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***Toxoplasma gondii* HLA-B*0702 restricted GRA7_{20–28} peptide with adjuvants and an universal helper T cell epitope elicits CD8⁺ T cells producing IFN- γ and reduces parasite burden in HLA-B*0702 mice¹**

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Abstract

Ability of CD8⁺ T cells to act as cytolytic effectors and produce IFN- γ was shown to mediate resistance to *Toxoplasma gondii* in murine models due to recognition of peptides restricted by murine MHC Class I molecules. However, no *T. gondii* specific HLA-B07 restricted peptides were proven protective against *T. gondii*. Recently, two *T. gondii*-specific HLA-B*0702-restricted T cell epitopes, GRA7_{20–28} (LPQFATAAT) and GRA3_{27–35} (VPFVFLVA), displayed high-affinity binding to HLA-B*0702, and elicited IFN- γ from PBMCs of seropositive HLA-B*0702 persons. Herein, these peptides were evaluated to determine whether they could elicit IFN- γ in splenocytes of HLA-B*0702 transgenic mice when administered with adjuvants and protect against subsequent challenge. Peptide-specific IFN- γ producing T cells were identified by ELISPOT and proliferation assays utilizing splenic T lymphocytes from HLA transgenic mice.

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Appendix. Supplementary data

Supplementary data associated with this article (Table S1 and Table S2) can be found, in the online version.

When HLA-B*0702 mice were immunized with one of the epitopes identified, GRA7_{20–28} in conjunction with a universal CD4⁺ T cell epitope (PADRE) and adjuvants (CD4⁺ T cell adjuvant, GLA-SE, and TLR2 stimulatory Pam₂Cys for CD8⁺ T cells), this immunization induced CD8⁺ T cells to produce IFN- γ and protected mice against high parasite burden when challenged with *T. gondii*. This work demonstrates feasibility of bioinformatics followed by an empirical approach based on HLA binding to test this biological activity for identifying protective HLA-B*0702 restricted *T. gondii* peptides and adjuvants that elicit protective immune responses in HLA-B*0702 mice.

Keywords

Toxoplasma gondii; HLA-B07 epitopes; PADRE; adjuvant; vaccine

1. Introduction

Toxoplasma gondii is an intracellular parasite which infects a broad range of mammalian hosts and can cause severe disease in immune-compromised or immunologically immature persons. The infection can cause severe ocular, neurologic, and systemic disease in these settings (1–3). Since no medicines eradicate all life cycle stages of this parasite (4), development of a vaccine against *T. gondii* would be extremely useful, especially if a vaccine could prevent acquisition of parasite completely.

The ideal vaccine to protect against toxoplasmosis in humans would include epitopes that elicit a protective Th1 biased-immune response, characterized by the generation of long-lived IFN- γ -producing CD8⁺ T cells (5). CD4⁺ and CD8⁺ T cells have a critical role in protective immunity to *T. gondii* in murine models and humans (6, 7). CD8⁺ T cells and interferon gamma (IFN- γ) are significant effectors mediating resistance to acute and chronic *T. gondii* infection (8). Peptide-based vaccines derived from CD8⁺ T cell epitopes are a promising strategy to mobilize the immune system against *T. gondii* in humans. However, until now no *T. gondii* specific HLA-B07 restricted CD8⁺ T cell epitopes that were proven to be protective had been identified.

Previously, HF10 (HPGSVNEFDF), a single decapeptide, derived from the dense granule protein 6 (GRA6) was identified as the protective, immunodominant L^d-restricted epitope in H-2^d mice infected with type II *T. gondii* parasite (9). Our earlier work had definitively proven that L^d was essential for restriction of cyst number and brain pathology in L^d mice. Our recent study also demonstrated that immunization of H-2L^d mice with the lipopeptide constructed by HF10 and PADRE or HF10 with monophosphoryl lipid A (GLA-SE, #2573) protected Balb/c mice. However, HF10 administered to HLA-B07 transgenic mice in a similar manner was not immunogenic despite a common L^d and B7 peptide binding motif (10). Therefore, in order to find effective peptides for *T. gondii* derived HLA-B07⁺ restricted CD8⁺ T cells, we utilized bioinformatic algorithms to identify novel, *T. gondii*-derived, CD8⁺ T cell epitopes restricted by the HLA-B07 supertypes, which will collectively provide coverage for 30% of the human population worldwide (11, 12). We screened the amino acid sequences from nine surface and secreted proteins (SAG1, SUSA1, GRA2, GRA3, GRA6, GRA7, ROP2, ROP16, ROP18) of the type II *T. gondii* strain, ME49, for HLA-B*0702- specific peptide binding scores using bioinformatic analysis. Peripheral blood mononuclear cells from seropositive and seronegative persons were tested for response to these peptides using IFN- γ Elispot assay. Strikingly, two *T. gondii*-specific HLA-B*0702- restricted T cell epitopes, one derived from GRA7_{20–28} (LPQFATAAT), the other derived from GRA3_{27–35} (VPFVVFLVA) both displayed high-affinity binding to

