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Mutating the anchor residues associated with MHC binding inhibits and deviates CD8+ T cell mediated protective immunity against malaria

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Abstract

We investigated whether immune responses induced by immunization with plasmid DNA are restricted predominantly to immunodominant CD8+ T cell epitopes, or are raised against a breadth of epitopes including subdominant CD8+ and CD4+ T cell epitopes. Site-directed mutagenesis was used to change one or more primary anchor residues of the immunodominant CD8+ T cell epitope on the *Plasmodium yoelii* circumsporozoite protein, and *in vivo* protective efficacy and immune responses against defined PyCSP CD8+ and/or CD4+ epitopes were determined. Mutation of the P2 but not P9 or P10 anchor residues decreased protection and completely abrogated the antigen-specific CD8+ CTL activity and CD8+ dependent IFN-γ responses to the immunodominant CD8+ epitope and overlapping CD8+/CD4+ epitope. Moreover, mutation deviated the immune response towards a CD4+ T cell IFN-γ dependent profile, with enhanced lymphoproliferative responses to the immunodominant and subdominant CD4+ epitopes and enhanced antibody responses. Responses to the subdominant CD8+ epitope were not induced. Our data demonstrate that protective immunity induced by PyCSP DNA vaccination is directed predominantly against the single immunodominant CD8+ epitope, and that although responses can be induced against other epitopes, these are mediated by CD4+ T cells and are not capable of conferring optimal protection against challenge.

Keywords: Malaria; Circumsporozoite protein; Protective immunity; MHC binding; T cells; Immune deviation

1. Introduction

Immunization with radiation-attenuated *Plasmodium* spp. confers sterile protection against sporozoite challenge in both human and animal models (reviewed in Hoffman et al., 2002). In mice, CD8+ T cells have been implicated as critical effector cells in this sporozoite-induced protective immunity. *In vitro*, MHC-restricted and Ag-specific elimination of *Plasmodium yoelii* infected hepatocytes from *in vitro* culture by CD8+ CTL has been demonstrated (Hoffman et al., 1989). *In vivo*, the protective immunity induced by immunization of BALB/c mice with irradiated *P. yoelii* sporozoites can be abrogated by *in vivo* depletion of CD8+ T cells but not CD4+ T cells (Weiss et al., 1988), and adoptive transfer of CD8+ CTL against a single epitope on the *P. yoelii* circumsporozoite protein (PyCSP) can protect against sporozoite-induced malaria in the absence of other parasite-

Abbreviations: APC, antigen presenting cell; CI, confidence interval; IFAT, indirect fluorescent antibody test; MAP, multiple antigen peptide; *P. yoelii*, *Plasmodium yoelii*; *P. berghei*, *Plasmodium berghei*; PyCSP, *P. yoelii* circumsporozoite protein; S.I., stimulation index

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specific immune responses (Rodrigues et al., 1991; Weiss et al., 1992). Evidence that CD8+ T cells are also critical in protective immunity against *Plasmodium falciparum* in humans has accumulated from studies of humans immunized with irradiated sporozoites or naturally exposed to malaria (reviewed in Aidoo and Udhayakumar, 2000; Doolan et al., 1996b). In addition to CD8+ CTL, IFN-γ has been demonstrated as another critical effector mechanism mediating the inhibition of pre-erythrocytic forms of the parasite in the liver (Ferreira et al., 1986).

Previous studies have established that the protective immunity induced in BALB/c mice by immunization with a plasmid DNA construct encoding the PyCSP is absolutely dependent upon CD8+ but not CD4+ T cells (Doolan et al., 1996a; Sedegah et al., 1994) and IFN-γ (Doolan et al., 1996a). A single immunodominant CD8+ CTL epitope located at residues 280–288 (SYVPSAEQI) has been identified on the PyCSP (Weiss et al., 1990). *In vitro* CTL activity and IFN-γ production specific for this minimal nine amino acid epitope correlate with *in vivo* protective immunity in BALB/c mice (Sedegah et al., 1994). In addition, an epitope located at residues 59–79 of the PyCSP (YNRNIVNRLLGDALNGKPEEK) was shown to prime for specific T cell proliferation (Del Giudice et al., 1990) and CD8+ mediated elimination of *P. yoelii* infected hepatocytes from *in vitro* culture (Renia et al., 1991). Finally, immunization of mice with a multiple antigenic peptide containing the Th epitope PyCSP 57–70 (KIYNRNIVNRLLGD) induces T cell proliferation and a CTL response capable of eliminating infected hepatocytes *in vitro*, and confers partial protection against sporozoite challenge (Franke et al., 1997). The CTL response is specific for a subdominant CD8+ T cell epitope mapped to residues 58–67 (IYNRNIVNRL) that is recognized following immunization with MAP (57–70) but not following immunization with either the whole organism (irradiated sporozoite) or whole Ag (PyCSP plasmid DNA).

To mediate protective immunity, CD8+ T cells must recognize parasite-derived peptides that are presented on the surface of infected hepatocytes in association with class I MHC molecules and either directly or indirectly destroy the infected hepatocyte, or inactivate the intra-hepatic parasite. Since sporozoites and merozoites in circulation are extracellular, and mature human erythrocytes do not express class I MHC molecules on their surface, infected hepatocytes are considered the likely target of CD8+ T cells against *Plasmodium* spp. (Good et al., 1988). Accordingly, there have been major efforts to develop pre-erythrocytic stage vaccines that induce protective CD8+ T cells.

