefore of interest to determine whether ERAAP would influences outcomes of congenital toxoplasmosis. If it did so, it would provide further evidence that MHC Class I presentation and antigen processing are important for protection/resistance in humans with congenital toxoplasmosis. Intriguingly, humans express 2 ERAAP genes, *ERAP1* and *ERAP2*, while mice have only 1 known ERAAP gene. ERAP1 closely resembles murine ERAAP in its substrate specificity and function, but ERAP2, which has 49% homology with ERAP1, exhibits a significantly different substrate specificity from ERAP1 [49]. Activity and contribution of ERAP1 and ERAP2 towards shaping of final peptide-MHC repertoire in humans remain poorly understood, although both proteases might act in tandem or as heterodimers to trim precursor antigens [49,50]. Blanchard et al. demonstrated, ERAAP-deficient H-2^d mice generate significantly lower frequencies of HF10-specific $CD8^+$ T cells and are consequently not protected from *T.*

gondii infection. Herein, we provide evidence that polymorphisms in the human ERAAP gene, *ERAP1*, also confer increased susceptibility to congenital toxoplasmosis in humans.

We propose that an effective approach for development of a vaccine against *T. gondii* infection is an immunosense vaccine; one that considers not only adjuvants and $CD8^+$ T cell epitopes but also MHC-restriction of putative epitopes, antigen specificity of different archetypal clonal and nonarchetypal strains and of different life-cycle stages of the parasite, as well as other genetic factors that influence *in vivo* generation of immunogenic epitopes and quality of immune responses. It will be useful to identify a panel of immunogenic epitopes derived from all 3 strains of *T. gondii* that are presented by the broad-coverage HLA supertypes, such as HLA-A02, HLA-A03, HLA-B07, HLA-A-24, and HLA-B44. The putative secretome of *T. gondii*, which includes the rhoptry and dense granule proteins, and surface antigens, represents a central source of antigens that can access the MHC class I processing pathway. Consistent with this, our peptides that have been identified as immunogenic appear to be derived from secreted proteins and a surface antigen, SAG1. In addition, some $CD4^+$ T cell epitopes bound by the major HLA Class II alleles, have been identified [31,51] and are more promiscuous so they could be included to induce specific *T. gondii* $CD4^+$ T cells, and epitopes that induce humoral immunity are known as well. We propose that collectively, these epitopes, properly adjuvanted, will elicit an immune response that is sufficiently potent and comprehensive to protect against toxoplasmosis in humans. Support for this approach comes from several lines of evidence: It appears that sterile infections have occurred naturally when an initial infection of pigs completely protected some immunized animals against subsequent infection [52]. A robust experience with comprehensive maternal serologic screening to detect seroconversion and congenital *T. gondii* infection in France for immunologically normal pregnant women demonstrated that, with very rare exception, only those who acquire the infection for the first time while pregnant transmit the infection to their fetus (Desmonts G, personal communication to McLeod R, 1990). The present work begins to provide a foundation for determining whether an immunosense vaccine will be protective.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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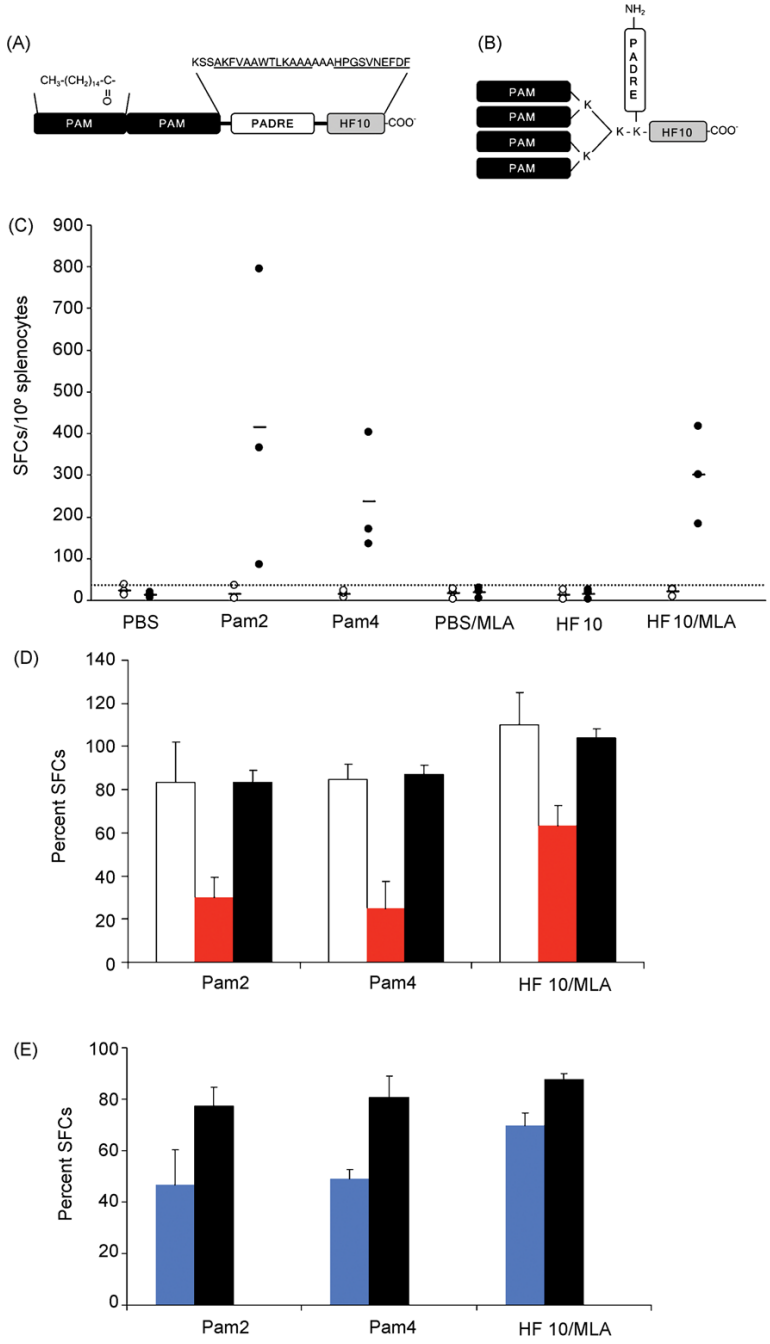