HLA-B*0702 and stimulated IFN- γ production by peripheral blood cells from HLA-B*0702 persons infected with *T. gondii*, but not seronegative controls (10).

Herein, we evaluated these peptides identified earlier using bioinformatics (13–17) and *in vitro* analyses as vaccine components administered in conjunction with pan DR epitope (PADRE), a universal helper T lymphocyte epitope in HLA-B*0702 transgenic mice (13, 14). Immunization of HLA-B*0702 transgenic mice with the peptide LPQFATAAT with the help of PADRE and GLA-SE and Pam₂Cys as adjuvants activated CD8⁺ T cells to produce IFN- γ and protected against subsequent challenge with a high burden of a type II parasite.

2. Materials and Methods

2.1 Peptides and adjuvants

HLA-B*0702-restricted peptides – GRA_{720–28} (LPQFATAAT) and GRA_{327–35} (VPFVVFLVA), PADRE (AKFVAAWTLKAAA) and Pam₂Cys (Pam₂-KSS) (18) were synthesized by Synthetic Biomolecules, San Diego at >90% purity in lyophilized form. The TLR4 agonist was a GLA-SE mimetic that was synthesized by the Infectious Diseases Research Institute (IDRI, Seattle, WA), used as previously described (19).

2.2 Mice

Female HLA-B*0702 transgenic mice express a chimeric HLA-B07/H2-D^b MHC Class I molecule, are on a C57BL/6 x Balb/C background backcrossed through many generations and have been previously described (11), which were produced at Pharmexa-Epimmune (San Diego, CA) and bred at the University of Chicago. HLA 0311 mice also were utilized in an experiment. All studies were conducted with the approval of the Institutional Animal Care and Use Committee at the University of Chicago.

2.3 Analysis of peptide solubility

Peptides were dissolved in PBS followed by brief vortexing, to give a concentration of 5 mg/ml. Samples were centrifuged at 15,000 rpm at room temperature for 1h. Supernatant concentrations were determined by measuring absorbance at 280 nm and by HPLC. The percentage solubility of the samples was calculated, although no detailed studies of optimal concentrations of peptides or adjuvant were performed herein.

2.4 Immunizations and challenge

HLA-B*0702 mice were inoculated subcutaneously (s.c.) at the base of the tail using a 30-gauge needle with single peptides or a mixture of two of the identified B7 CD8⁺ T cell peptides, (amino acid sequence LPQFATAAT [LT9] and amino acid sequence VPFVVFLVA [VA9]) (50 μ g of each peptide per mouse), with or without 50 μ g PADRE and 50 μ g Pam₂Cys emulsified in 20 μ g of GLA-SE (TLR4 agonist). Control mice were injected with PBS. Mice were boosted twice at 2-week intervals with a total of 50 μ g of each peptide per mouse with each immunogen. For challenge studies, mice were challenged 14 days post immunization with peptide emulsions via intraperitoneal (i.p.) inoculation with Prugniaud – a type II strain luciferase expressing parasite (20). In an experiment to compare treatment with PBS, GLA-SE and PADRE, and GLA-SE, PADRE and LT9, a Prugneaud parasite transfected with luciferase that produced more cysts was kindly provided by Laura Knoll (Madison, Wisconsin) and used for challenge.

