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Adjuvanted multi-epitope vaccines protect HLA-A*1101 transgenic mice against *Toxoplasma gondii.*

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ABSTRACT

We created and tested multi-epitope DNA or protein vaccines with TLR4 ligand emulsion adjuvant (gluco glucopyranosyl lipid adjuvant in a stable emulsion (GLA-SE)) for their ability to protect against *Toxoplasma gondii* in HLA transgenic mice. Our constructs each included five of our best down selected $CD8^+$T cell eliciting epitopes, an universal $CD4^+$ helper T lymphocyte epitope (PADRE), a secretory signal, all arranged for optimal MHC Class I presentation. Their capacity to elicit immune and protective responses was studied using immunization of HLA-A*1101 transgenic mice. These multi-epitope vaccines increased memory $CD8^+$ T cells that produced IFN-γ and protected mice against parasite burden when challenged with *T. gondii*. Endocytosis of emulsion-trapped protein and cross presentation of the antigens must account for the immunogenicity of our adjuvanted protein. Thus, our work creates a novel adjuvanted platform assembly of peptides resulting in cross presentation of $CD8^+$ T cell eliciting epitopes in a vaccine that prevents toxoplasmosis.

Keywords: *Toxoplasma gondii,* HLA-A*1101, vaccine, DNA, multi-epitope protein.

INTRODUCTION

Toxoplasma gondii is an intracellular parasite that can cause severe ocular and neurological diseases in the fetus, newborn infants and immunocompromised individuals (1). The acute infection is characterized by proliferation of tachyzoites, which replicate rapidly within host cells and lyse their host cells within 24–48 hours to release large numbers of progeny. In response to immune pressure, the parasite differentiates into a slow growing form called bradyzoites, which resides within intracellular cysts. Formation of tissue cysts normally occurs in long-lived cells such as muscle or neuronal cells.

Although anti-parasitic medicines such as sulfadiazine and pyrimethamine are effective against tachyzoites, they are associated with toxicity or hypersensitivity and do not eliminate the latent, the cyst form of the parasite (2). Thus, there is a need for development of a safe, protective vaccine. Our recent work has focused on development of an epitope-based vaccine for toxoplamosis to enhance host immunity by stimulating efficacious responses of $CD4^+$ helper T lymphocytes (HTLs) and $CD8^+$ IFN-γ-producing T lymphocytes (3-6) for humans. These $CD8^+$ T cells recognize the octamer/nonamer peptides presented by HLA supermotif major histocompatibility complex class I (MHC-I) molecules on antigen presenting cells (APCs) in humans (3-6). Considerable effort has been made to identify promising vaccine candidate antigens for *T. gondii*. Using bioinformatic analyses, multiple predicted $CD8^+$ epitopes of tachyzoite- and bradyzoite-specific antigens have recently entered our vaccine development pipeline (3-6). Candidate amino acid sequences are from surface antigens (SAG1 and SUSA1) and secreted proteins, including dense granule proteins (GRA2, GRA3, GRA6, GRA7) and

rhoptry proteins (ROP2, ROP16, ROP18). We reported in our previous studies $CD8^+$ epitopes derived from these antigens that can be presented by HLA-A02, HLA-A03 and HLA-B07 human major histocompatibility complex (MHC) molecules (3-6). These $CD8^+$ T cell-restricted peptides administered with a universal helper T cell epitope, PADRE and adjuvants elicit IFN-γ and reduce parasite burden in HLA-B*0702, HLA-A*1101 and HLA- A*0201 transgenic mice.

In the present study, we selected five peptide epitopes, KSFKDILPK ($SAG1_{224-232}$), STFWPCLLR ($SAG2C_{13-21}$), AVVSLLRLLK ($GRA5_{89-98}$), SSAYVFSVK ($SRS52A_{250-258}$), and AMLTAFFLR ($GRA6_{164-172}$) that elicited IFN-γ from peripheral blood mononuclear leukocytes (PBMCs) from *T. gondii* seropositive HLA-A03 supertype humans. SAG1 is a surface protein expressed in tachyzoites, and mediates adhesion of the parasite to the host cell prior to invasion (7, 8). It is highly conserved in *T. gondii* strains, and shown to induce cellular immune responses in mice (9). SAG2C appears to be expressed exclusively on the surface of bradyzoites. SRS52A is one of the surface antigen-1-related sequences (SRSs), which comprise a gene family with similar structure to the major tachyzoite immunodominant surface antigen, SAG1. GRA6 is expressed and secreted by both the tachyzoite and the bradyzoite stages of the parasite. GRA5 another dense granule protein is present in bradyzoites and is strongly concentrated in the cyst wall (10).

As identification of protective epitopes increases, novel ways to present these immunogenic peptide epitopes to a host's immune system are needed. Development of

multi-epitope DNA vaccines has been utilized to protect against other pathogens. However, immunogenicity of epitope-based DNA vaccines is not yet optimal, as immune responses induced by epitope DNA vaccines are often weak (11). In our previous studies we produced a prototypic *Toxoplasma* vaccine based on a highly versatile self-assembling protein nanoparticle platform that can repetitively display PADRE-GRA7$_{20–28}$ antigenic epitopes and found a 72% reduction of cysts in type II *T. gondii* infected HLA-B*0702 transgenic mice (12). Thus, our current study is intended to develop new tools to add to a rational design that might be adapted to other platform approaches with $CD8^+$ epitopes that bind to human major histocompatibility complex (HLA) class I molecules or be sufficient by themselves to elicit robust protection in humans.

Herein, we selected five potentially protective octamer $CD8^+$ T cells eliciting epitopes that interact with HLA-A*1101. We generated two polypeptides containing the five HLA-A*1101 peptides and PADRE linked with 2 different linkers. We found better protection with the K/NAAA linker. This polypeptide administered with TLR4 ligand containing emulsion, GLA-SE was more effective than ALUM adjuvant. Furthermore, we examined the immunogenicity of a synthetic DNA vaccine encoding the five octamer $CD8^+$ T cells eliciting epitopes of *T. gondii*. DNA was delivered by electroporation as previously described (13, 14) into HLA-A*1101 transgenic mice and the immunization was followed two weeks later by two protein boosts with the multi-epitope recombinant protein adjuvanted with a universal $CD4^+$ helper T lymphocyte epitope, PADRE, and GLA-SE. Immunization of these mice activated $CD8^+$ T cells to produce IFN-γ, increase memory $CD8^+$ T cells and protected against subsequent challenge with a high inoculum

of type II parasites. Our results highlight the potential for the use of GLA-SE adjuvanted $CD8^+$ T cell-restricted epitopes in multi-epitope assemblies as a platform for vaccine approach to protect against toxoplasmosis.

RESULTS

*Identification of new candidate T. gondii specific HLA-A*1101-restricted epitopes*

We have searched candidate epitopes from *T. gondii* that show high affinity binding to HLA-A*1101 molecules (Table 1). Five peptide epitopes derived from SAG1, SRS52A, SAG2C, GRA6 and GRA5 have representative affinities for HLA-A*1101 molecules, a haplotype that covered 16-30% population in China; 7-16% in Europe and North America; 1.5-10% in South America (www.allelefrequencies.net) (Table 1).

To determine which of these peptides would be recognized in the context of *Toxoplasma* infection, peripheral blood mononuclear cells (PBMC) from *T. gondii*-seropositive HLA-A03 individuals were tested for response to these peptides in pools or individually by using an IFN-γ ELISpot assay. Candidate peptides were considered immunogenic if they induced IFN-γ-secreting spot formation that was significant compared to an irrelevant HLA-A*1101-restricted peptide. As shown in Figure 1, there were five peptide pools which stimulated significant response by PBMC derived from *Toxoplasma* seropositive HLA-A03 individuals. These were: one from $SAG1_{224-232}$ (KSFKDILPK), $SAG2C_{13-21}$ (STFWPCLLR), $GRA5_{89-98}$ (AVVSLLRLLK), $SRS52A_{250-258}$ (SSAYVFSVK), and $GRA6_{164-172}$ (AMLTAFFLR).

*Specificity of epitopes for HLA A*1101.*

The peptides we selected are HLA-A*1101 specific. This specificity is shown in our binding assay where our peptides do not significantly bind to HLA-A*0201 or B*0702 molecule (Table 1). HLA-A*0201 and B*0702 are among the other most prevalent

supermotifs, and our results are consonant with in silico predictions for these haplotypes. In table 1, none of the peptides would be considered an A*02.01 or B*07.02 binders. In Table 2, relative to the background genes in the C57BL/6J mice, there is specificity of presentation of peptides to those that are restricted by HLA-A*1101 by macrophages from the transgenic HLA-A*1101 mice, but not presented by macrophages from their wild type mouse controls. In earlier work by Tan, McLeod et al; .Cong, McLeod et al. (3, 5) for all 5 peptides utilized herein, PBMC from 3 *T. gondii* seropositive HLA-A 03-11 persons with serum antibody to *T. gondii* (seropositive) and 3 persons without serum antibody to *T. gondii (*seronegative) were tested. PBMC from all the seropositive persons responded to the peptides and no seronegative persons had PBMC that responded with IFN-γ production demonstrated by ELISPOT. For peptides and TLA differences were highly significant for seropositive and seronegative persons ($p < 0.001$) whereas differences for Concanavalin A were not significant. The response to the peptides is specific to the parasite in seropositive persons and not present in those who are seronegative. Herein, the peptides were re-tested at the same time for the same HLA A3-11 seropositive human donor used for our subsequent studies herein. HLA A2, B7 haplotype seropositive persons (and seronegative persons) whose cells were without response to individual peptides or the polyepitope in the ELISPOT for IFNγ in two replicate experiments (data not shown).