It is well established that T cells do not recognize foreign Ag directly, but instead recognize a form of the Ag presented in the context of class I or class II molecules encoded by genes within the MHC (Zinkernagel and Doherty, 1979; Bjorkman et al., 1987a,b; Jardetzky et al., 1991; Saper et al., 1991). A stringent size requirement for peptide binding to class I MHC molecules has been defined (Cerundolo et al., 1991) and it is apparent that the different class I molecules require a specific combination of two main "anchor" residues crucial for binding within their peptide ligands, typically at position 2 and the C-terminus (Rotzschke and Falk, 1991). Binding of these primary anchor side chains in specific MHC pockets constrains the conformation of class I-bound peptides at both ends but leaves the center relatively free to adopt a conformation that optimizes secondary anchor and other interactions with the MHC molecule.

Using the well established *P. yoelii*-BALB/c rodent model of pre-erythrocytic stage immunity, we have investigated the effect that mutating one or more of the primary anchor residues of the single immunodominant PyCSP 280–288 CD8+ CTL epitope has on immunogenicity and protection against *P. yoelii* sporozoite challenge induced by DNA vaccination. The *P. yoelii*-BALB/c model is considered a good model for *P. falciparum* in humans and to have a negative predictive value for potential success in the clinic, in that vaccine technologies or strategies that do work in this model may not necessarily work in humans but vaccines that do work in humans do work in this model. Based on the previously described K_d peptide-binding motif (Haskins et al., 1984), positions 2 (Y_{281}) and 9 (I_{288}) represent the primary anchor residues of the immunodominant PyCSP CTL epitope. However, position 10 (L_{289}) may potentially substitute for position 9 (I_{288}) as the C-terminus primary anchor residue. Previous studies (Weiss et al., 1992) have demonstrated that truncation of the epitope to remove position Y_{281} abrogates the CTL response. Herein, site-directed mutagenesis was employed to specifically mutate one or more of positions 2 (Y_{281}), 9 (I_{288}) and 10 (L_{289}) to alanine, where the small size of the latter amino acid would not be expected to interfere with peptide binding to the MHC. We determined whether this epitope is absolutely required for the CD8+ T cell mediated protection in BALB/c mice, and whether immunization with plasmid DNA encoding the PyCSP would preferentially induce protective immune responses directed against this epitope. Our studies establish that protective immunity induced by PyCSP DNA vaccination is directed predominantly against the single immunodominant CD8+ epitope, and that although responses can be induced against other epitopes, these are mediated by CD4+ rather than CD8+ T cells and are not capable of conferring optimal protection against parasite challenge.

2. Materials and methods

2.1. Oligonucleotide primers

Oligonucleotide primers (Integrated DNA Technologies Inc., Caralville, IA) were designed for site-directed mutagenesis to specifically change amino acid Y_{281} representing the position 2 primary anchor residue of the previously defined 10-mer CTL epitope $PyCSP_{280–289}$ (SYVPSAEQIL, encoded by TCA TAT GTC CCA AGC GCG GAA CAA ATA CTA) (Weiss et al., 1990) from tyrosine to alanine (^{888} AAT GGT AAT AAT AAT GAA GAT TCA **GCT** GTC CCA AGC GCG GAA ^{929}, restriction enzyme sites underlined, mutated positions in bold; translated as ^{273} NGNNNEDSYVPSAE ^{286}, immunodominant CTL epitope underlined), and amino acids I_{288} and L_{289} representing positions 9 and 10 primary anchor residues from isoleucine and leucine, respectively, to alanine (^{915} GTC CCA AGC GCG GAA CAA GCA GCA GAA TTC GTT AAA CAG ATA AG ^{958}; translated as ^{282} VPSAEQILEFVKQI ^{295}). Thus, the TAT codon encoding the P2 anchor residue Y_{281} was mutated to GCT (Ala),

and the codons encoding the P9 I_{288} (ATA) or P10 L_{289} (CTA) anchor residues were both mutated to GCA (Ala). For each mutation, two primers containing the desired mutation, each complementary to opposite strands of the nkCMVintPolyli/CSP plasmid, were synthesized. Primers 1 (forward) and 2 (reverse) (42 bp) incorporated a *Pvu*II restriction enzyme site to confirm the mutagenesis of position 2. Primers 3 (forward) and 4 (reverse) (44 bp) incorporated an *Eco*RI restriction enzyme site to confirm the mutagenesis of positions 9 and 10. Primers were designed so that the thymidine (T) and guanidine (G) content was approximately 40%, with melting temperatures approximately 10 °C greater than the extension temperature (68 °C) to encourage binding of the primers to the DNA template. Mutations were mapped to the middle of the primer sequence.

2.2. Site-directed mutagenesis of plasmid DNA

Four DNA plasmids were constructed. The DNA construct encoding the non-mutated PyCSP gene (nkCMVintPolyli/CSP) has been described previously (Sedegah et al., 1994). This construct was mutated at position Y_{281} representing the position 2 primary anchor residue of the previously defined CTL epitope, or at positions I_{288} and L_{289} representing positions 9 and 10 primary anchor residues respectively, or at positions Y_{281}, I_{288} and L_{289} representing positions 2, 9 and 10 primary anchor residues. Site-directed mutagenesis was carried out using the QuikChange™ (Stratagene, La Jolla, CA) as described by the manufacturer. The cycling parameters outlined in the mutagenesis kit protocol were optimized for the specific primer-DNA combinations as one cycle at 95 °C for 30 s, followed by 18 cycles at 95 °C for 30 s, 55 °C for 1 min, 60 °C for 2 min, and then 68 °C for 12 min, using 15 ng DNA template and a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). For position 2 mutagenesis, or positions 9 and 10 mutagenesis, the template was the nkCMVintPolyli/CSP plasmid. For positions 2, 9 and 10 mutagenesis, the template was the nkCMVintPolyli/CSP plasmid mutated at positions 9 and 10, and cycled with primers 1 and 2. Oligonucleotide primers were extended during the temperature cycling by the action of *Pfu* DNA polymerase and copies of the plasmid incorporating the desired mutation(s) generated by linear amplification. The methylated non-mutated parental DNA template was digested by treatment with *Dpn*I endonuclease, resulting in selection of synthesized DNA containing the desired mutation(s). DNA was then transformed into *Epicuran Coli* XL1-Blue or DH10B ultracompetent cells by heat shock. Plasmid DNA from transformed colonies was prepared using the QIAprep Spin Plasmid kit (QIAGEN Inc., Chatsworth, CA) and digested with *Pvu*II and/or *Eco*RI to confirm the mutagenesis.