Figure 1. Schematic representation of lipopeptide constructs used and data demonstrating that immunization of H-2^d mice with lipopeptides and HF10/MLA elicits significant L^d-restricted, CD8$^+$ T cell-mediated IFN-γ production

(A) Pam2 lipopeptide contains 2 covalently linked palmitoyl moieties at the N terminus and 2 spacers comprising 3 amino acids each. (B) Pam4 lipopeptide consists of PADRE and HF10 separated by a fan-like structure that contains 4 lysines and 4 branching palmitoyl groups. Amino acid sequence of PADRE, HF10, and intervening spacers are indicated in single-letter code above the peptides, with those for PADRE and HF10 underlined. The N and C termini of each lipopeptide and the α- and ε-amino groups of certain lysines in Pam4 are also indicated for clarity. Pam: palmitoyl moiety. (C) Splenocytes from immunized (Pam2 and Pam4

lipopeptides and HF10/MLA) and control H-2^d mice (PBS, PBS/MLA, or HF10 alone) were stimulated with HF10 (filled circles) or irrelevant peptide YL9 (unfilled circles) and frequency of IFN-γ-producing cells in each case was determined using an *ex vivo* ELISPOT assay. Each symbol represents an individual mouse; small horizontal bars indicate the mean of each group. The dotted line represents the cutoff value determined as the maximal mean of the control mice + 3 S.D. (D) Splenocytes were incubated with antibodies to CD4 (unfilled bars), CD8α (red bars), and their common isotype control (filled bars) for 60–90 min prior to stimulation with HF10. Data are normalized to the number of spots generated in the absence of antibody and presented as the percent production of SFCs. (E) As in (D), except that anti-L^d (blue bars) Ab and its isotype control (filled bars) were added instead. *, $p<0.05$; **, $p<0.01$.