*HLA-A*1101-transgenic mice as a model to assess cellular immunogenicity of 5 identified HLA-A*1101-restricted CD8+ T cell epitope peptides*

To address the HLA-A11-specific genetic restriction of the 5 HLA-A*1101 epitopes (plus a GRA7 peptide) identified to prime for IFN-γ responses, HLA-A*1101-transgenic mice were immunized subcutaneously. Immunization was with these peptides mixed with PADRE, and adjuvants or the adjuvants alone As shown in Table 2, robust IFN-g responses from CD8+ T cells from spleens of mice immunized with peptides plus adjuvants were observed following stimulation by the pool of peptides in the presence of APC from the HLA-A*1101 transgenic mice but not of APC from wild-type C57BL/6 mice (Table 2). Immunization of HLA-A*1101-transgenic mice with adjuvants alone did not elicit IFN-g when the $CD8^+$ T cells were stimulated either with or without peptides (i.e. background levels) (Table 2).

Vaccination with peptide pools, PADRE and GLA-SE adjuvant protects mice and increase memory against type II parasite challenge

HLA-A*1101-transgenic mice were immunized with peptide pools combined with GLA-SE adjuvant and PADRE three times at intervals of two weeks. PBS was used as control. Five weeks after the last immunization, mice were challenged with type II parasites. Differences in brain cyst numbers between control and immunized mice were significant ($p< 0.013$) as shown in Figure 2A-B, with the immunized mice having a reduced cyst burden. Spleens from unchallenged immunized and control mice were tested for the ability of the immunization to induce $CD8^+$ T cell memory response. As shown in Figure 2C-D, there is an increase of memory $CD8^+$ T cells in the immunized group.

LO and AZ multi-epitope polypeptide immunogenicity in vitro

The $CD8^+$ T cell-epitopes identified are intended to form the basis of *Toxoplasma* vaccine for persons with the HLA-A*1101 supertype. We expressed and purified from *E. coli* a protein composed of the five epitopes linked in a sequence with the universal $CD4^+$ T cell epitope, PADRE (AKFVAAWTLKAAA) and the murine Igκ-chain signal sequence for targeting protein to secretory pathway at the N-terminus. The epitopes were linked together with N/K alanines or GPGPG as linker, named as LO and AZ, respectively (Figure 3A-B). Both proteins were purified via Ni-NTA affinity column and the molecular weight was verified by SDS-PAGE analysis (Figure 3C). Immunogenicity of the two proteins compared to the pool of the 5 individual A11-restricted $CD8^+$ T cell epitope peptides were tested in vitro. Briefly, PBMCs from *T. gondii* seropositive HLA-A03 supertype humans were tested for their ability to generate IFN-γ in response to the stimulation with either LO or AZ protein or a pool of the peptides for 2 days to allow time for processing the proteins and presentation thereof to MHC class I. The data in Figure 4A-C demonstrate IFN-γ secretion was significantly enhanced by stimulation with either LO or AZ multi-epitope polypeptide compared with a pool of the individual 5 epitope peptides ($p<0.001$).

Herein, the peptides and polyepitope were re-tested at the same time for the same HLA A3-11 seropositive human donor used for our subsequent studies herein and HLA A2, B7 haplotype seropositive persons (and seronegative persons) whose cells were without response (data not shown).

*Immunization with LO and AZ multi-epitope polypeptides with GLA-SE adjuvant confers a potent protection in HLA-A*1101 transgenic mice against T. gondii.*

HLA-A*1101 transgenic mice were immunized with a combination of LO and AZ multi-epitope polypeptides with GLA-SE or ALUM adjuvant. As a control, mice were immunized with adjuvant alone or PBS. Mice were then challenged 2 weeks after the last immunization with type II strains of *T. gondii*. As shown in Figure 5, 80% of mice immunized with LO and AZ multi-epitope polypeptides emulsified in GLA-SE adjuvant survived parasite challenge. In contrast, only 30% of mice immunized with the ALUM adjuvant plus polypeptides survived parasite challenge ($p<0.05$). As a control, neither mice immunized with the adjuvant alone nor those immunized with PBS increase their survival after challenge (Figure 5).

Prime/ boost strategy: LO and AZ DNA plus multi-epitope polypeptide immunogenicity in vivo

We next addressed whether a prime/boost strategy using DNA encoding the five individual epitopes plus PADRE and the polypeptides would confer better protection against parasite challenge in HLA-A*1101 mice. We constructed synthetic DNAs in which the 5 poly-epitope nucleotides with the N/K alanines or GPGPG linkers plus PADRE were cloned in *EcoRI* and *BglII* sites of the vaccine vector pMB75.6 (Figure 6A). During DNA vaccinations, mice were immunized intramuscularly (i.m.) two times at 2 weeks interval with 100 µg of LO or AZ DNA vectors followed by another two injections of 50 µg of the polypeptides at 2 weeks interval. They were challenged with 2,000 ME49 (Fluc) 2 weeks after the last immunization. Brains from these mice were

imaged 21 days after the challenge using a Xenogen in vivo imaging system to assess parasite burden in the brain. As shown in Figure 6B-C, as an additional measure of efficacy for protection against challenge with luciferase-expressing parasites, the numbers of these parasites in HLA-A*1101 mice immunized with LO or AZ DNA plus multi-epitope polypeptide or polypeptides alone were significantly reduced compared to the mice immunized with control empty vector or PBS. This correlates with the reduction of the number of cysts per brain (Figure 6D). We then analyzed the effect of LO or AZ DNA plus multi-epitope polypeptide on IFN-γ expression in vitro. Briefly, mice were immunized two times at 2 weeks interval with LO or AZ DNA followed by another two injections of either LO polypeptide, AZ polypeptide or ovalbumin peptide as control. Negative controls mice were vaccinated twice with empty vector followed by saline. Two weeks after the last immunization, mice were sacrificed and splenocytes were harvested for immune responses analysis. As shown in Figure 7A, considerable amount of IFN-γ expressing $CD8^+$ cells were observed following immunization with AZ DNA or LO DNA compared with mice immunized with the empty vector. An even more robust response was achieved when mice immunized with the vectors expressing the polypeptides were stimulated in vitro by the corresponding AZ or LO protein, although the difference was only significant in the LO treated mice. In contrast, there was no increase in the amount of $CD8^+$ IFN-γ producing cells observed when the mice were immunized with AZ or LO DNA followed by a challenge with a non-relevant protein, ovalbumin. There was a very modest increase in protection when DNA administration precedes administration of protein with the LO construct as in survival curves and dot plots (Figure 7B).

*LO DNA plus multi-epitope LO polypeptide are protective against Toxoplasma challenge in HLA-A*1101 transgenic mice*

As shown in Figure 7B, a majority, 7 of 8 (87%) HLA-A*1101 mice immunized with *LO DNA plus LO polypeptide* emulsified in GLA-SE adjuvant survived parasite challenge. In contrast, only 1 of 8 (12%) unimmunized mice or immunized with empty vector survived parasite challenge.

LO DNA plus multi-epitope polypeptide increases memory $CD8^+$ T cell response

We then analyzed the effect of *LO DNA plus multi-epitope polypeptide* on the *T. gondii*–specific $CD8^+$ T cell memory response. This was performed by quantifying the levels of memory T cells in the spleen from HLA-A*1101 mice at 35 days after the last immunization. As shown in Figure 7C-D, $CD8^+$ memory T cells were significantly increased in mice immunized with either LO protein alone or LODNA plus LO protein compared with mice immunized with the empty vector or PBS.

GLA-SE is a TLR4 ligand that elicits cytokines.

Demonstration that GLA-SE is a TLR4 ligand is shown schematically and with data in Figure 8A eliciting IL6, IL2, and TNFα. We determine whether GLA-SE induces cytokine production. As shown in Figure 8A, human whole blood was stimulated with GLA-SE for 24 hours. There was an increase of cytokine production in plasma from these cultures. Difference between stimulated and unstimulated cultures are significant ($p<0.05$) (Figure 8B).

DISCUSSION

There is a need for improved vaccination and delivery approaches to induce cellular immune responses against *T. gondii.* Earlier, bioinformatics, immunosense approaches and empiric studies with human peripheral blood mononuclear cells facilitated identification of protective peptide epitopes for HLA-A*1101 supertypes. The most immunogenic of these epitopes (KSFKDILPK ($SAG1_{224-232}$), STFWPCLLR ($SAG2C_{13-21}$), AVVSLLRLLK ($GRA5_{89-98}$), SSAYVFSVK ($SRS52A_{250-258}$), and AMLTAFFLR ($GRA6_{164-172}$)) were then utilized in comparative studies with differing adjuvants and delivery methods in the HLA-A*1101 supermotif transgenic mice to characterize immune responses and study protection. Herein, we present a novel way to present immunogenic peptide epitopes to a host's immune system based on the assembly of five protective $CD8^+$ T cell epitope for HLA-A*1101-restricted supertypes. These epitopes were constructed with the universal $CD4^+$ T cell epitope, PADRE linked with N/KAAA or GPGPG spacers and a secretory signal. These vaccine design features were incorporated to optimize proteasome processing and, subsequently, epitope and vaccine immunogenicity.

Improved delivery approaches are important for successful vaccine. This was showed by previous studies with another transgenic mouse supertype, HLA-B*07, where *T gondii*–specific HLA-B07–restricted $CD8^+$ T cell epitope LPQFATAAT derived from $GRA7_{20–28}$ elicited $CD8^+$ T cell specific IFN-γ with the help of a universal $CD4^+$ epitope and adjuvant GLA-SE confered protection of HLA-B07 mice from type II parasite challenge (4). The same epitope used in a novel nanoparticle platform was found to have better

immunogenicity (12), with 72% reduction of cyst burden in the brains of $GRA7_{20–28}$ nanoparticles-immunized mice compared to 54% reduction with immunization with $GRA7_{20–28}$ peptide in combination with PADRE and the adjuvant GLA-SE. This work provided a foundation for the present study, which is intended to improve vaccination and delivery approaches against *T. gondii* to protect humans.