2.5 ELISPOT assay

Mice were euthanized 10 to 14 days after the last immunization. Spleens were harvested, pressed through a 70 μ m screen to form a single-cell suspension, and depleted of

erythrocytes with AKC lysis buffer (160mM NH₄Cl, 10mM KHCO₃, 100μM EDTA). Cultures were prepared separately, each with the spleen cells from an individual mouse. Cells were cultured for 24 hours as described previously(10). Murine ELISPOT assays were performed using anti-mouse IFN-γ mAb (AN18) and the biotinylated anti-mouse IFN-γ mAb (R4-6A2) and 5×10⁵ 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 10⁶ murine splenocytes. Murine splenocytes were incubated with the relevant blocking antibody for 1.5 to 2 h at 37°C and 5% CO₂ before they were seeded at 5×10⁵ cells per well. Anti-CD4⁺ antibody (RM4-5, BioLegend), anti-CD8 monoclonal antibody (53-6.7, BD Biosciences), and their relevant isotype control (Rat IgG_{2a}, BD Biosciences) were added to each sample at a final concentration of 10μg/ml. To assay for B7-restricted IFN-γ production, splenocytes were incubated with 0.5μg of FcBlock™ (BD Biosciences) per 10⁶ cells for 10–15 min at 4°C and washed before anti-HLA-B*0702 (BioLegend) mAb or its isotype control (mouse IgG_{2a}, BioLegend) was added at a final concentration of 20μg/ml.

2.6 T cell Proliferation assay

A splenocyte suspension containing 5×10⁶ cells/ml from immunized mice was plated into 96-well U-bottomed tissue culture plates (100μl per well) along with 100μl of each stimulant diluted to appropriate concentrations in complete RPMI1640. The stimulant used was peptide at 10μg/ml. Concanavalin A at 10μg/ml was used as a positive control and cells cultured with media alone were used as negative controls. The plates were incubated for 3 days in 5% CO₂ at 37 °C and pulsed with 1μCi [³H] thymidine per well for the final 18 h. The cells were then harvested onto a unifilter 96-well plate using a cell harvester (Packard Instrument, Meriden, CT). After drying, 25μl of Microscint was added to each well and the filter plate was sealed and counted in a Top Count (Packard Instrument) to determine radioactivity incorporated into DNA. Results are expressed as the stimulation index (SI), calculated as the ratio between the mean counts per minute (cpm) for triplicate stimulated cultures and the mean counts per min for triplicate unstimulated cultures. SI values 2.0-fold greater than the SI of the control groups were considered to be significant.

2.7 In vivo bioluminescence imaging

The transgenic *T. gondii* strain used for *in vivo* challenges was derived from the type II Prugniaud (Pru) strain and expresses the Firefly luciferase (FLUC) gene constitutively by tachyzoites and bradyzoites. It was created, maintained and utilized as previously described (20). Mice infected with Pru-FLUC tachyzoites were imaged at 6 days post-challenge using the *in vivo* imaging system (IVIS; Xenogen, Alameda, CA) as previously described (21). Briefly, mice were injected i.p. with 200μl of D-luciferin (15.4 mg/ml) and immediately anesthetized in an O₂-rich induction chamber with 2% isoflurane. After 12 min, mice were imaged in ventral positions and photonic emissions were assessed using Living image® 2.20.1 software (Xenogen). Separately, to compare the effects of immunization with PADRE-GLASE versus PADRE-GLA-SE-Peptide, imaging of brains *ex vivo* was performed at day 21 after FLUC Prugneaud strain(kindly provided by Laura Knoll, Madison,Wisconsin).challenge. Each mouse had received and intraperitoneal dose of 200 microliters of D-luciferin 15 minutes earlier and then the brains were removed, submerged in luciferin (15.4mg.ml) and then image for bioluminescence. Data were presented as pseudocolor representations of light intensity and mean photons/s/region of interest (ROI).

2.8 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. Assays were performed as

described previously (22, 23). Briefly, 1 to 10nM concentrations of radiolabeled peptides were coincubated with 1 M to 1 nM concentration of purified MHC in the presence of 1 to 3uM human β 2-microglobulin. After 2 days, the binding of radiolabeled peptide to the corresponding MHC Class I molecule was determined by capturing MHC-peptide complexes on Greiner Lumitrac 600 microplates coated with the W6/32 antibody. The bound counts per minutes was measured using a Topcount microscintillation counter 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 (24, 25).

2.9 Statistical analyses

There were 3–5 mice per group. PBMC were obtained from 4 seropositive and 3 seronegative donors. Each experiment was repeated at least twice. Statistical analyses for all *in vitro* assays were performed using a 2-tailed student's T-test. Two-tailed P values <0.05 were considered to be statistically significant. Peptides were considered immunogenic in mice if they induced IFN- γ spot formation or proliferation SI from immunized mice that was significant (P <0.05) compared with spot formation from control mice.