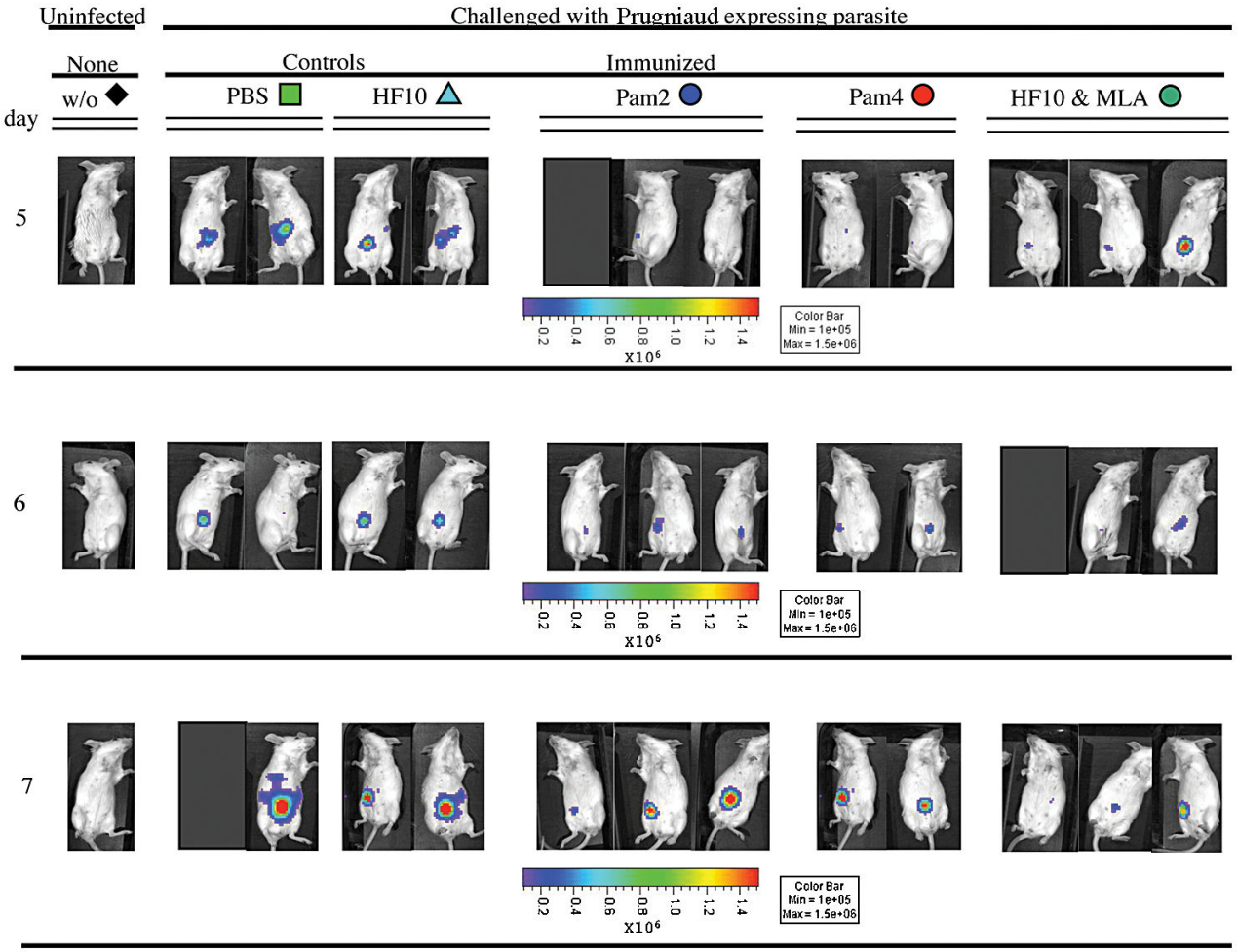

Figure 2. Dissemination and replication of *T. gondii* luciferase expressing parasites were partially reduced in H-2^d mice immunized with lipopeptides and MLA/HF10 when assayed from 5–7 days after challenge

Different mice were imaged on each day. Mice from each of the groups of immunized mice that have T cells that produced IFN-γ in response to HF10 were protected compared with unimmunized control mice that did not produce IFN-γ. There were a total of 5–9 mice tested in each control or immunization group.