Herein, we examined the immunogenicity of a multi-epitope protein and synthetic consensus DNA, clinically approved mammalian expression vector encoding five *T. gondii* specific HLA-A*1101-restricted epitopes. The DNA plasmid vaccine is encoding the five $CD8^+$ T cell epitopes restricted by HLA-A*1101 supertype alleles and the universal HTL epitope, PADRE. These DNA constructs were optimized using codon optimization, leader sequence addition, plasmid production at high concentration and the DNA was delivered by electroporation as described (13). Immunization of HLA-A*1101 mice with recombinant multi-epitope formulated with a Toll-like receptor 4 ligand (TLR4)-containing adjuvant (gluco glucopyranosyl lipid adjuvant in a stable emulsion [GLA-SE]) induced antigen-specific IFN-γ-producing $CD8^+$ T cells in their spleens, increase memory $CD8^+$ T cells population, and conferred a potent protection against *T. gondii* challenge. The adjuvant ALUM formulated to the recombinant multi-epitope was less effective in conferring protection as when compared to the vaccine preparations containing GLA-SE. The present study also demonstrated that DNA prime followed by a multi-epitope protein-GLA-SE boost is more protective than either of the DNA prime followed by ovalbumin (unrelated protein) boost or the recombinant multi-epitope alone.

The major limitation of DNA vaccination in the past has been its relatively weak immunogenicity in vivo for humans. Electroporation has successfully aided in enhancing the delivery of DNA vaccines, leading to the generation of stronger immune responses (10–100-fold) compared with naked DNA vaccine delivery alone to muscle or skin (15). Interestingly, a multi-epitope DNA prime-protein boost also appeared to drive a more functionally divergent T-cell response (16). Previous studies have shown that protein and DNA only vaccination produced similar numbers of IFN-γ^+ cells in both $CD4^+$ and $CD8^+$ T-cells, as well as similar numbers of IL-2^+ cells in both $CD4^+$ and $CD8^+$ T-cells (16). In contrast, a multi-epitope DNA prime-protein boost resulted in four times more IFN-γ^+-secreting $CD8^+$ T-cells than IFN-γ^+-secreting $CD4^+$ T-cells and higher IL-2^+ -secreting $CD4^+$ and $CD8^+$ T-cells (16). Notably, our present study showed that protein prime and boost with GLA-SE was also quite effective. The *ex vivo* stimulation of spleen cells from HLA-A*1101 mice and PBMC from HLA-A03 seropositive individuals showed $CD8^+$ T cells were more responsive to the composite polypeptide than to the pooled or single constituent peptides. Processing and presentation of AZ and LO poly-epitope polypeptide in human cells occur with high efficiency in LO poly-epitope-stimulated cells. These cells demonstrate stronger responses with N/K alanines linker compared to GPGPG.

The polyepitope protein induces a more robust response, compared to single peptides with GLA-SE, or lipopeptides with single or three of the peptides, linked, but in a different order. In these linked and single peptide lipopeptides, responses are much less or absent (3, 5). Other arrangements of sequence in DNA vaccines encoding peptides in DNA vaccine or peptides in a non-ionic surfactant vesicles (NISV) also elicited less

robust response or were or absent (data not shown). In future studies, when constructs for all haplotypes are fully defined and optimized, it will be important to utilize models we have developed for ocular, congenital, peroral encysted bradyzoite and oocyst infections, as well as immune compromised models of infections (17-24), along with intranasal immunizations (Gigly, Dubey, Zhou, McLeod et al., In preparation).

There are a number of aspects of our current approach that make our work herein unique and valuable: This work makes a step change forward towards building immunosense vaccines for humans. These paradigm changes include: The GLA-SE emulsion leads to delivery of peptides from an artificial, polypeptide-epitope protein to both Class I and Class II MHC pathways. This is demonstrated by epitope content and effector functions they elicit. The polyepitope with a secretory sequence and with peptides arranged so cleavage is optimized in such a way that it includes the intended peptides in the protein is another step change. This arrangement allows for optimal processing and presentation. Computer-based modeling was used to design our vaccine component in this manner (25, 26). It was designed to optimize proteasome-mediated epitope processing and to minimize the creation of junctional epitopes. Junctional epitopes occur by the juxtaposition of two epitopes. Altering epitope order and introducing selected amino acid spacers at the C terminus of individual epitopes were used to control these properties (25, 26). This is, to our knowledge, the first and only creation of a artificial protein based on immunosense in conjunction with GLA-SE that successfully immunizes HLA transgenic, or any, mice, against *Toxoplasma gondii*. Our use of mouse strains with differing HLA supermotif transgenes also makes our work pertinent to begin to protect people globally

against *Toxoplasma* infection with vaccines rather than simply protecting mice against this infection. It presents a new paradigm for an immunosense approach for protection against human *T. gondii* and other infections and diseases. Further, we found earlier that protection and IFN-γ production following immunization with individual peptides pooled or organized with alanine linkers, and linked in lipopeptides, are substantially less robust in eliciting IFN-γ and protection (3, 5) than the approach herein with DNA and protein, or protein and protein immunizations, in HLA-A*011 transgenic mice. Our earlier attempt to include these and many other epitopes that would bind to other supermotifs in a "chain of pearls" DNA vaccine (El Bissati, Dubey, Mui, Roberts, Gigly, McLeod, et al., unpublished) administered with electroporation or a peptide in NISV (Roberts, Woods, Mui, El Bissati, Dubey, Gigly, McLeod, unpublished data) were unsuccessful other than a very small response with the DNA vaccine to the N terminal encoded peptide, conferring very modest protection. This was despite finding the peptides by mass spectroscopy when the full length construct was delivered as a vector transfected into a monocytic cell line (El Bissati, McLeod, unpublished data).

We tried deconvoluting our peptides in a separate study to characterize whether adding a homologous mouse $CD4^+$ T cell stimulating peptide would enhance protection. We present some of these data in another manuscript (El Bissati, McLeod et al, in preparation 2016). In this work we learned something quite unexpected. Surprisingly, in this other study we found that in female mice, this $CD4^+$ T cell eliciting nonamer peptide epitope which added protection in the combination of peptides, when tested as a single peptide administered with GLA-SE, as we deconvoluted peptides, was lethal in the booster,

second immunization (El Bissati, Zhou, McLeod et al., manuscript in preparation). This lethality was associated with intestinal, hepatic, spleen, and lymph node pathology due to the $CD4^+$ T cell elicited (El Bissati, Zhou, McLeod et al., manuscript in preparation). Without the $CD8^+$ T cell eliciting peptides or PADRE, there was a cytokine storm with IL6 especially prominent. In contrast to administration of this one peptide with GLA-SE, with inclusion of our $CD8^+$ peptides or addition of only PADRE there was enhanced protection. What we learned is that it is not only the presence of the particular peptide but the company it keeps that leads to harmful or enhanced protective immune response. Some other recent similar studies make this point a different way. For example, based on the recent work of Blanchard et al. (27) it is also location in the entire construct that determines immunogenicity and robustness of protection. Similarly, background genes of the host, and also the microbiome (from the work of others), influences robustness and skewing of immune responses (28-30). We had thought to confirm safety we should deconvolute the constituents of our vaccines. What we learned was that immunogenicity and protection will depend on the host's genetics and epigenetics, and company the peptides keep in the final vaccine for humans. Function of the assembled vaccine constituents in the human, not just murine host, will be critical. Our recent work, and very recent work of others (27), indicates that efficacy and toxicity are likely to be more complex, and ultimately require safety and efficacy testing in humans.

The in vitro and in vivo murine work in the present study indicates that the GLA-SE nano-emulsion must provide protein for cross presentation by antigen presenting cells as shown schematically in Figure 8. As shown in our experiments herein (Figure 8B) GLA-

SE is a TLR4 ligand and in multiple other experimental systems and moving into the clinic, triggering MYD88 and downstream signaling pathways (31, 32). The GLA-SE emulsion entraps proteins in nanoparticle structures of approximately 100 nm with multiple associated GLA molecules on their surfaces, although the percentage entrapment and precise location of the GLA-SE was not studied herein. Such particles as well as proteins that are endocytosed enter a MHC Class II pathway stimulating $CD4^+$ T cells (33). Herein, we demonstrate both MHC Class I and Class II responses to peptides in our constructed protein. It is likely the small particle size (100 nm) is responsible for this unusual trajectory to MHC Class I presenting bound peptides to $CD8^+$ T cells. The effector mechanism restricted by MHC Class I for a peptide defines the cross presentation of these antigens. From a polyepitope, that must enter its host cell by endocytosis, protein or peptides must leave the endocytic vacuole, and therefore be "cross presented". Further, since completion of the present work, we also placed this polyepitope protein construct with $CD8^+$ T cell eliciting epitopes in a Self-Assembling Protein Nanoparticle (SAPN) (El Bissati, Zhou, Burkhard, McLeod, *in submission*). Recent electron microscopy studies also demonstrated a malaria SAPN eliciting $CD8^+$ T cells with localization to the proteasome and delayed kinetics of antigen processing and presentation with a time frame consistent with cross presentation (34). Further, we deliver PADRE, which is a universal $CD4^+$ T cell eliciting epitope, in the same polyepitope protein as our $CD8^+$ T cell eliciting epitopes that trigger an MHC Class I eliciting immune response. PADRE from this constructed protein also does enter a Class II pathway so either some polyepitope is not entrapped or our delivery system must bifurcate to cross presentation to MHC Class I and II pathways. This is a novel and

important finding with broad implications for vaccine development when induction of immune responses with protective $CD8^+$ T- cells are critical.

Despite the protection afforded by vaccination regimens developed in this study, no preparation provided complete protection against *T. gondii* and some brain cysts and parasites in peritoneal fluid were still detected. There is room to improve the vaccine by adding the parasite immunogenic peptides, which generate parasite specific $CD4^+$ T cells. In addition the inclusion of B cell epitopes to induce antibodies might also help develop more robust vaccines against *T. gondii*. Antibodies have not been considered to be the primary protective mechanism for *T. gondii,* when compared to cell mediated immunity with the induction of cytolytic T cells and IFN-γ production (35, 36). Nonetheless, antibodies, including IgM have been implicated in killing extracellular parasites via inducing complement-dependent lysis or by blocking entry of parasites into host cells (37-39).