3. RESULTS

3.1 Cellular Immunogenicity of two identified B7 restricted CD8⁺ T cell epitope peptides in HLA B*0702 transgenic mice

To address the capacity of two identified B7 epitopes, GRA7_{20–28} (LPQFATAAT) and GRA3_{27–35} (VPFVVFLVA), to prime for IFN- γ responses, HLA-B07 transgenic mice were immunized s.c. with each of peptides alone and peptides with PADRE or peptides with PADRE and Pam₂Cys. Each of these regents was mixed with GLA-SE. A mixture of these two peptides with PADRE, or plus Pam₂Cys mixed with GLA-SE were also used as immunogens. As shown in Fig. 1A, little IFN- γ responses were observed following immunization by peptides mixed with GLA-SE alone. When PADRE was added for immunization, a significant amount of IFN- γ was produced following stimulation by one B7 epitope peptide LT9 (LPQFATAAT) *in vitro* (P =0.0003, compared to peptide mixed with GLA-SE alone). An even more robust response was achieved when Pam₂Cys was added to the immunogen. In contrast, no significant amount of IFN- γ was produced using splenocyte groups immunized with another B7 epitope peptide VA9 (VPFVVFLVA) (P >0.05, compared to control group), when they were stimulated by this peptide. In 4 separate experiments, immunizations of HLA-B07 mice with GLA-SE plus PADRE did not elicit IFN- γ when Splenocytes were tested without peptide (background) or with LT9 or VA9. Details of this experiment were reported previously (10).

Similar results were found with peptide pool immunizations. Splenocytes from the mice immunized with the mixtures of two peptides LT9 and VA9 with PADRE or plus Pam₂Cys mixed with GLA-SE produced significant amount of IFN- γ when stimulated by LT9 (P <0.001, compared to the PBS immunization group), but not by VA9 (P >0.05, compared to the PBS immunization group) (Fig. 1B).

To assess relative contributions of CD8⁺ T cells to overall IFN- γ response, the activity of splenic CD8⁺ T cells was blocked using antibody specific to the CD8⁺ T cell receptor. IFN- γ spot formation was significantly reduced in the presence of α CD8 incubated with the spleen cells from mice immunized with LT9 included in the vaccination and stimulated by LT9 (Fig. 1C).

3.2 Relation between solubility and immunogenicity

The correlation between the solubility of the peptides and their immunogenicity is summarized in Table 1. The VA9 was much less soluble with overall percent solubility of 10%. In contrast, LT9 had 99% solubility. LT9 was effective in the peptide vaccine construct, but VA9 was not. Reduced solubility of this peptide in the vaccine correlated with its reduced immunogenicity. There was more than a log difference in solubility for the ineffective (much less soluble VP9) and effective (much more soluble LP9) peptide preparations. Solubility of VP9 was 0.4 mg/ml and solubility of LP9 was 4.73mg/ml. The R^2 values for the standard curves were 0.98 and 0.99 respectively.

3.3 Evaluation of cellular immune responses to LT9 formulated peptide vaccine

Since VA9 peptide formulation vaccination of HLA-B*0702 transgenic mice could not effectively elicit CD8⁺ T cell to produce IFN- γ , only LT9 peptide formulations were evaluated for protection in HLA-B07 transgenic mice in subsequent studies.

The ability of GLA-SE and Pam₂Cys to adjuvant the LT9 peptide also was evaluated. Mice were immunized with LT9 peptide alone, LT9/PADRE, LT9/PADRE/GLA-SE, LT9/PADRE/GLA-SE/Pam₂Cys. As shown in Fig. 2A, immunization with LT9 alone did not induce effective IFN- γ production, but when the PADRE helper epitope was included in the immunogens, it induced a small amount of IFN- γ but this was not significant ($P=0.057$, when compared to peptide alone). When GLA-SE was added, robust CD8⁺ T cell production of IFN- γ was achieved ($P=0.0001$, compared to peptide alone; $P=0.00013$ compared to peptide/PADRE). Greater responses were observed when Pam₂Cys was added in the vaccine ($P=0.000009$, compared to peptide alone; $P=0.000013$, compared to peptide/PADRE; $P=0.09$, compared to peptide/PADRE/GLA-SE). However, the addition of Pam₂Cys to PADRE/GLA-SE did not significantly enhance the LTP specific IFN- γ response. Thus, GLA-SE is an effective adjuvant to enhance IFN- γ production in response to CD8⁺ T cell eliciting peptides in HLA-B*0702 transgenic mice.