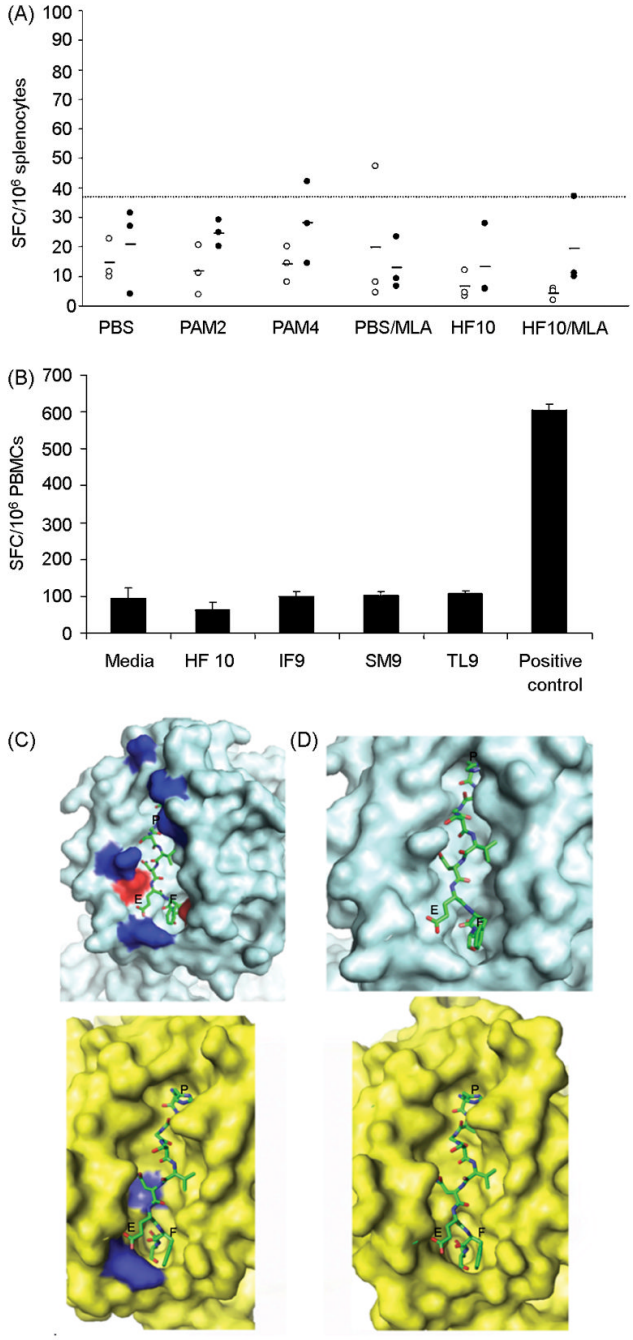

Figure 3. HF10 was not immunogenic in HLA-B*0702 mice and L^d-restricted peptides HF10, IF9, SM9, and TL9 were not recognized by *T. gondii*-infected HLA-B07$^+$ humans, likely due to differences between L^d and HLA-B07 surface and electrostatic complementarity for HF10 and size of peptide-binding pockets

(A) Splenocytes from immunized (Pam2 and Pam4 lipopeptides and HF10/MLA) and control HLA-B*0702 mice (PBS, PBS/MLA, or HF10 alone) were stimulated with HF10 (filled circles) or irrelevant peptide LF9 (unfilled circles) and frequency of IFN-γ-producing cells in each case was determined using an ELISPOT assay. Each symbol represents an individual mouse; small horizontal bars indicate mean of each group. Dotted line represents cutoff value determined as maximal mean of control mice ± 3 S.D. (B) PBMCs from *T. gondii*-seropositive

HLA-B07^{+} humans were stimulated with 10 μg/ml of 4 L^{d}-restricted peptides (HF10, IF9, SM9, TL9) previously shown to be immunogenic in mice. Frequency of SFCs was measured using an ELISPOT assay. Data (mean ± s.e.m.) are from 1 seropositive individual and representative of 2 to 4 individuals per peptide tested. Media: negative control containing equivalent concentration of DMSO relative to peptide-stimulated wells; Positive control: 10 μg/ml of an immunogenic HLA-B07-restricted peptide derived from Epstein-Barr virus (EBV). (C,D) Predicted structures of murine L^{d} and human HLA-B07 supertype pMHC complexes were modeled by superimposing HF10 peptide (presented as a 'ball-and-stick') into previously determined peptide-binding pocket of L^{d} (cyan) and HLA-B*3501 (yellow), a HLA-B07 supertype member. (C) Surface electrostatics of peptide-binding pocket of both MHC molecules are indicated in blue (basic patches) and red (acidic patches). Remaining regions represent nonpolar, hydrophobic patches. (D) A close-up view of pMHC region shown in (C). Relative positions of Pro, Glu, and C-terminal Phe of HF10 (HPGSVNEFDF) are indicated in single-letter code above corresponding residues of peptide.