In summary, our study shows a composite protein, with a secretory signal, five $CD8^+$ MHC class I epitopes from *T. gondii*, and PADRE, a universal $CD4^+$ eliciting peptide, can be assembled and elicits protective MHC Class I, restricted responses. Using HLA-A*1101 transgenic mice, we demonstrate the capacity and specificity of 5 HLA-A*1101 restricted epitopes to prime and boost an IFN-γ response. In addition, the recombinant multi-epitope polypeptide emulsified in GLA-SE adjuvant confers more protection and increases memory $CD8^+$ T cell response against *T. gondii* in HLA-A*1101 transgenic mice. Previous studies have found that GLA-SE, formulated with the recombinant fatty

acid binding protein Sm14 enhances Th-1 type responses. Production of IFN-γ was the basis of Sm-14 mediated protective immunity both in humans with schistosomiasis and animal models (40). This Sm14 anti-schistosome vaccine is safe and entering phase 2 trials in Brazil and Africa. In our study, it is likely that the GLA-SE emulsion encloses the protein and presents an optimum configuration decorated with the TLR4 ligand GLA that induces a powerful $CD8^+$ and $CD4^+$ T cell immune response (Figure 8). In addition, DNA encoding the multi-epitope delivered by electroporation and followed by protein boosts is useful for the induction of a strong immune response against *T. gondii*. Thus, our data provide important support suggesting that enhanced electroporation-delivered DNA prime-protein boost, and protein prime protein boost which would be considerably better tolerated, are useful strategies for delivery of a multi-epitope anti-apicomplexan parasite vaccine. As these epitopes for immunization are tailored to HLA and the efficacy of vaccination with these epitopes is evaluated in mice expressing an HLA molecule, they provide a foundation and promise as a vaccine for humans. Potential improvements to this vaccine might be made with the addition of epitopes from various *T. gondii* proteins encompassing several of the parasite life stages and polymorphogenetic strains in a single platform that could elicit a protective immune response for the HLA class I and II supermotifs present for all the human population.

METHODS

Bioinformatic predictions and MHC-peptide binding assays

Protein sequences derived from SAG1, SRS52A, SAG2C, GRA6, and GRA5 were analyzed for $CD8^+$ T cell epitopes based on predicted binding affinity to HLA-A*1101 molecules using algorithms available at the Immune Epitope database (IEDB) http://www.iedb.org (41, 42). Quantitative assays to measure binding of peptides to HLA class I molecules are based on inhibition of binding of radiolabeled standard peptide. Assays were performed as described previously (43, 44). Concentration of peptide yielding 50% inhibition of binding of radiolabeled probe peptide (IC_{50}) was calculated. Under the conditions utilized, were where [label]<[MHC] and IC50, [MHC], the measured IC50 values are reasonable approximations of the true Kd values.

Human PBMC and ELISpot assay

PBMC were obtained from individuals seropositive to *T. gondii*, and their HLA haplotype was determined. These cells were processed and cryopreserved as described previously (3). ELISpot assays with human PBMCs used anti-human IFN-γ mAb (1-D1K) with biotinylated anti-human IFN-γ mAb (7B6-1) with 2×10^5 PBMCs per well (5, 6). All antibodies and reagents used for ELISpot assays were from Mabtech (Cincinnati, OH). The PBMC were plated in at least 3 replicate wells for each condition. Results were expressed as number of spot forming cells (SFCs) per 10^6 PBMCs.

Epitope peptides

KSFKDILPK ($SAG1_{224-232}$), STFWPCLLR ($SAG2C_{13-21}$), AVVSLLRLLK ($GRA5_{89-98}$),

SSAYVFSVK ($SRS52A_{250-258}$), AMLTAFFLR ($GRA6_{164-172}$) and PADRE-derived universal CD4 helper epitope (AKFVAAWTLKAAA) were used in the vaccine constructs (45). GLA-SE adjuvant (TLR4 agonist), synthesized by the Infectious Diseases Research Institute (Seattle, Washington) was used as a stable oil-in-water emulsion with specified epitopes during immunization.

Multi-epitope DNA vaccine design

To maximize epitope immunogenicity in vivo, the peptides encoding minigene included starting codon ATG and the mouse Ig k signal sequence at the 5' end of the construct, and spacer sequences N/AAA (LO construct) and GPGPG (AZ construct) residues flanking the C-terminus of all epitopes (Figures 3 A-C). Whereas the former facilitates processing of the CTL epitopes in the ER, the latter favors proper proteasomal processing and prevents the formation of junctional HLA epitopes. The order of the CTL and HTL epitopes in the minigene and type of spacer sequences that favor proper proteasomal cleavage were determined by a customized computer software program (Epimmune) that identifies the most favorable sequence for epitope processing and simultaneously minimizes the creation of new junctional HLA-A11 determinants.

Purification of multi-epitope protein vaccine

LO and AZ DNA were PCR-amplified and cloned in the expression vector pET-22 (Novagen). The multi-epitope protein was expressed in the *Escherichia coli* BL21-CodonPlus strain (Stratagene). Expression clones were grown at 37°C in Luria broth medium containing 50 μg/μl kanamycin and 34 μg/μl chloramphenicol. A 1-liter culture

of *E. coli* was grown to an A_{600} of 0.6, and protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (1mM final concentration). Recombinant protein was extracted under native conditions by using the BugBuster protein extraction reagent (Novagen, 6 ml/g of cell pellet) containing a protease inhibitor mix (Roche Diagnostics) and 10µg/ml lysozyme. All purification steps were performed under 8 M urea denaturing conditions. The His-tagged polypeptides were purified by using nickel-affinity chromatography and followed by Q-Sepharose, which used to capture the endotoxin. The eluate, which contains 8 M urea, was dialyzed against a buffer containing 5 mM Hepes-KOH (pH 7.8) and 0.5 mM DTT. The purity of the protein was determined by SDS-PAGE, and the protein concentration was measured by the method of Bradford using BSA as a standard. Using E-TOXATE Kits (Sigma-Aldrich, USA), endotoxin concentration in these proteins is <25EU/ug of protein.

Plasmid and gene cloning of multi-epitopes DNA vaccine construct

The clinically approved mammalian expression vector pMB75.6 (from Inovio Pharmaceuticals, Blue Bell, PA) was used as a DNA vaccine vector (25). Briefly, the plasmid contains elements essential for expression in mammalian cells: a cytomegalovirus (CMV) promoter, intron, and gene of interest followed by the simian virus 40 (SV40) polyadenylation signal.

The oligonucleotides of 5 individual *T. gondii* peptides shown in Table 1 plus PADRE-derived universal CD4 helper epitope were linked with different spacers and synthesized using 9 overlapping 50-nucleotide oligonucleotides. *LO* and *AZ* were first assembled and

amplified as three small fragments that were subsequently used as templates to amplify the whole gene. The full-length constructs were cloned into the vaccine vector pMB75.6 using *EcoRI/BglII* restriction sites. Neither the pMB75.6 vector backbone nor the epitope-encoding region shares significant homology with known human genomic sequences. All recombinant plasmids were propagated in *Escherichia coli* TOP10 and confirmed by restriction analysis and PCR sequencing. Large-scale plasmid DNA was prepared using the endotoxin-free Mega kit according to the manufacturer's instructions (Qiagen, Hilden, Germany), and the DNA concentrations were determined by A260/A280 absorption measurements. Plasmid DNA was dissolved in sterile endotoxin-free PBS and stored at -20°C until use.

Mice

HLA-A*1101/K^b transgenic mice were produced at Pharmexa-Epimmune (San Diego, CA), embryo-rederived at Taconic and JAX laboratories and bred at the University of Chicago. These HLA-A*1101/K^b transgenic mice express a chimeric gene consisting of the 1^{st} and 2^{nd} domains of HLA-A*1101 and the 3^{rd} domain of H-$2K^b$, and were created on a C57BL/6 background. Mice were maintained in SPF conditions throughout. All studies were conducted with the approval of the Institutional Animal Care and Use Committee at the University of Chicago.

Immunizations of mice and challenge

To evaluate multi-epitope protein immunogenicity, HLA-A*1101 transgenic mice were inoculated subcutaneously (s.c.) at the base of the tail using a 30-gauge needle with 50 μg

LO or AZ recombinant protein emulsified in 20 μg of GLA-SE (TLR4 agonist) three times at two weeks intervals. For immunization by DNA, mice were inoculated by injection of 50 μl of PBS containing 100 μg of DNA into each quadriceps muscle using a G26 gauge needle at weeks 0, 2 and 4. In a bid to enhance delivery of DNA, we used an electroporation device. Briefly, following injection of DNA, the surface dermal device was applied to the site of injection as described (46). The array was "wiggled" at the injection site to ensure good contact and electro-transfer achieved through pulse generation from the ELGEN 1000 (Inovio Pharm., San Diego) pulse generator. The parameters used were three 15 V pulses of 100 ms duration. Negative control mice were vaccinated with 100μg empty vector or 50μl saline. For challenge studies, immunized mice were challenged intraperitoneally (i.p.) 14 days post-immunization using 2,000 or 20,000 *T. gondii* ME49-Fluc (Type II) parasites that express firefly luciferase.

ELISpot assay to determine immune responses with murine splenocytes

Mice were euthanized 14 days after immunization. Spleens were harvested, pressed through a 70 μm screen to form a single-cell suspension, and erythrocytes were lysed with AKC lysis buffer (160 mM NH_4Cl, 10 mM $KHCO_3$, 100 mM EDTA). 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 (Life technologies). Murine ELISPOT assays were performed using anti-mouse IFN-γ mAb (AN18) and the biotinylated anti-mouse IFN-γ mAb (R4–6A2) and $2.5\text{-}5\times10^5$ 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 murine splenocytes.