LT9-specific proliferative responses (Fig. 2B) with a SI of ~3.5 were observed following immunization with LT9/PADRE/GLA-SE/Pam₂Cys. When the same peptide epitope was used alone for immunization, SI was 1.5 (Fig. 2B). Magnitude of lymphocyte blastogenesis and IFN- γ elicited by LT9 with adjuvants had the same pattern (Figs. 2A and 2B).

3.4 PADRE delivers help for peptide- specific IFN- γ production

In order to determine function of the CD4⁺ T cell epitope PADRE in this peptide vaccine, PADRE-specific T cell responses were evaluated by IFN- γ ELISPOT assay and a proliferative response assay using splenocytes from the immunized mice. As shown in Fig. 3A, immunization with the CD8⁺ T cell epitope alone did not induce an IFN- γ response, whereas when the CD4⁺ T cell epitope PADRE was added to the immunogen, CD8⁺ T cells could be activated to secrete IFN- γ ($P=0.0006$ compared to PBS group; $P=0.001$ compared to peptide alone immunized group). A proliferative response of SI ~ 4, was observed following immunization with LT9 plus PADRE and Pam₂Cys in GLA-SE. However, blocking the CD4⁺ T cell receptor using anti-CD4⁺ antibody significantly reduced IFN- γ production (Fig. 3C), thus indicating that PADRE is an effective CD4⁺ Th epitope which contributes to the peptide vaccine construct. However, in some instances (eg. Fig4A), PADRE elicited only a small enhancement of the response to LT9.

3.5 LT9 Peptide elicited HLAB7-restricted CD8⁺ T cells responses in immunized mice

Splenocytes were isolated from peptide-immunized HLA-B07 transgenic mice 10 days after immunization and incubated with antibodies to α CD8 or HLA-B07 and their common isotype control prior to stimulation with LT9. Their abilities to generate IFN- γ and

lymphocyte proliferation in response to peptide were tested. To study the ability of α HLA-B*0702 to block response of CD8⁺ T cells in the same experiment, blocking α CD8 T cells and blocking α HLA-B*0702 were used with the same spleen cells in the same experiment. The data in Fig. 4A,B shows that blocking CD8⁺ T cell receptors using α CD8⁺ antibody significantly reduced LT9-specific IFN- γ production and T cell proliferation ($P < 0.05$). (Fig. 1B)

Then, to further analyze the association of LT9 presentation by HLA-B*0702, blocking experiments using a monoclonal anti-HLA-B*0702 antibody (BB7.2) was performed. Results indicated that the IFN- γ secretion was significantly decreased by the anti-HLA-B07 antibody but not by the control antibody ($p < 0.05$) (Fig. 4C). These results demonstrate that the peptide specific IFN- γ response is HLA-B*0702 restricted and involves CD8⁺ T cells. The partial blocking by the HLA B7 antibody is likely a function of the antibody's capacity for blocking, and not reactivity of the peptide with the murine H2 haplotypes. This is because the algorithms predict poor affinity of all of the background Balb/c and B6 H2 molecules with these peptides. Using the IEDB to predict, these peptides are predicted to bind poorly to these H-2 molecules (Table S1).

3.6 Peptide with adjuvant vaccination protects mice against type II parasite challenge

HLA-B*0702 transgenic mice were immunized with the LT9 peptide plus PADRE and Pam₂Cys in GLA-SE three times at two week intervals. Mice were challenged 2 weeks after the last immunization. They were imaged 5 days post-challenge with 10,000 Pru (Fluc) using the Xenogen *in vivo* imaging system. As shown in the representative experiment in figure 5, the numbers of luciferase expressing parasites in immunized HLA-B*0702 mice were significantly reduced compared to the numbers of parasites in unimmunized mice, Figure 5A. There was also a significant reduction of cysts in the brain when mice were immunized with LT9 peptide vaccine, Figure 5B. There was a 54% reduction from a mean of 335 to 155 cysts per brain.