2.3. Sequencing of DNA constructs

The DNA sequence of each construct was determined from both strands using the ABI PRISM™ Ready Reaction Dye Deoxy™ Terminator Cycle Sequencing kit (Perkin-Elmer Corporation, Norwalk, CT), under conditions described by the manufacturer. Sequencing primers mapped to the vector DNA sequence adjacent to the polycloning site, or within the PyCSP gene. Large-scale preparations of the DNA constructs were purified by standard alkaline lysis followed by cesium chloride gradient centrifugation (Hedstrom and Doolan, 2002).

2.4. In vitro expression

Expression of the encoded gene was confirmed *in vitro* by transient transfection of UM449 cells (kindly provided by Vical Inc., San Diego, CA) or P815 mastocytoma cells (American Type Culture Collection, TIB 64) with 2.5 μg of QIAGEN purified plasmid DNA and 10 μl lipofectin (Gibco BRL), as described by the manufacturer. Subsequent analysis of the UM449 cell lysate was done by Western blot using the PyCSP specific mAb NYS1 (Charoenvit et al., 1987). Subsequent analysis of the P815 transfected cells was done by the indirect fluorescent antibody test (IFAT) using NYS1 as the primary Ab and FITC-conjugated goat-anti-mouse as the secondary Ab (Kierkegaard and Perry, Gaithersburg, MD).

2.5. Mice and parasites

Female 5–8-week-old BALB/cByJ mice were obtained from The Jackson Laboratory, Bar Harbor, ME. *P. yoelii* (17× NL non-lethal strain, clone 1.1) was maintained by alternating passages of the parasites in *Anopheles stephensi* mosquitoes and CD-1 mice. *P. yoelii* sporozoites were obtained 14 days after an infectious blood meal by hand-dissection of *P. yoelii* 17× NL infected mosquito glands in M199 medium containing 5% normal mouse serum (Rockland, Gilbertsville, PA). The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals", Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996 (Office of Laboratory Animal Welfare Assurance #A4117-01; AAALAC Accreditation Unit# 00847).

2.6. Immunizations

Mice were immunized 3 times at 3-week interval i.m. in each tibialis anterior muscle with 50 μg of each plasmid DNA construct in a total volume of 50 μl saline, or unmodified empty plasmid. Two weeks after the third immunization, mice were challenged by tail-vein injection with infectious sporozoites. Two, 4 or 7 weeks after the third immunization, mice were sacrificed for T cell studies.

2.7. Synthetic peptides

Synthetic peptides representing each of the non-mutated or mutated T cell epitopes, S**Y**VPSAEQ**IL**, S**A**VPSAEQIL, SYVPSAEQ**AA**, and S**A**VPSAEQ**AA** were synthesized commercially (Research Genetics, Huntsville, AL). Peptides were assessed for affinity of binding to purified MHC molecules

Table 1
PyCSP epitope sequences and H-2K_d binding affinity

Peptide sequences	Length	Comments	K_d IC_{50} (nM)
S**Y**VPSAEQ**I**	9	PyCSP 280–288: dominant CTL epitope (9-mer)	2.2
S**Y**VPSAEQI**L**	10	PyCSP 280–289: dominant CTL epitope (10-mer)	280
S**A**VPSAEQ**I**	9	PyCSP 280–288: mutated at P2	>10,000
S**Y**VPSAEQ**A**A	10	PyCSP 280–288: mutated at P9, P10	8,600
S**A**VPSAEQ**A**A	10	PyCSP 280–288: mutated at P2, P9, P10	>10,000
S**Y**VPSAEQ**I**LEFVKQI	16	PyCSP 280–295: dominant CD4+/CD8+ T cell epitope (nested dominant CD8+ T cell epitope)	463
IYNRNIVNR**L**	10	PyCSP 58–67: subdominant CD8+ T cell epitope	16
K**I**YNRNIVNR**L**LGD	14	PyCSP 57–70: dominant CD4+ T cell epitope (nested subdominant CD8+ T cell epitope)	8,250
YNRNIVNRLLGDALNGKPEEK	21	PyCSP 59–79: subdominant CD4+ T cell epitope	1,104

Primary anchor residues are indicated in bold type, underlined.

in vitro (Epimmune Inc., San Diego, CA). Additional peptides used for *in vitro* stimulation and for antigen presenting cell (APC) sensitization in the T cell assays are detailed in Table 1.