In vivo bioluminescence imaging for determining outcomes of challenge with type II parasites

Mice infected with 2,000 *T. gondii* ME49-Fluc (Type II) tachyzoites were imaged 21 days post-challenge using the in vivo imaging system (IVIS; Xenogen, Alameda, CA). Mice were injected retroorbitally with 200 µl (15.4mg/ml) of D-luciferin, anesthetized in an O_2-rich induction chamber with 2% isoflurane, and imaged after 12 minutes. Photonic emissions were assessed using Living image® 2.20.1 software (Xenogen). Data are presented as pseudocolor representations of light intensity and mean photons/region of interest (ROI). All mouse experiments were repeated at least twice.

Enumeration of cysts in mouse brains following type II parasite challenge

Mice were euthanized at 21 days after infection with 2,000 of Me49-Fluc, and brains were collected, homogenized with 1 ml of saline (0.85% NaCl). Tissue cysts were counted microscopically in 50 µl of the homogenate, and the count was multiplied by 20 to obtain the number of tissue cysts per brain. This number was confirmed by staining brain cysts with fluorescein-labeled *Dolichos biflorus* agglutinin (Vector Laboratories) and quantitation using fluorescence microscopy.

Flow cytometry.

Splenocytes were manually processed using 70μm filters in DMEM media supplemented with 5% FCS and red blood cells were lysed with ACK lysis buffer. Cells were stained with CD3 APC (145-2C11), CD4 PE (GK1.5), CD8 PerCP (53-6.7), and CD44 AF780 (IM7), CD45RB FITC (C363.16A). All antibodies were purchased from eBioscience (San Diego, CA). Memory T cells were defined as $CD44^{hi}CD45RB^{lo}$. All flow cytometry data was collected on LSRII flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software 10.0 (Tree Star, Ashland, OR).

Demonstration that GLA is a TLR 4 ligand.

Stimulation of human whole blood with GLA-SE. Heparinized whole blood was collected from six healthy donors, and 200 μl was stimulated with 5 μg GLA-SE in 96-well plates at $37^{o}C$-CO_2. After 24 hours, plasma was removed and assayed for IL-6, IL-12(p40), and TNF by a custom Luminex-based multiplex immunoassay kit (Affymetrix eBioscience, San Diego, CA). Data was analyzed using the Masterplex QT software (Miraibio, San Francisco, CA). The cytokine production stimulated by adjuvant was statistically significant ($p<0.05$) compared to the unstimulated groups as assessed by the Mann-Whitney t-test (GraphPad Prism software, San Diego, CA).

Statistical analyses

Data for each assay were compared by One-way ANOVA or a Student *t* test using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). Differences between the groups were identified by ANOVA and multiple comparison procedures, as we

previously described (6). Data are expressed as the means ± SD. Results were considered to be statistically significant at $p < 0.05$.

Study approval

Experiments and handling of mice were conducted under federal, state, and local guidelines under an IACUC protocol and with approval from the University of Chicago IACUC.

Institutional Review Board (IRB) approval was obtained at the University of Chicago for this study. This study also is in compliance with all Health Insurance Portability and Accountability Act of 1996 (HIPAA) regulations.

Author Contributions

KE, AC, PK, YZ, SW, JD, CR, EM, YS, QS, SB, NC, and LF performed experiments. LV and KB contributed essential reagents or tools. JS, SR, JA, TV, AS analyzed and interpreted data. KE and RM supervised the work, interpreted the data, and wrote the manuscript.

The authors declare no competing interests.

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Table 1. HLA-A*1101 restricted $CD8^+$ T-cell epitopes predicted IC_{50} within the *T. gondii* protein GRA6, SAG2C, SAG1, GRA5 sequences and utilized for recombinant DNA and protein constructs and affinity of binding.

Peptide	Sequence[a]	MW	Affinity (IC_{50}, nM)		
			A*1101	A*02.01	B*07.02
$SAG2C_{13-21}$	STFWPCLLR	1122	9.3	26987	>50,000
$GRA5_{89-98}$	AVVSLLRLLK	1111	6.5	11326	>50,000
$SRS52A_{250-258}$	SSAYVFSVK	987	9.3	>50,000	>50,000
$SAG1_{224-232}$	KSFKDILPK	1075	35	>50,000	>50,000
$GRA6_{164-172}$	AMLTAFFLR	1069	3.6	10563	>50,000

[a] Position of each peptide relative to the full length proteins (GRA6, SAG2C, SAG1, GRA5, and SRS52A) including the signal sequence, their molecular weight (MW), and the predicted IC_{50} as calculated by in vitro binding affinity to purified HLA-A*1101 molecules expressed as means of binding capacities (IC_{50} nM) determined from two independent experiments, as previously described (5) and for A*02.01 and B*07.02. None of the peptides would be considered an A*02.01 or B*07.02 binder.

Table 2. IFN-γ production of $CD8^+$ T cells purified from the spleens of HLA-A*1101 transgenic mice immunized three times with *T. gondii* epitope peptides at two weeks intervals following stimulation with the peptides in vitro.

HLA-A*1101 Mice Immunized in vivo with	Stimulated in vitro with	APC*	IFN-γ** (ng/mL)
Peptides + GLA-SE^+	Peptides	HLA-A*1101	3.06 ± 0.90
GLA-SE^+	Peptides	HLA-A*1101	0.17 ± 0.23
PBS	Peptides	HLA-A*1101	0.12 ± 0.12
Peptides + GLA-SE^+	PBS	HLA-A*1101	0.31 ± 0.20
GLA-SE^+	PBS	HLA-A*1101	0.030 ± 0.015
PBS	PBS	HLA-A*1101	0.24 ± 0.17
Peptides + GLA-SE^+	Peptides	C57BL/6	0.040 ± 0.038
GLA-SE^+	Peptides	C57BL/6	0.025 ± 0.038
PBS	Peptides	C57BL/6	0.030 ± 0.040
Peptides + GLA-SE	PBS	C57BL/6	0.020 ± 0.031
GLA-SE	PBS	C57BL/6	0.00 ± 0.00
PBS	PBS	C57BL/6	0.095 ± 0.038

Mice were immunized with PBS, peptide pool (KSFKDILPK ($SAG1_{224-232}$), STFWPCLLR ($SAG2C_{13-21}$), AVVSLLRLLK ($GRA5_{89-98}$), SSAYVFSVK ($SRS52A_{250-258}$), AMLTAFFLR ($GRA6_{164-172}$), RSFKDLLKK ($GRA7_{134-142}$) with PADRE, $^+(Pam)_2KSS$ (5) plus in GLA-SE, and GLA-SE alone. * Adherent cells from spleens of mice. ** Concentrations in the culture supernatants.

Figure 1

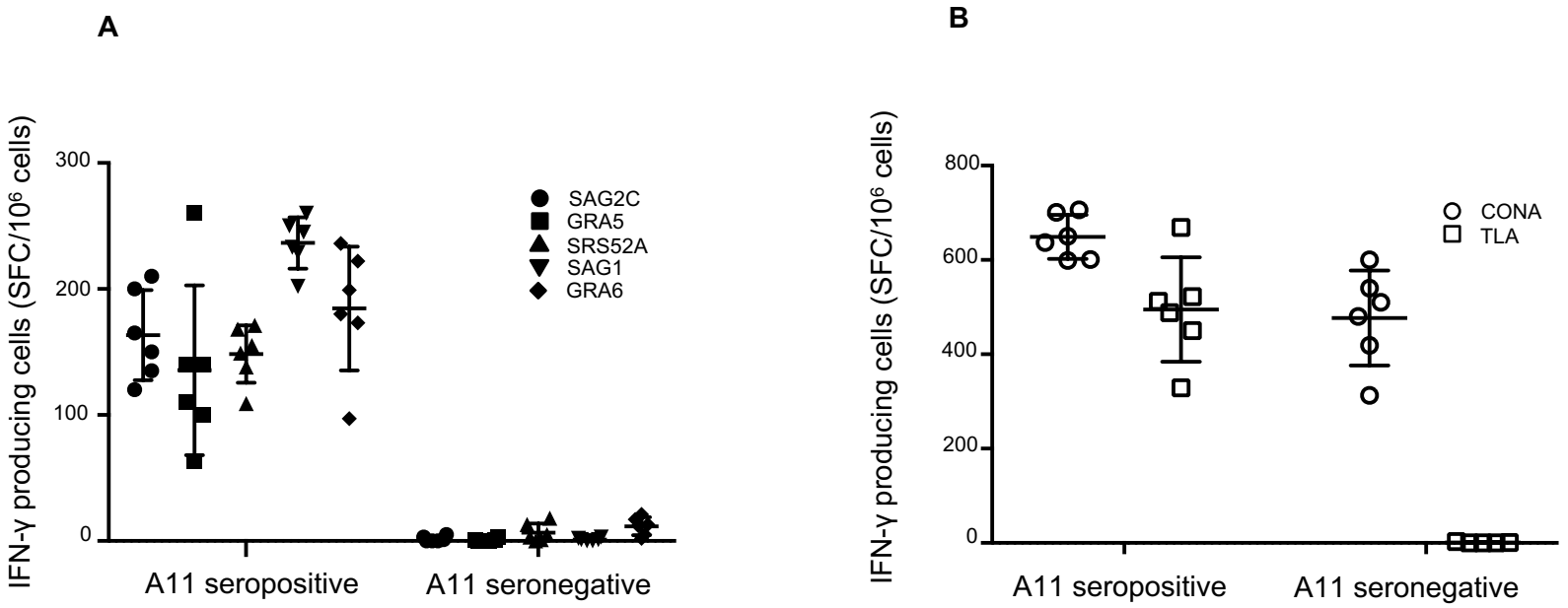

Figure 1: New peptides tested with PBMCs from HLA-A03 seropositive and seronegative donors. The peripheral blood mononuclear cells from seropositive *T. gondii* donors were tested for response to these predicted HLA-A03 restricted $CD8^+$ T cell epitope individual peptides by using IFN-γ ELISpot assay. Concanavalin A (Con A) and Tachyzoite Antigen Lysates (TLA) were used as controls.