Separate experiments were performed in which immunization with GLA-SE –PADRE was compared to GLA-SE-PADRE-LT9. In these experiments GLA-SE-PADRE-LT9 consistently resulted in Splenocytes which produced IFN- γ (3 experiments). Challenges with the fluc parasite demonstrated no protection with GLA-SE-PADRE. Another strain of transgenic mouse HLA A 11 also was use and confirmed that GLA-SE with PADRE neither elicits IFN- γ nor is protective in another strain of HLA transgenic mouse (Table S2). We found in B7 mice that brain luminescence was less in the peptide-PADRE- GLA-SE group ($p < 0.05$) but not less in the PADRE- GLA-SE group ($p > 0.05$).

4. DISCUSSION

Herein, two *T. gondii*-specific HLA-B*07 restricted CD8⁺ T cell epitopes LT9 (LPQFATAAT) and VA9 (VPFVVFLVA) derived from GRA7_{20–28} and GRA3_{27–35} elicited IFN- γ from human PBMC cell, 4 HLA-B07 seropositive and 0 of 3 seronegative persons (10). Upon immunization of mice, only LPQFATAAT elicited CD8⁺ T cell specific IFN- γ with the help of a universal CD4⁺ epitope and adjuvants, GLA-SE and Pam₂Cys, enhanced the response. This peptide vaccine plus adjuvants protect HLA-B07 transgenic mice from type II parasite challenge.

CD8⁺ T cell epitopes identified were from dense granule (GRA) proteins of *Toxoplasma gondii*. Many studies have shown that dense granule antigens (GRA) are involved in parasite survival and virulence (26). The proteins are secreted in abundance and circulate in the blood stream during the first hours following infection and appeared 2–5 days after mice

infected with parasite (27, 28). The GRA proteins have thus been considered to be attractive vaccine candidates for the prevention and control of toxoplasmosis.

Two peptides were recognized by T cells from *T. gondii* specific HLA-B07 restricted PBMC. However, although peptide VPFVVFLVA was recognized by T cells from *Toxoplasma gondii*-specific HLA-B07-restricted human PBMC, it did not induce a significant IFN- γ response in HLA-B*0702 transgenic mice. We therefore concluded that VA9 was presented by HLA-B07 supertype molecules to CD8⁺ T cells in humans, but was not present in significant quantity to be presented by HLA-B07 supertype molecules to CD8⁺ T cells in the transgenic mice. We speculate that this seems most likely to be due to the relative insolubility of the VA9 peptide although other differences in formulation could also explain this result. The VA9 peptide was 90% insoluble with overall percent solubilities of 10% in PBS. In contrast, LT9 was soluble, with 99% solubility in PBS. LT9 was effective in the peptide vaccine construct, but VA 9 was not, reduce solubility of peptides in the vaccine peptides might correlate with reduced immunogenicity.

The inclusion of PADRE, a synthetic nonnatural Pan HLA DR binding Epitope peptide that binds promiscuously to variants of the human MHC Class II molecule DR and is effective in human and HLA transgenic mice, can augment CD8⁺ T cell effector functions by inducing CD4⁺ T helper cells (29). The PADRE epitope was demonstrated to deliver help for a MHC Class I restricted peptide-specific CTL response to the *T. gondii* peptide herein. Requirement for CD4⁺ T cell help in the generation of a CD8⁺ T cell response also has been proved in viral, bacterial, and parasite models (30, 31). In studies of *Trypanosoma cruzi* infections (32), it was demonstrated that CD4⁺ T cell help is required during the primary response to immunodominant epitopes. Our results suggest that in a vaccine, both CD4⁺ and CD8⁺ epitopes should be targeted in order to drive a protective immune response.