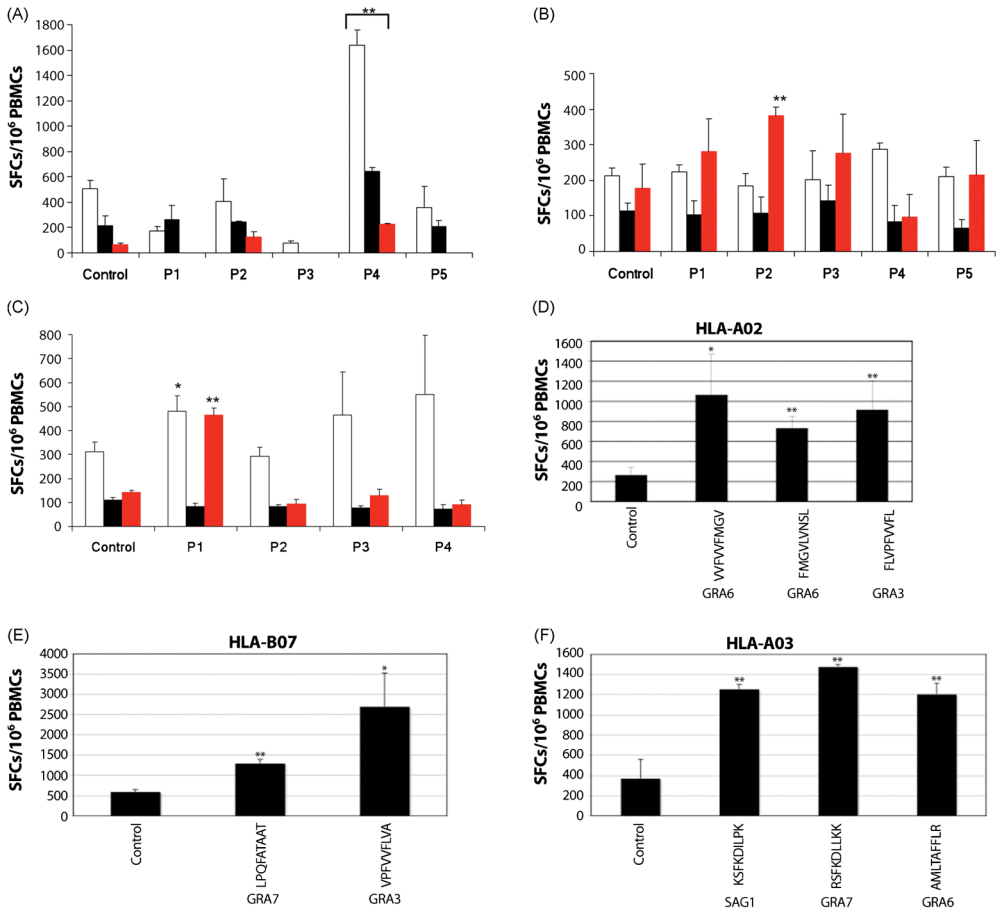

Figure 4. Peptide screening in MHC-matched *T. gondii*-infected individuals identified 2 immunogenic peptide pools restricted by the HLA-A02 and -A03 supertypes and peptides eliciting IFN-γ in persons with HLA-A02, -A03, and B07

(A) 42 HLA-A02-restricted peptides were divided in 5 pools with 8 to 9 peptides per pool and tested for reactivity in PBMCs isolated from HLA-matched *T. gondii*-seropositive individuals in an *ex vivo* ELISPOT assay. (B) As in (A), except that 40 HLA-A03-restricted peptides were used instead. (C) As in (B), except that 36 HLA-B07-restricted peptides were used instead. Data (mean ± s.e.m.) are from 3 *T. gondii*-seropositive individuals per HLA supertype; bars of the same color represent the same individual in each HLA supertype group. Control: media containing an equivalent concentration of DMSO relative to the peptide-stimulated wells; P1 to P5: peptide pools as described in Tables A1–A3. *, p=0.087; **, p<0.01. (D,E,F) In D, E and F, representative data for 1 person of each haplotype are shown. Three A02, 2 B07, and 4 A3-11 seronegative control persons did not respond to these peptides. Thus, individual peptides for each HLA supertype that can elicit high IFN-γ production were identified. Controls are the same as in A, B, and C. Peptide sequences and proteins from which they are derived are shown. *, p=0.06; **, p<0.05.