2.8. CTL assay

Spleen cells from mice collected after the third immunization were incubated at a concentration of 5×10^6 cells in 2 ml of RPMI 1640 medium supplemented with 10% heat inactivated FBS (Sigma), 10 mM HEPES, 2 mM L-glutamine (Gibco), 50 μM 2-ME, 50 U/ml penicillin, and 50 U/ml streptomycin (Gibco) (cRPMI) in a 24-well plate, in the presence of 2.5 μM of synthetic peptide PyCSP 280–295 containing the previously defined 9/10-mer CTL epitope, or in the presence of the minimal PyCSP 280–288 peptide. Alternatively, spleen cells were stimulated *in vitro* at a responder:stimulator ratio (number of responder spleen cells stimulated *in vitro* per added transiently transfected stimulator cell) of 50:1 with irradiated MHC-matched (H-2d) P815 mastocytoma cells (American Type Culture Collection, TIB 64) or MHC-mismatched (H-2b) EL4 lymphoma cells (ATCC TIB 39) transiently transfected 48 h previous with 2.5 μg of non-mutated or mutated plasmid DNA and 10 μl lipofectin (Gibco BRL), as described by the manufacturer; cells were incubated at a concentration of 30×10^6 cells per 10 ml volume of cRPMI in an upright 25 cm^2 Roux flask. In both cases, Rat T-stim (Collaborative Biomedical Products) (2.5%) was added at 48 h as a source of IL-2. After 7 days of incubation, cells were used as effectors in a standard 6 h chromium release assay. Target cells were P815 or EL4 cells transiently transfected with non-mutated or mutated plasmid DNA 48 h previous, or pulsed overnight with PyCSP 280–288, PyCSP 280–295, PyCSP 57–50, an unrelated control peptide, or no peptide, and labeled with 100 μCi ^{51}Cr (sodium chromate solution). Expression of the transfected cells was confirmed by IFAT analysis using the PyCSP mAb NYS1 (data not presented). Percent lysis was determined as [(experimental release − medium control release)/(maximum release − medium control release)] × 100. Percent specific lysis (net) was determined as % lysis (test) − % lysis (control). Responses were classified as positive if percent specific lysis [% lysis (test) − % lysis (control)] was greater than 10%.

2.9. In vitro depletions of effector cells

MACS magnetic beads (Miltenyi Biotec, Auburn, CA) were used to deplete CD4+, CD8+, or CD56+ cells from mouse splenocytes, in accordance with manufacturer's instructions. MACS CD4 (L3T4), CD8a (Ly-2) and anti-NK (DX5) MicroBeads (Miltenyi Biotec) positive selection columns were used to specifically remove selected cell populations from splenocyte suspensions immediately prior to IFN-γ ELIspot or intracellular cytokine staining assays. The depleted flow-through portions were used as effector cells in the *in vitro* T cell studies to evaluate the requirement for CD4+ T cell, CD8+ T cell and NK cell in IFN-γ production. Efficiency of depletion was >95–99% in all cases.

2.10. Interferon γ ELIspot

Multiscreen MAHAS 4510 plates (Millipore, Bedford, MA) were coated with 50 μl/well of sterile carbonate/bicarbonate buffer containing 10 μg/ml of anti-murine IFN-γ (R4, Pharmingen, San Diego, CA) and incubated overnight at room temperature. Plates were washed twice with 200 μl/well RPMI medium and twice with cRPMI medium containing penicillin/streptomycin, L-glutamine and 10% FBS, and incubated with 200 μl/well of cRPMI medium in 5% CO_2 at 37 °C for at least 3 h. After blocking, the plates were washed once more with cRPMI before the addition of effector cells and APCs. A20.2J (e.g., ATCC clone HB-98) and P815 (ATCC TIB 64) APCs were washed once with cRPMI, incubated at 5×10^6 cells/ml with or without PyCSP peptide (10 μg/ml) for 1 h at 37 °C in 5% CO_2, and irradiated in a ^{137}Cs gamma irradiator (A20.2J at 16,000 rads and P815 at 10,000 rads). Next, APCs were washed three times with cRPMI, diluted to 1.0×10^6 cells/ml (P815) or 1.5×10^6 cells/ml (A20.2J) in cRPMI. To obtain splenocytes, immunized mice were sacrificed 2, 4 or 7 weeks after the boost (three mice/group), their spleens removed to a sterile tissue screen and ground with glass pestle into a sterile Petri dish using cRPMI. The spleen cell suspensions were washed three times, counted and diluted to 5×10^6 cells/ml and 2.5×10^6 cells/ml. Effector cells and APCs were plated in quadruplicates at 100 μl/well, and incubated in 5% CO_2 at 37 °C for 36 h. Plates were washed three times with PBS followed

by four times with PBS-T (PBS 0.05% Tween20). 100 μl/well of biotinylated anti-IFN-γ (XMG1.2, Pharmingen, San Diego, CA) at 2 μg/ml in PBS-T were added to the plates and incubated overnight at 4 °C. Plates were washed six times with PBS-T and 100 μl/well peroxidase conjugated streptavidin (Kirkekaard & Perry, Gaithersburg, MD) was added at 1:800 dilution in PBS-T. After 1 h incubation at room temperature, plates were washed six times with PBS-T followed by three times with PBS alone, and developed with DAB reagent (Kirkekaard & Perry, Gaithersburg, MD) according to manufacturer's instructions. After 15 min, the plates were rinsed extensively with dH_2O to stop the colorimetric substrate, dried and stored in the dark. Spots were counted with a KS ELIspot reader (Carl Zeiss Vision, Germany).