Figure 2

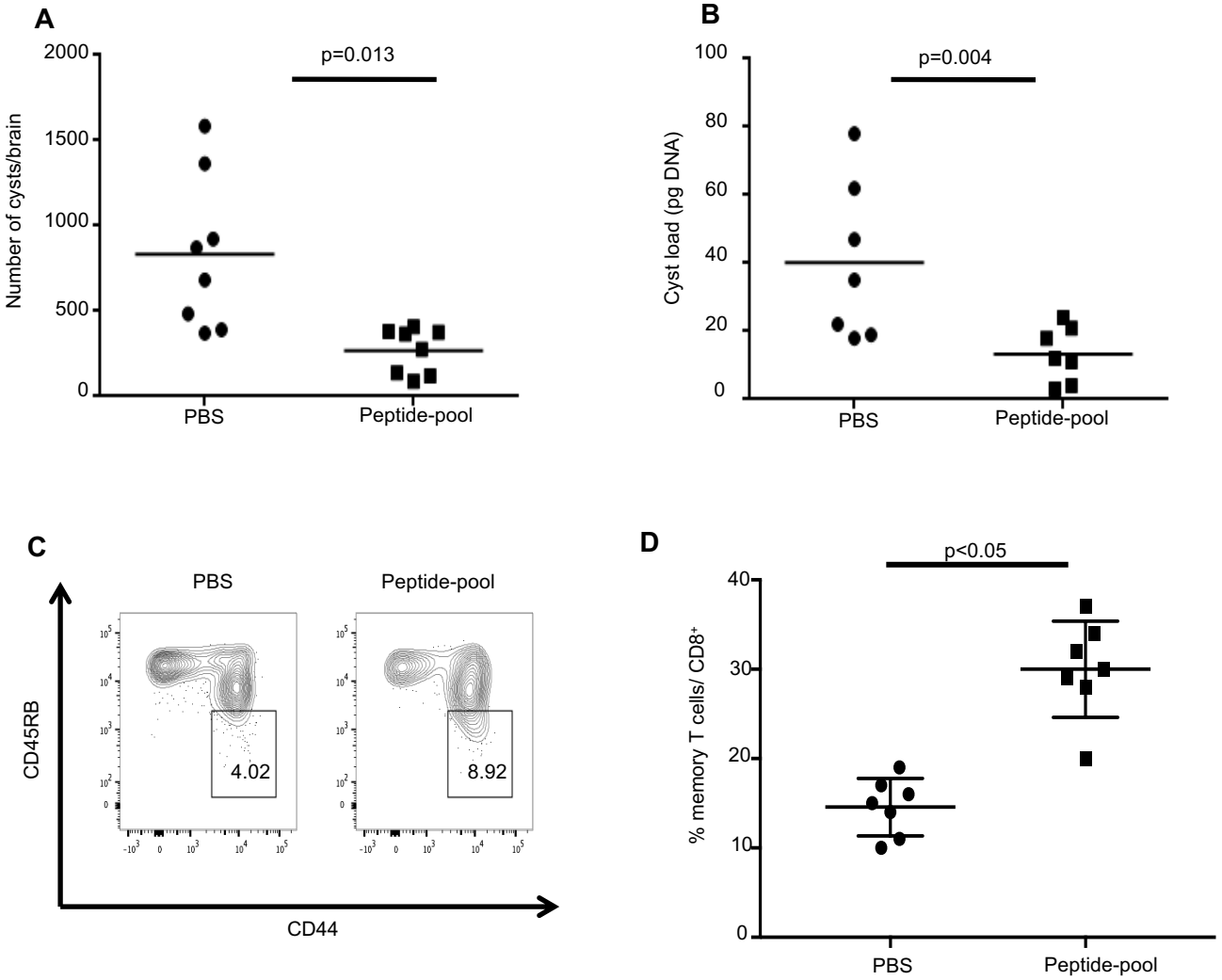

Figure 2: Immunogenicity and efficacy of 5 identified A11-restricted CD8⁺ T cell epitope pool peptides in combination with PADRE and GLA-SE adjuvant in HLA-A*1101 transgenic mice. A, *T. gondii* brain cysts was significantly reduced in HLA-A*1101 mice immunized with pool of peptides plus PADRE and GLA-SE adjuvant at 21 days after challenge with 2000 *T. gondii* ME49-Fluc (Type II) parasites. B, Quantitative real time PCR for the parasite burden in the *T. gondii* challenged HLA-A*1101 mice brains. C-D, Flow cytometry gating for $CD8^+$ memory T cells 11 weeks after immunization of HLA-A*1101 mice with pooled adjuvanted peptides. Cells are gated on $CD3^+CD8^+$ T cells. Memory T cells were defined as $CD44^{hi}CD45RB^{lo}$. For each group, a representative FACS plot is shown with the percent of $CD8^+$ memory T cells shown. For each group, n=3.

Figure 3

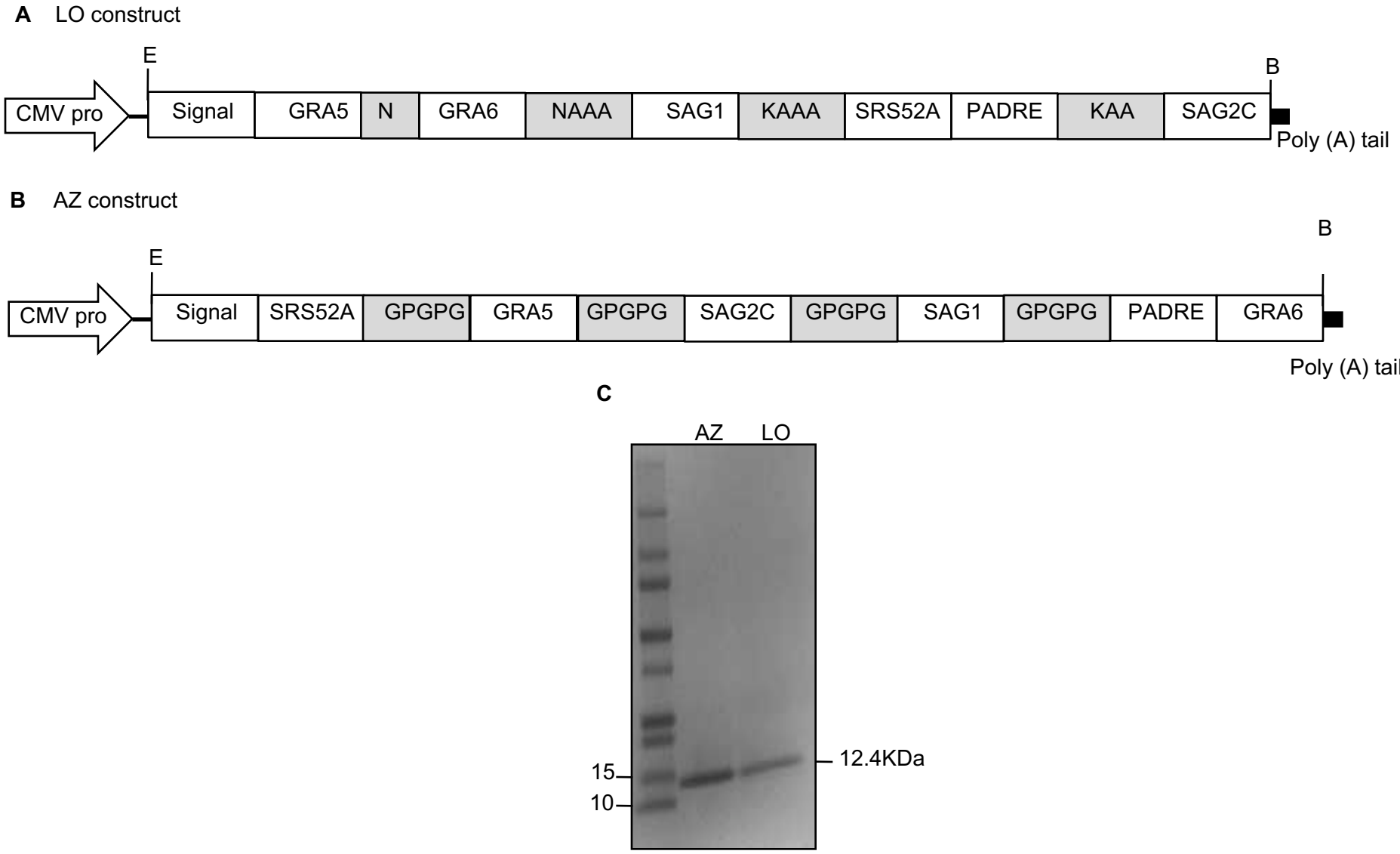

Figure 3: Schematic diagram of the DNA vaccine construct. A and B, The orientation of the HLA-A*1101-restricted $CD8^+$ T cell epitopes and PADRE in the synthetic gene is shown with two different types of spacers, called LO and AZ for respectively N/KAAA and GPGPG linker. C, SDS-PAGE 4-20% of the purified LO and AZ proteins.