Although peptide vaccines including CD4⁺ and CD8⁺ epitopes could prime cell-mediated immune responses, low-molecular-weight synthetic peptide antigens are not highly immunogenic by themselves. These observations have led to investigations to co-administer additives known as adjuvants with vaccine antigens to potentiate activity of vaccines and the weak immunogenicity of the synthetic epitopes (33, 34). Adjuvanting antigens contribute to the success of vaccination. For example, 3-deacylated monophosphoryl lipid A (MLA, #2573) a detoxified derivative of the lipopolysaccharide (LPS) from *Salmonella minnesota* R595 is a Toll-like receptor 4 (TLR4) agonist and thus a potent activator of Th1 responses (35–37). It has been used as an adjuvant in human vaccine trials for several infectious diseases and cancer (38). It has been very effective as an adjuvant providing CD4⁺ T cell help for immunizations against other protozoan infections such as leishmaniasis (39). In our study, a robust response was observed when GLA-SE was included in preparation for immunization of mice. Pam₂Cys (S-[2,3-bis(palmitoyloxy)propyl]cysteine) is a lipid component of macrophage-activating lipopeptide. Pam₂Cys binds to and activates dendritic cells by engagement of Toll-like receptor 2 (TLR-2) (40, 41). Toll-like receptors (TLRs) function as pattern-recognition receptors in mammals (42). It is suggested that both TLR2 and TLR4 receptors might participate in the host defense against *T. gondii* infection through their activation by the GPIs and could work together with other MyD88-dependent receptors, like other TLRs, to obtain an effective host response against *T. gondii* infection (43). In our study, an even more robust response was observed in the Pam₂Cys co-administration group and robust protection was achieved in the transgenic mice challenged with Type II parasites following immunizations. The background of these HLA-B07 mice is a multigeneration back-cross of C57BL/6J mice and BalbK mice (11). There are no comparable mice without this transgene.

The work presented herein indicates that an HLA-B07 restricted epitope LT9 (LPQFATAAT) derived from GRA7_{20–28} that can elicit IFN- γ from T cells of seropositive HLA-B07 persons elicits IFN- γ and CD8⁺ T cells when administered with a universal DR epitope PADRE for CD4⁺ help and GLA-SE and Pam₂Cys to adjuvant MHC Class I-restricted CD8⁺ T cells. This peptide vaccine reduced parasite burden in immunized mice challenged with type II parasites. To our knowledge, this is the first report of a *T. gondii* specific HLA-B07 restricted CD8⁺ T cell epitope based vaccine that protects HLA-B07 transgenic mice against *T. gondii*. These findings facilitate development of an immunosense (i.e., based on a bioinformatics, followed by empiric testing approach based on HLA binding effects with human cells *in vitro* and testing in HLA transgenic mice [44]) epitope-based vaccine for human use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Immunogenicity of LT9 and VP9 in HLA transgenic mice

(A) HLA-B*0702-transgenic mice immunized with PBS as a control, peptides (LT9: LPQFATAAT; VA9: VPFVVFLVA) prepared as follows: peptide alone, peptide plus PADRE, peptide plus PADRE and Pam₂Cys, all these immunogens mixed with GLA-SE. Immunization was given three times at two week intervals. 11.5 $\pm$ 0.5 days after the last immunization, spleen cells were obtained from immunized mice and stimulated by the specified peptide in an *ex vivo* IFN- γ ELISPOT assay. This experiment was performed 3 (4) times. (B) HLA-B*0702 mice immunized with peptide pools and tested for recall responses with LT9 or VA9 or PADRE. HLA-B*0702 transgenic mice immunized with mixtures of two identified peptides (LT9 and VA9), PADRE or Pam₂Cys mixed with GLA-SE, three

times at two week intervals. Fourteen days after the last immunization, spleens were removed from immunized mice and splenocytes were cultured with peptides, LT9 or VA9 or PADRE. The results are expressed as IFN- γ spot formation by one million cells. Data presented are a representative example from three independent experiments. (C) IFN- γ spot formation was significantly reduced in the presence of anti-CD8 antibody. Splenocytes from immunized mice were incubated with antibodies to CD8, and their isotype control for 60–90 min prior to stimulation with LT9 or VA9. *, $P < 0.05$; **, $P < 0.01$. IFN- γ spot formation was tested for splenocytes from mice that received different vaccine formulations in the absence or presence of blocking antibody.

Figure 2. LT9-specific response induced by LT9 immunization

Mice were immunized with LT9 peptide alone, LT9/PADRE, LT9/PADRE/GLA-SE, LT9/PADRE/GLA-SE/Pam₂Cys. IFN- γ production (A) and lymphocyte proliferation (B) were tested 2 weeks after last immunization. *, $P < 0.05$; **, $P < 0.01$.

Figure 3. PADRE-specific T cell responses were evaluated in immunized group
 (A,B) PADRE-specific IFN- γ spot formation and spleen cell proliferation were tested by splenocytes from LT9 formulation immunized B7 mice exposed to PADRE peptide. *, $P < 0.05$; **, $P < 0.01$. (C) Blocking CD4⁺ T cell receptor using anti-CD4⁺ Ab significant decreases IFN- γ production in the following group.