2.11. Intracellular cytokine staining and FACS analysis

A20.2J cells were pulsed with or without 10 μM PyCSP peptide (10 μg/ml) for 1 h at 37 °C in 5% CO_2, and irradiated as above. Then, 100 μl/well of spleen cells (5×10^6 cells/ml) and 100 μl/well A20.2J cells (1.5×10^6 cells/ml) pulsed with or without PyCSP peptide were incubated in duplicates in U-bottom 96-well plates (Costar) in the presence of 1 μM Brefeldin A (GolgiPlug™, Pharmingen, San Diego, CA) in 5% CO_2 at 37 °C for 16 h. Plates were spun at 1200 rpm for 5 min, the supernatant flicked, and the cell pellet resuspended by gentle vortexing. Cell surface markers were stained with 0.3–0.5 μl/well of anti-CD8-APC, anti-CD4-PERCP, anti-DX5-FITC or anti-CD62L-FITC Abs (Pharmingen, San Diego, CA) in a final volume of 100 μl in FACS wash. Plates were incubated with a combination of three Abs on ice in the dark for 20 min. After the surface staining, cells were washed with FACS wash twice, gently resuspended, and incubated with 90 μl of Cytofix/Cytoperm buffer (Pharmingen, San Diego, CA) for 20 min on ice in the dark. Next, cells were washed with 100 μl of Perm/Wash buffer and intracellular IFN-γ or TNF-α were stained with 0.5 μl/well of anti-IFN-γ-PE or anti-TNF-α-PE Abs (Pharmingen, San Diego, CA) in a final volume of 100 μl in Perm/Wash buffer. After 20 min incubation on ice in the dark, cells were washed twice with Perm/Wash, once with FACS wash, resuspended in 100 μl of FACS wash and stored at 4 °C prior to analysis. The efficiency of anti-CD4+, anti-CD8+ and anti-DX5 (NK cells) Ab depletion *in vivo* and *in vitro*, as well as peptide-specific IFN-γ and TNF-α intracellular production, was determined in a four-color FACSCalibur™ (Becton Dickinson Immunocytometry Systems, San Jose, CA) with CellQuest software.

2.12. Lymphocyte proliferation

Spleen cells from immunized mice were cultured in quadruplicate at a concentration of 125, 250 or 500×10^3 cells in 0.2 ml of cRPMI medium in a flat-bottom 96-well tissue culture plate in the presence of peptide (concentrations ranging from 0.625 to 40 μg/ml) without peptide, or with mitogen (ConA at 5 μg/ml) for 5 days. Wells were then pulsed with 1.0 μCi ^{3}H-methyl thymidine (Dupont NEN) overnight, and uptake assessed by liquid scintillation spectroscopy (Beckman LS6800). Results were expressed as a stimulation index (S.I.) (cpm sample/cpm control without peptide).

2.13. Antibody response

Mice were bled for serum approximately 2 weeks after each immunization. Abs were assessed by ELISA against recombinant PyCSP protein (0.1 μg/ml), as previously described (Charoenvit et al., 1987), either individually or pooled as indicated in the text.

2.14. Cytokine analysis

Individual sera from experimental mice ($n = 12$–13 per group) collected 14 days post last immunization, immediately prior to challenge, were assayed in duplicate using the Pierce Searchlight Multiplexed Sample Analysis Service (Pierce Biotechnology Inc., Woburn, MA) against a custom 16-plex mouse panel comprising the following cytokines: IFN-γ, TNF-α, IL-1α, IL-1β, IL-2, hIL-2r, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, IL-18, and hIL-15.

2.15. In vivo depletions

Anti-CD4+ mAb GK1.5 (rat IgG2a) (Dialynas et al., 1983) and anti-CD8+ mAb 2.43 (mouse IgG2a) (Sarmiento et al., 1980) were obtained from ATCC (TIB207, TIB210). Anti-IFN-γ mAb XMG-6 (rat IgG1) (Cherwinski et al., 1987) was kindly provided by Dr. F. Finkelman. All Igs were purified from ascites (Harlam Bioproducts for Science, Indianapolis, IN) by 50% ammonium sulphate precipitation and final Ab concentrations determined by OD. Cell depletions were done immediately prior to the evaluation of liver-stage protection. For CD8+ T cell depletion, mice received a single i.p. dose of 0.5 mg/0.1 ml of mAb 2.43 on days −3, −2, and −1. For CD4+ T cell or IFN-γ depletion, mice received a single i.p. dose of 1.0 mg/0.1 ml of mAb GK1.5 or XMG-6, respectively, on days −3, −2, and −1. The control group received no treatment. *In vivo* depletion regimes reported here are routinely used in our laboratory and have been designed so as to ensure that the treatments are effective and reproducible (data not presented) (Doolan and Hoffman, 1999).

2.16. In vivo protection

For evaluation of sterile protection at the blood-stage, infectious sporozoites were diluted to a final concentration of 50 sporozoites per 0.2 ml. Mice were challenged by tail-vein injection and giemsa-stained blood smears were examined on days 5–14, up to 50 oil-immersion fields being scanned for parasites. Protection was defined as the complete absence of blood-stage parasitemia. For evaluation of partial protection at the liver-stage, sporozoites were diluted to a final concentration of 50,000 sporozoites per 0.2 ml and injected i.v. in the tail vein. Forty-two hours after challenge, mice were euthanized and the livers collected in RNAlater™ solution (Ambion, Austin, TX), homogenized in Trizol® (Invitrogen, Carlsbad, CA), and stored at −80 °C. Parasite RNA was extracted and quantified

by TaqMan® quantitative real time PCR, as described elsewhere (Witney et al., 2001). An estimate of *P. yoelii* 18S rRNA "plasmid equivalents" and mouse GAPDH (housekeeping gene) "plasmid equivalents" were derived from the Ct (threshold cycle) measured for each PCR target for each unknown sample (Witney et al., 2001).

2.17. Statistical analysis

Mean Ab titers were expressed as OD units 0.5 (serum dilution corresponding to an OD value of 0.5) and analyzed by the *t*-test of independent samples. Serum cytokines were reported as median and percentiles (25% and 75%) and analyzed by the non-parametric Mann–Whitney *U*-test. To assess blood stage protection, comparison between groups was performed by the chi square test (two-tailed) (EpiInfo, CDC). To assess liver stage protection, quantitative parasite burden data was expressed as the ratio of *P. yoelii* 18S rRNA plasmid equivalents over the mouse GAPDH RNA plasmid equivalents for each sample (Witney et al., 2001). Assessment of statistical significance was performed by Student's unpaired *t* testing on log (base2) transformed data. Significance was defined at the 5% level.