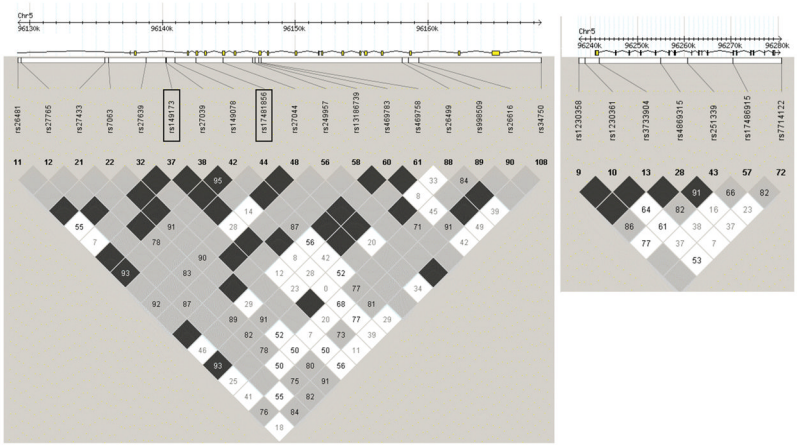

Figure 5. Gene structure and LD plots for human *ERAP1* and *ERAP2*

Upper diagrams show the positions of genotyped SNPs relative to the intron/exon structure of each gene. Lower diagrams show the LD plots generated in Haploview using data for each gene from our North American patient cohort. LD values (D′ × 100) between markers are indicated at the intercept of the 2 markers on the matrix. Where there is no value, D′=1 (i.e. 100). The black (high) through grey to white (low) shading indicates the degree of confidence in the estimate of LD between the markers. The 2 SNPs in *ERAP1* that were found to associate with congenital disease (rs149173 and rs17481856) are outlined in black rectangles. Left panel, *ERAP1*; right panel, *ERAP2*.

Table 1

Immunogenicity of predicted Me49-derived epitopes in seropositive individuals

Patient number	HLA haplotype[a]		Peptide pool tested[d]	Immunogenicity[e]				
	HLA-A[b]	HLA-B[c]		Pool 1	Pool 2	Pool 3	Pool 4	Pool 5
1	A*02	B*07	A2	−	−	−	+	−
2	A*01, A*02	B*07, B*08	A2	−	−	ND	+	−
3	A*02	B*27, B*62	A2	ND	−	ND	+	ND
4	A*02, A*11	B*07, B*51	A3	−	+	−	−	−
5	A*01, A*03	B*07, B*08	A3	−	−	−	−	−
			B7	−	−	−	−	−
6	A*03, A*24	B*07, B*61	A3	−	−	−	−	−
			B7	+	−	−	−	−
7	A*03, A*31	B*07, B*51	B7	−	−	−	−	−

[a] HLA-A and HLA-B alleles of the seropositive individual.

[b] HLA-A*02 alleles are classified under the HLA-A02 supertype; HLA-A*03, -A*11, and A*31 alleles are classified under the HLA-A03 supertype.

[c] HLA-B*07 and -B*51 alleles are classified under the HLA-B07 supertype.

[d] The HLA supertype-restriction of the peptide pool tested in each individual. Peptide pools were screened only in HLA supertype-matched people. A complete list of peptide pools and their constitutent peptides can be found in Tables A1 to A3.

[e] Each peptide pool (pool 1–5) was tested for its ability to elicit IFN-γ spot formation from PBMCs of a HLA supertype-matched individual using an ELISPOT assay.

+, statistically significant response ($p < 0.01$);

−, response not statistically significant ($p > 0.05$); ND, not done.

Table 2

Toxoplasma gondii peptide binding assays

A2-supertype peptides

				HLA A02-supertype binding capacity (IC50 nM)					
Peptide ID	**Sequence**	**Len**	**Protein**	**A*0201**	**A*0202**	**A*0203**	**A*0206**	**A*6802**	**Alleles bound**
7103.0001	VVFVVFMGV	9	GRA6	**135**	3451	**19**	**0.92**	**39**	4
7103.0002	FMGVLVNSL	9	GRA6	**239**	**5.3**	**2.8**	553	4335	3
7103.0003	FLVPFVVFL	9	GRA3	**<0.1**	**<0.1**	**0.11**	**3.5**	**1.5**	5