Figure 4. LT9 peptides with adjuvants induce HLA-B07-restricted CD8⁺ T cell responses in immunized mice

LT9-specific IFN- γ response and lymphocyte proliferation were measured by blocking CD8 (A,B) and HLA-B07 (C). Splenocytes were isolated from peptide-immunized HLA-B07 transgenic mice 10 days after immunization and incubated with antibodies to CD8 α or HLA-B07 and their common isotype control for 60–90 min prior to stimulation with LT9. Their abilities to generate IFN- γ and lymphocyte proliferation in response to peptide were tested. *, $P < 0.05$; **, $P < 0.01$.

Figure 5. Replication of *T. gondii* luciferase expressing parasites were significant reduced in HLA-B07 mice immunized with LT9/PADRE/GLA-SE/Pam₂Cys assayed from 6 days after challenge and cyst formation in day 30

HLA-B07 transgenic mice were either not immunized or immunized with LT9/PADRE/GLA-SE/Pam₂Cys three times 14 days apart. Two weeks after vaccine administration, transgenic mice were challenged with 10,000 Pro(Fluc)-type II *T. gondii* luciferase expressing parasites. At 6 days after challenge infection, ventral image of mice were shown in Fig 5A. Average cyst enumeration was performed in the brains of mice challenged 30 days after final immunization (Fig 5B). The data represent the means. There were a total of 5–9 mice tested in each control or immunization group. Asterisks indicate significant differences compared to the control. These mice are on a C58BL/6 x Balb/c background backcrossed. There are no comparable mice without the HLA B-07 transgene. In separate experiments GLA-SE and PADRE were used but conferred no protection nor led to production of interferon gamma(Fig.6).

Figure 6. Xenogen imaging of brain *ex vivo* following the injection of luciferin intraperitoneally and then exposure of the brain to luciferin solution

Twenty one days earlier mice had been challenged with 20,000 FLUC Prugneaud parasites. They did not appear ill when they received three doses of GLA-SE-PADRE-LT9. However, when they received three doses of PBS or three doses of GLA-SE-PADRE they appeared ill 8 days after challenge. There were 5 mice per treatment group. FLUC challenge followed immunization by 2 weeks. Neither GLA-SE-PADRE nor PBS elicited splenocytes that produced IFN- γ when re-stimulated with LT9 whereas GLA-SE-PADRE-LT9 immunization did (data not shown). This was also shown in other HLA transgenic mouse strains (Table S2). Neither PBS nor GLA-SE-PADRE led to decrease in luciferase detection but GLA-SE-PADRE-LT9 did.

Table 1

The correlation between the solubility of the peptides and their immunogenicity.

Peptides ¹	Peptide Sequences	Predicted IC ₅₀ nM ²	Affinity ³ HLA-B*0702	Solubility ⁴ in PBS (%)	Elicits IFN- γ ⁵ in		Immunogenicity ⁶ in Mice	Protective ⁷ in Mice
					Seropositive Human	Seronegative Human		
GRA7 ₂₀₋₂₈	LPQFATAAT	62.4	14	99	+	-	+	+
GRA3 ₂₇₋₃₅	VPFVVELVA	373.9	18	10	+	-	-	-

¹ Peptides derived from two proteins and the position within the protein

² Peptide binding affinities to HLA-B*0702 were predicted using the ARB algorithms from immunopeptide database (IEDB). IC₅₀ is 50% inhibition of binding by a competitor.

³ Binding affinity was performed by MHC binding assay.

⁴ Percent solubility, values are determined by absorbance at 280nm and Bradford assay.

⁵ PBMC cells from four *T. gondii*-seropositive HLA-B07⁺ humans and three seronegative ones were stimulated with GRA7₂₀₋₂₈ (LPQFATAAT) and GRA3₂₇₋₃₅ (VPFVVELVA); the T cells that produce IFN- γ were tested by Elispot Assay.

⁶ Splenic T cells were isolated from HLA-A*0702 mice 2 weeks after peptide immunization and tested for their ability to generate IFN- γ in response to peptide. (-), not immunogenic at any dose.

⁷ Protective HLA-B*0702 in transgenic mice.