3. Results

3.1. In vitro and in vivo expression of plasmid DNA constructs

IFAT analysis of transfected P815 cells confirmed the *in vitro* expression of all non-mutated and mutated plasmid DNA constructs (data not presented). The *in vitro* expression of the DNA constructs was reconfirmed by analysis of transiently transfected UM449 cells by Western blot (data not presented).

3.2. Binding affinity of non-mutated and mutated peptides

The capacity of synthetic peptides representing the non-mutated (native) and mutated epitopes to bind *in vitro* to the MHC molecule was quantitated as a coefficient of binding (K_d). Binding efficiencies for the respective peptides are reported in Table 1. K_d values of 1–50 nM are associated with high binding affinity, 50–500 nM with intermediate binding affinity, and >500 nM with little or no binding. Previous studies (Sette et al., 1994) have revealed an association between binding affinity and immunogenicity.

3.3. Effect of mutations on induction of CTL to defined peptide epitopes or intact protein

Spleen cells of mice immunized with non-mutated PyCSP DNA stimulated *in vitro* with either the PyCSP 280–288 or PyCSP 280–295 synthetic peptides containing the non-mutated 9/10-mer CTL epitope induced robust Ag-specific CTL responses directed against the immunodominant CD8+ T cell epitope (residues 280–288) (Fig. 1 and Table 2). Results obtained with splenocytes harvested at different time points (2, 4 or 7 weeks) post third immunization were consistent between time-points. Effectors failed to recognize the epitope mutated at positions P2, P9 and P10, but did recognize the epitope with the original residue at P2 but mutated residues at P9 and P10 (Table 2). These data show that the primary P2 N-terminal anchor, but not the P9 or P10 C-terminal anchors, are critical for CTL recognition. Consistent with previous data (Franke et al., 1997), these effectors failed to recognize the subdominant CD8+ T cell epitope at residues 58–67 (Table 2).

Immunization of mice with PyCSP DNA containing the P2, P9/P10 mutations, the P2 mutation, or the P9/P10 mutations, did

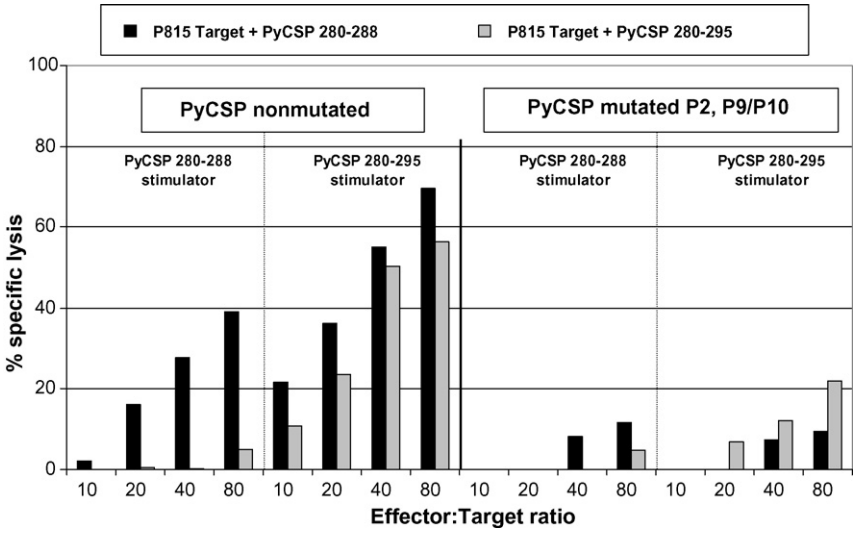

Fig. 1. Antigen-specific, MHC-restricted CTL activity to immunodominant CD8+ and CD8+/CD4+ epitopes induced by immunization with non-mutated and mutated PyCSP plasmid DNA. Effector cells were splenocytes from BALB/c mice immunized with non-mutated or P2, P9/P10 mutated PyCSP DNA, harvested 7 weeks after the third immunization ($n = 3$ per group, pooled), and cultured *in vitro* for 6 days in the presence of synthetic peptides representing the immunodominant CD8+ T cell epitope (residues 280–288) or the overlapping CD8+/CD4+ T cell epitopes (peptide 280–295). Effectors were then assayed against MHC-matched P815 cells or MHC-mismatched EL4 cells (not shown) target cells pulsed with the 280–288 or 280–295 peptides, or not pulsed, in a conventional chromium release assay.

Table 2
Antigen-specific, MHC-restricted CTL activity to native and mutated PyCSP peptide epitopes

Effector	Target	CTL response
Native (non-mutated)	Non-mutated dominant CD8+ 280–288 peptide epitope	Yes
	280–288 peptide epitope mutated at P2, P9, P10	No
	CD8+ 280–288 peptide epitope mutated at P2	Not done
	280–288 peptide epitope mutated at P9, P10	Yes
	CD4+(57–70)/subdominant CD8+(58–67) peptide epitope	No
Mutated P2, P9/P10	Non-mutated CD8+ 280–288 peptide epitope	No
	280–288 peptide epitope mutated at P2, P9, P10	No
	CD8+ 280–288 peptide epitope mutated at P2	Not done
	280–288 peptide epitope mutated at P9, P10	No
	CD4+(57–70)/subdominant CD8+(58–67) peptide epitope	No
Mutated P2	Non-mutated CD8+ 280–288 peptide epitope	No
	CD8+ 280–288 peptide epitope mutated at P2	Not done
	CD8+ 280–288 peptide epitope mutated at P9, P10	No
	CD4+(57–70)/subdominant CD8+(58–67) peptide epitope	No
Mutated P9, P10	Non-mutated CD8+ 280–288 peptide epitope	No
	CD8+ 280–288 peptide epitope mutated at P2	No
	CD8+ 280–288 peptide epitope mutated at P9, P10	Not done
	CD4+(57–70)/subdominant CD8+(58–67) peptide epitope	No
MAP 57–70	Non-mutated CD8+ 280–288 peptide epitope	No
	CD8+ 280–288 peptide epitope mutated at P2, P9, P10	No
	CD4+(57–70)/subdominant CD8+(58–67) peptide epitope	Yes