Figure 4

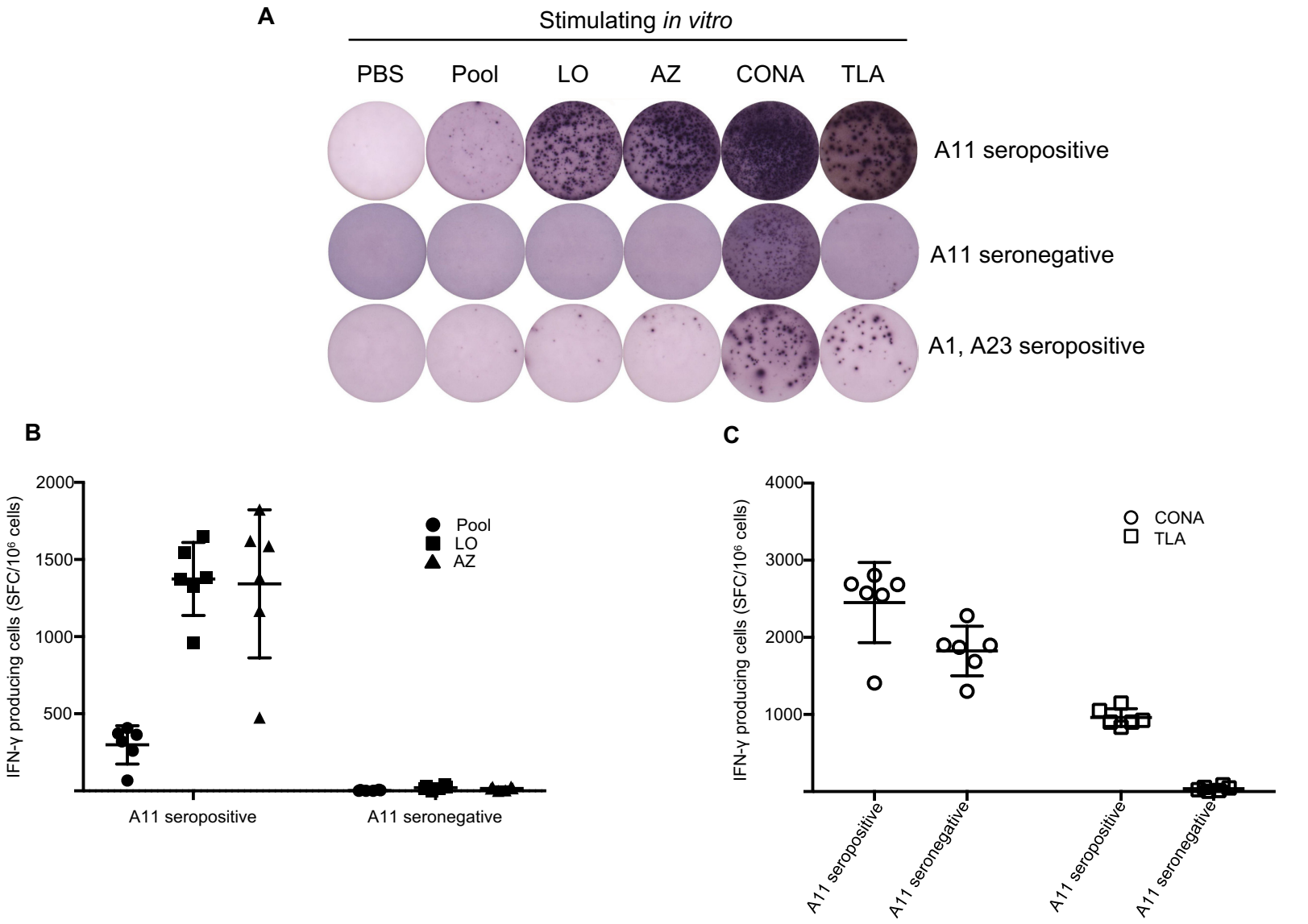

Figure 4: LO and AZ elicit specific immune responses in HLA-A03 seropositive and seronegative donors. A and B, ELISpot showing IFN-γ spot formation. PBMC's were tested using LO, AZ, and pool of peptides. C, CONA and TLA were used as controls.

Figure 5

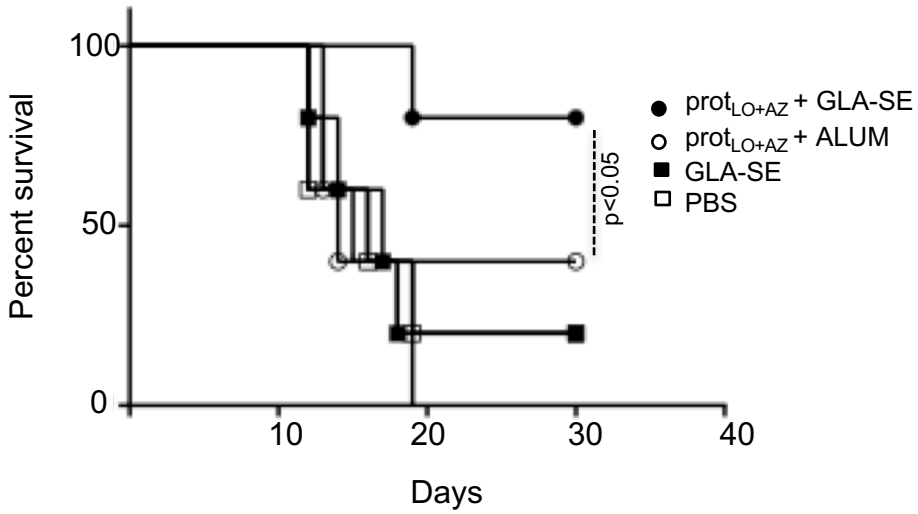

Figure 5: Multivalent polypeptide LO and AZ protective efficacy *in vivo* HLA-A*1101 transgenic mice survival curve after challenge with Type II parasites. Two weeks after last immunization, the transgenic mice immunized with pooled LO and AZ proteins in combination with either adjuvant GLA-SE or ALUM adjuvant or injected with PBS were infected with 2,000 Me49 (Fluc) parasites. The survival rates of the two groups were recorded. This figure shows data from mice in both of the replicate experiments combined (n=5 control and 5 immunized mice).

Figure 6

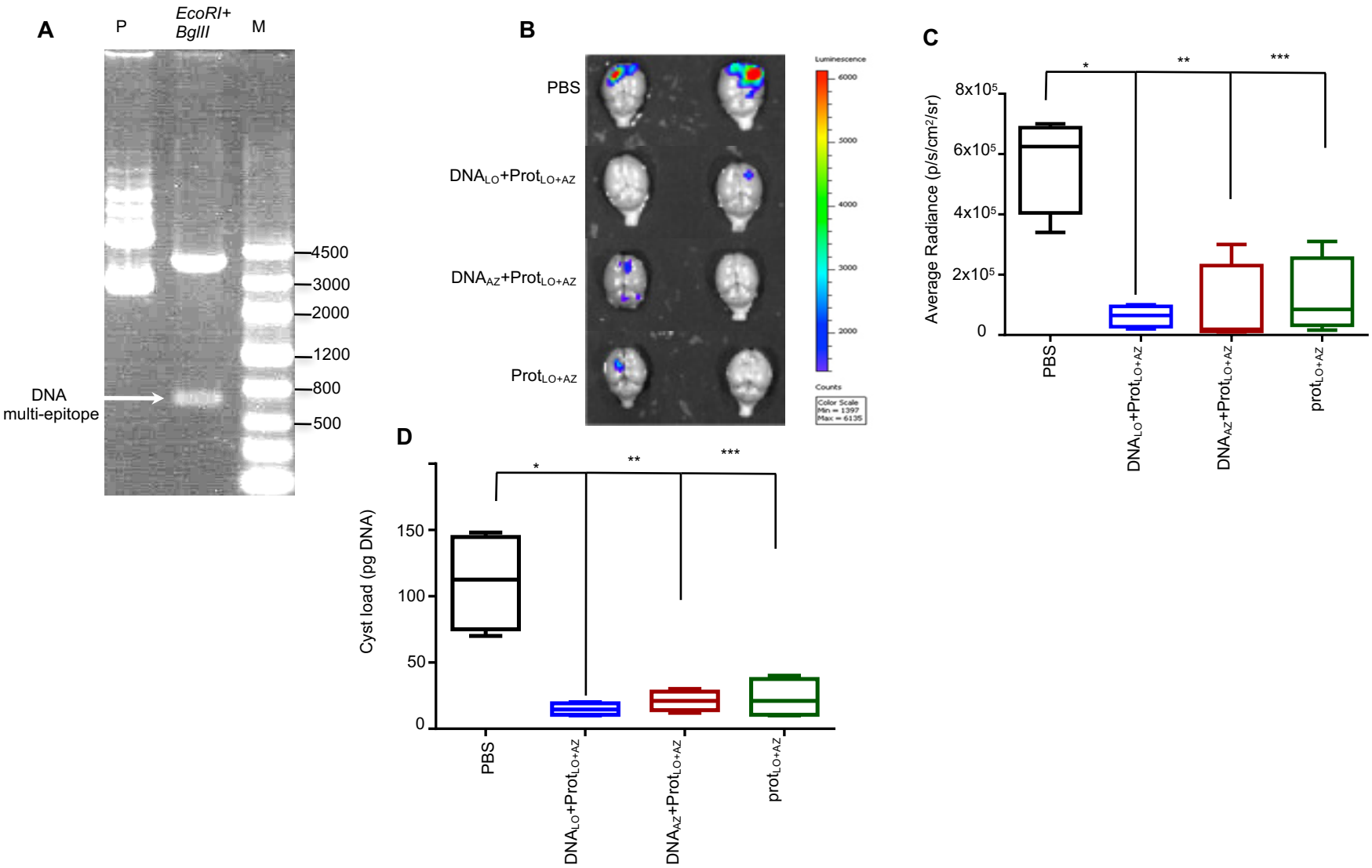

Figure 6: DNA prime-protein boost regimen. A, pMB75.6 vector used as a DNA vaccine vector for this study. Lane P: PMB75.6 plasmid, lane 2: PMB75.6 plasmid digested with *EcoRI* and *BglII*, lane M: KB ladder. B, *T. gondii* brain cysts luciferase expression was significantly reduced in HLA-A*1101 mice immunized with DNA/ protein boost at 21 days after challenge with 2000 *T. gondii* ME49-Fluc (Type II) expressing luciferase. C, Xenogen imaging of brain *ex vivo* following the injection of luciferin into the retroorbital plexus and then exposure of the brain to luciferin solution. D, Enumeration of cyst was performed with brains of mice challenged 21 days after final immunization. These experiments were performed at least two times, and each (n=5 control and 5 immunized mice). *,**,*** = $p<0.05$.