A3-supertype peptides

				HLA A03-supertype binding capacity (IC50 nM)						
Peptide ID	**Sequence**	**Len**	**Protein**	**A*0301**	**A*1101**	**A*3101**	**A*3301**	**A*6801**	**A*3001**	**Alleles bound**
7103.0004	KSFKDILPK	9	SAG1	**20**	**54**	**129**	-	11870	**200**	4
7103.0005	AMLTAFFLR	9	GRA6	**36**	**3.6**	**0.31**	**26**	**263**	12056	5
7103.0006	RSFKDLLKK	9	GRA7	**14**	**14**	**303**	-	10553	**145**	4

B7-supertype peptides

				HLA B07-supertype binding capacity (IC50 nM)							
Peptide ID	**Sequence**	**Len**	**Protein**	**B*0702**	**B*3501**	**B*3503**	**B*4201**	**B*5101**	**B*5301**	**B*5401**	**Alleles bound**
7103.0007	LPQFATAAT	9	GRA7	**14**	4045	6630	**17**	15388	-	**106**	3
7103.0008	VPFVVFLVA	9	GRA3	**18**	61936	20589	**36**	3016	30896	**0.87**	3
7103.0009	HPGSVNEFDF	10	GRA6	3621	2093	11457	877	14738	5521	854	0

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Table 3

Detailed information on the 25 genotyped SNPs in *ERAP1* and *ERAP2* and their associated TDT *p* values.

SNP (dbSNP)	Bp Position	Position in Gene	AA change[a]	NCCCTS patient cohort		*P* value[d]
				Overtransmitted allele[b]	T/U frequency[c]	
***ERAP1* (reverse strand Chr 5q15)**						
rs26481	96129108	Intron 19	–	T/C	1.18333	0.5791
rs27765	96129345	Intron 19	–	G/A	0.29444	0.3657
rs27433	96135691	Intron 19	–	T/G	0.75903	0.3692
rs7063	96135967	Intron 19	–	T/A	0.93056	0.7576
rs27639	96138798	Intron 18	–	T/G	0.67292	0.1615
rs149173	96140949	Intron 18	–	T/C	1.09028	**0.0077**
rs27039	96140306	Intron 18	–	C/T	1.26319	0.1161
rs149078	96140949	Intron 18	–	T/C	0.96667	0.063
rs17481856	96142564	Exon 17	–	C/T	0.62847	**0.0253**
rs27044	96144608	Exon 15	Gln-730-Glu	C/G	1.05139	0.0782
rs249957	96146798	Intron 13	–	A/G	0.29653	1
rs13186739	96146999	Intron 13	–	T/C	0.80347	0.7389
rs469783	96147280	Exon 13	–	A	1.26319	0.1161
rs469758	96147471	Intron 12	–	T/C	1.26319	0.1161
rs26499	96158099	Intron 4	–	C/T	1.14375	1
rs998509	96158551	Intron 4	–	G/A	0.46458	0.6547
rs26616	96159348	Intron 3	–	C/G	0.88333	0.1172
rs34750	96168562	Intron 1	–	C/G	0.92986	0.6394
***ERAP2* (forward strand Chr 5q15)**						
rs1230358	96237497	5′ UTR	–	A/T	0.97014	0.3428
rs1230361	96238927	5′ UTR	–	C/A	0.88542	0.3173
rs3733904	96241929	Intron 1	–	A/G	0.84167	0.1573
rs4869315	96255028	Intron 5	–	C/G	1.34861	0.1736
rs251339	96260794	Intron 8	–	T/C	1.34653	0.0687
rs17486915	96270475	Exon 13	–	C/T	0.25347	0.763

SNP (dbSNP)	Bp Position	Position in Gene	AA change[a]	NCCCTS patient cohort		*P* value[d]
				Overtransmitted allele[b]	T/U frequency[c]	
rs7714122	96280773	3′ UTR	–	C	0.37986	0.6171

[a] Where applicable.

[b] Allele that was overtransmitted from heterozygous parents to affected offspring presented as overtransmitted allele/under-transmitted allele.

[c] The ratio of transmitted to under-transmitted alleles from heterozygous parents to affected offspring.

[d] *p* value for the degree of genetic association between the indicated SNP and ocular and/or brain disease in congenital toxoplasmosis was determined using TDT analysis in Haploview. Statistically significant associations ($p < 0.05$) were bolded