BALB/c mice were immunized with non-mutated PyCSP DNA, P2, P9/P10 mutated PyCSP DNA, P2 mutated PyCSP DNA, P9/P10 mutated PyCSP DNA, or a MAP based on the subdominant CD8+ T cell epitope (residues 58–67) nested in the dominant CD4+ T cell epitope (residues 57–70). Splenocytes were harvested 2 weeks after the third immunization and cultured *in vitro* for 6 days in the presence of synthetic peptides representing the immunodominant CD8+ T cell epitope (residues 280–288), the immunodominant CD8+ 280–288 epitope mutated at P2 or at P9/P10, or the subdominant CD8+ epitope (58–67)/dominant CD4+ T cell epitope (residues 57–70). Effectors were then assayed in a conventional chromium release assay against MHC-matched P815 cells or MHC-mismatched EL4 cells (not shown) target cells pulsed with the respective peptides, or not pulsed.

not induce CTL responses against the immunodominant CD8+ T cell epitope (residues 280–288) or the overlapping CD8+/CD4+ epitope (residues 280–295) (Fig. 1 and Table 2), consistent with the fact that mutation of any of these anchor residues markedly abrogates binding to the H-2K_d MHC molecule (Table 1). These effectors also failed to recognize the subdominant CD8+ T cell epitope at residues 58–67 (Table 2), indicating that responses were not skewed towards induction of subdominant CD8+ T cell responses. That CTL specific for the subdominant epitope could be induced by peptide immunization was demonstrated in parallel (Table 2).

Consistent with the peptide epitope data presented above, splenocytes of mice immunized with non-mutated PyCSP DNA stimulated *in vitro* with P815 cells transfected with the same non-mutated PyCSP plasmid induced Ag-specific CTL (Table 3). These CTL recognized the intact protein as expressed *in vitro* by plasmid DNA encoding the PyCSP containing the non-mutated 9/10-mer epitope as well as the immunodominant CD8+ CTL epitope (Table 3). In contrast, stimulation with P815 cells transfected with PyCSP DNA containing the CTL epitope mutated at P2, P9/P10 failed to induce CTL that recognized the intact protein or the defined CD8+ CTL epitope (Table 3). The lack of response to the intact protein indicated that there was no induction of CTL specific for the subdominant epitope nor any other CTL epitope located on the PyCSP. IFAT analysis demonstrated expression of both the non-mutated and mutated DNA in P815 transfected cells, indicating that the negative CTL results were due to a lack of recognition, not a lack of expression. Although the above data cannot exclude a more subtle failure of the mutant to be processed for Ag recognition, this possibility is excluded on the basis of demonstrated lymphoproliferative and Ab responses (see below).

To confirm that the lack of recognition of other epitopes on the PyCSP was not somehow related to plasmid DNA administration, mice were immunized with irradiated sporozoites and splenocytes evaluated for their capacity to recognize non-mutated (native) and mutated PyCSP protein or peptide epitopes. Sporozoite-immune splenocytes stimulated *in vitro* with P815 cells transfected with the non-mutated PyCSP DNA, or pulsed with the synthetic peptide representing the immunodominant CD8+ T cell epitope, induced CTL which recognized both the intact protein and the immunodominant CD8+ T cell epitope (Table 3). These CTL failed to recognize the epitope mutated at positions P2, P9 and P10, as expressed *in vitro* by plasmid DNA containing the mutated epitope. Moreover, consistent with the plasmid DNA studies, sporozoite immune effectors stimulated *in vitro* with PyCSP DNA containing the P2, P9/P10 mutations in the 280–288 epitope did not recognize the native PyCSP protein, nor the 280–288 epitope (Table 3).

Overall, these data support the importance of the immunodominant CD8+ T cell epitope and the P2 primary anchor within this epitope.

Table 3
Antigen-specific, MHC-restricted CTL activity to native and mutated PyCSP protein

Effector	Target	CTL response
Native peptide (280–288)	Non-mutated dominant CD8+ 280–288 peptide epitope	Yes
	Native protein	Yes
	Mutated protein	No
Native protein (irradiated sporozoites)	Non-mutated dominant CD8+ 280–288 peptide epitope	Yes
	Native protein	Yes
	Mutated protein	No
Native protein (PyCSP DNA)	Non-mutated dominant CD8+ 280–288 peptide epitope	Yes
	Native protein	Yes
	Mutated protein	No
Mutated protein (P2, P9/P10 mutated DNA)	Non-mutated dominant CD8+ 280–288 peptide epitope	No
	Native protein	No
	Mutated protein	No

Splenocytes from BALB/c mice immunized with non-mutated PyCSP DNA, harvested 2 weeks after the third immunization, were cultured *in vitro* for 6 days with P815 cells transfected with non-mutated or mutated PyCSP DNA or with a synthetic peptide representing the immunodominant CD8+ T cell epitope (residues 280–288). Effectors were then assayed in a conventional chromium release assay against MHC-matched P815 cells or MHC-mismatched EL4 cells target cells transfected with the non-mutated or mutated PyCSP DNA or control DNA, or pulsed with or without the CD8+ peptide epitope.