Figure 7

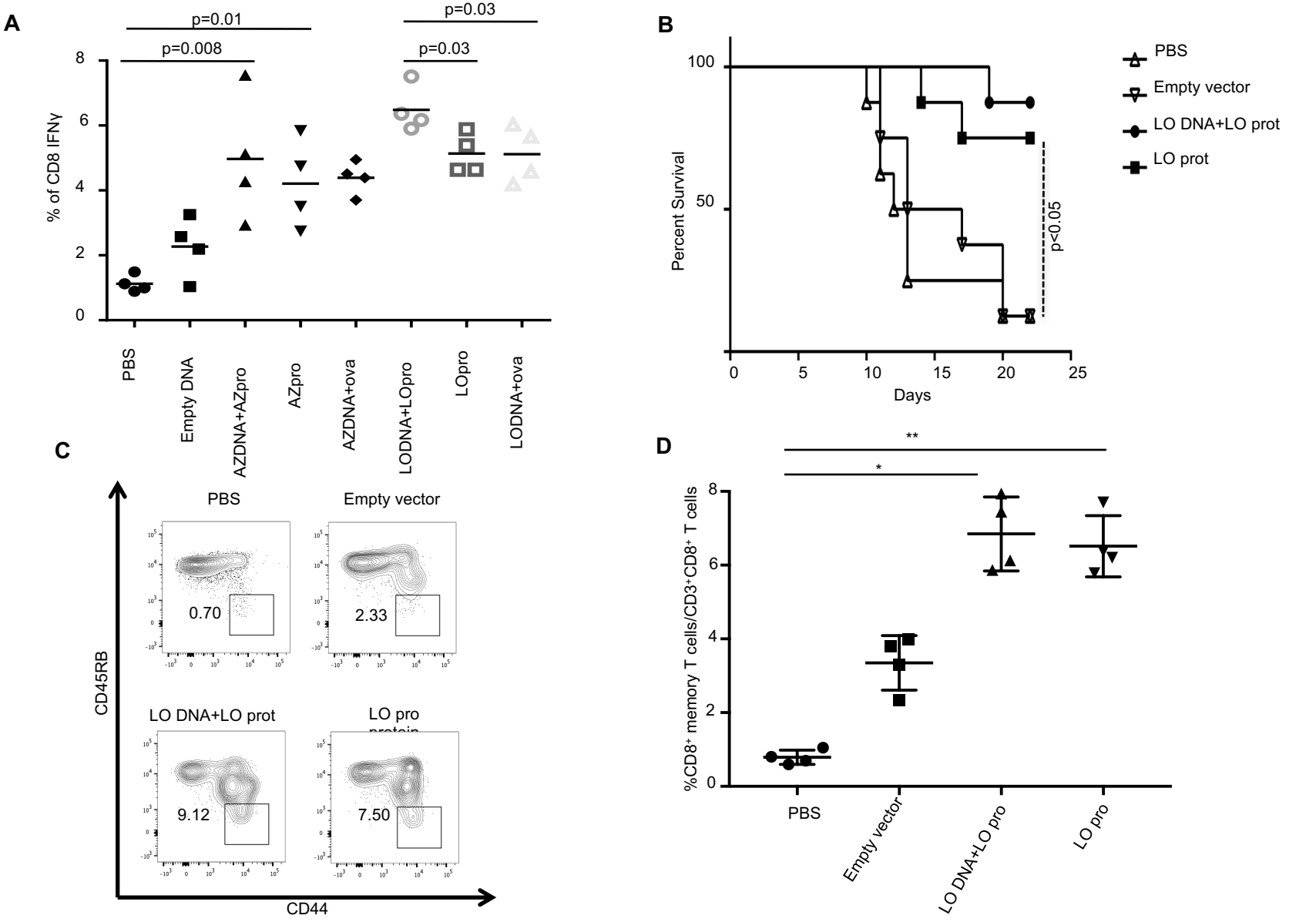

Figure 7: CD8⁺ T cell responses in HLA-A*1101 mice following immunization. A, splenocytes from immunized mice with LO DNA, AZ DNA, multi-epitope polypeptides alone or combined were harvested for 10–14 days post immunization and exposed to LO or AZ polypeptide in an *ex vivo* IFN-γ expression. B, (B) HLA-A*1101 transgenic mice survival curve after challenge with Type II parasites. Two weeks after last immunization, the transgenic mice immunized with empty vector, LO DNA plus LO polypeptide, LO polypeptide or injected with PBS were infected with 2,000 *T. gondii* ME49-Fluc (Type II) parasites. The survival rates of the two groups were recorded. Differences in survival were significant (p<0.03). This figure shows data from mice in one of the replicate experiments (n=8 control and 8 immunized mice). C-D: CD8⁺ memory T cells. Flow cytometry gating for CD8⁺ memory T cells. Spleen cells are gated on $CD3^{+}CD8^{+}$ T cells. Memory T cells were defined as $CD44^{hi}CD45RB^{lo}$. For each group, a representative FACS plot is shown with the percent of CD8⁺ memory T cells shown. All mouse experiments were repeated at least twice (n= 2-4 mice in each group).

Figure 8

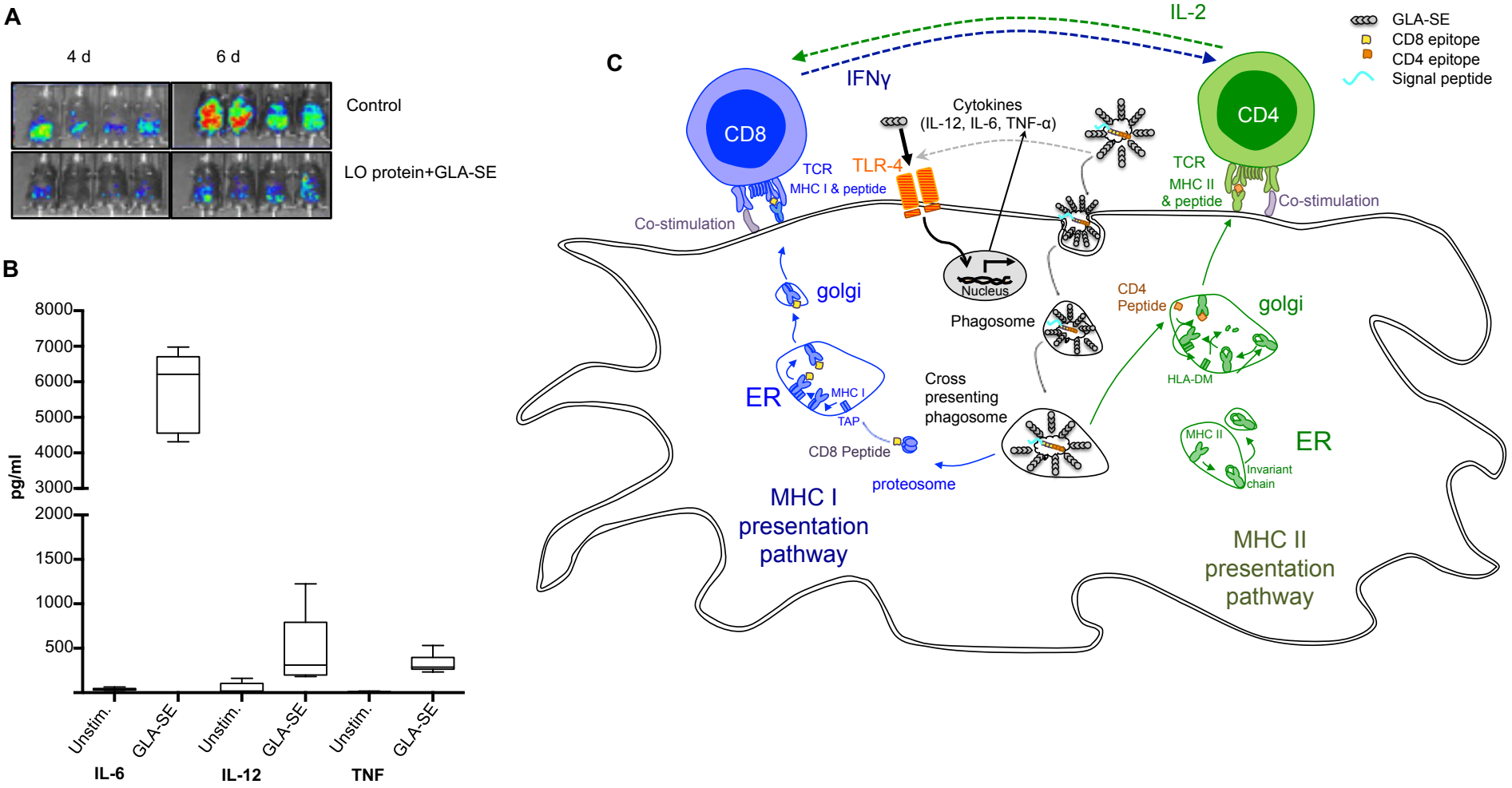

Figure 8: Multi-epitope adjuvanted to GLA-SE are captured and presented by MHC molecules on the on the APCs to T lymphocytes. A. HLA-A*1101 transgenic mice immunized with LO protein plus GLA-SE were protected compared to control mice inoculated with PBS when they were challenged with 10,000 *T. gondii* Prugneaud strain (Fluc) luciferase expressing parasites after 4 and 6 days. B. Stimulation of human whole blood with GLA-SE. Heparinized whole blood was collected from six healthy donors, and 200 µl was stimulated with 5 µg GLA-SE in 96-well plates at 37°C-CO_2. After 24 hours, plasma was removed and assayed for IL-6, IL-12(p40), and TNF by a custom Luminex-based multiplex immunoassay kit (Affymetrix eBioscience, San Diego, CA). Data was analyzed using the Masterplex QT software (Miraibio, San Francisco, CA). The cytokine production stimulated by adjuvant was statistically significant ($p<0.05$) compared to the unstimulated groups as assessed by the Mann-Whitney t-test (GraphPad Prism software, San Diego, CA). C. Multi-epitope proteins with GLA-SE are captured and presented by MHC molecules on the Antigen-presenting cells (APCs) to T lymphocytes. APCs are also activated through recognition of GLA-SE by TLR-4 receptors molecules. This activation leads to the production of proinflammatory cytokines (IL-12, IL-6, TNF-α) and the expression of co-stimulatory molecules on the cell surface.