3.4. Effect of mutations on IFN-γ and TNF-α responses to immunodominant and subdominant epitopes by intracellular cytokine staining

Both IFN-γ and TNF-α were produced by CD8+ T cells from mice immunized with the non-mutated PyCSP plasmid. These responses were Ag-specific, and directed to both immunodominant and subdominant CD8+ T cell epitopes PyCSP 280–288, 280–295 and 58–67 (Fig. 2). In contrast, no CD8+ T cell-mediated Ag-specific IFN-γ or TNF-α responses were detected to any of those epitopes when effectors were splenocytes from mice immunized with the mutated construct (Fig. 2). In this experiment, consistent with other studies from our laboratory, CD4+ T cell responses induced by immunization with PyCSP DNA and assessed by intracellular staining were low. Nonetheless, those CD4+ T cell responses that were detected (between 0.02% and 0.06%) were specific for the PyCSP 57–70, 58–67 and 59–79 epitopes (not for PyCSP 280–288 or PyCSP 280–295) and were detected in both mutated and non-mutated plasmid immunized mice (data not shown).

3.5. Effect of mutations on IFN-γ ELIspot response to immunodominant and subdominant epitopes and CD4+/CD8+ T cell dependence

In mice immunized with non-mutated PyCSP plasmid, *in vitro* depletion of CD8+ T cells but not CD4+ T cells, significantly reduced the IFN-γ responses to the PyCSP 280–288 and

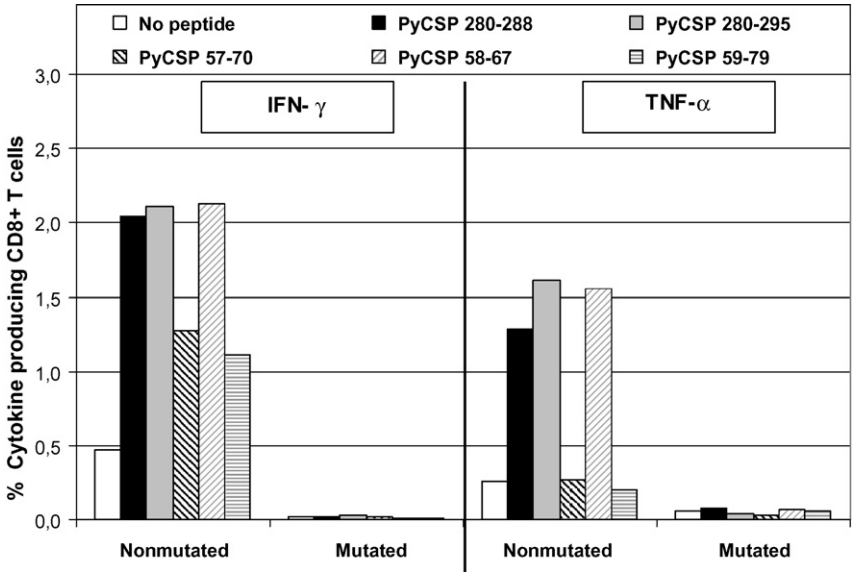

Fig. 2. Antigen-specific IFN-γ and TNF-α responses to immunodominant CD8+ and CD8+/CD4+ epitopes and subdominant CD8+ and CD8+/CD4+ epitopes induced by immunization with non-mutated and mutated PyCSP plasmid DNA. IFN-γ or TNF-α cytokines were assayed by intracellular cytokine staining and FACS analysis. Splenocytes from BALB/c mice immunized with non-mutated or P2, P9/P10 mutated PyCSP DNA harvested 4 weeks after the third immunization were used as effector cells, stimulated *in vitro* with MHC-matched A20.2J APCs pulsed with synthetic peptides representing defined CD8+ and CD8+/CD4+ T cell epitopes. Histograms represent the means of three mice per group, pooled.

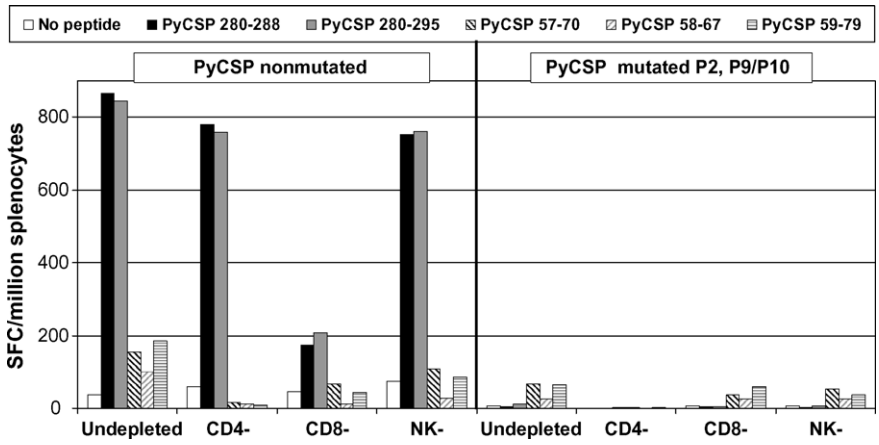

Fig. 3. T cell dependence of antigen-specific IFN-γ responses to immunodominant CD8+ and CD8+/CD4+ epitopes and subdominant CD8+ and CD8+/CD4+ epitopes induced by immunization with non-mutated and mutated PyCSP plasmid DNA. *Ex vivo* IFN-γ ELIspot was carried out using splenocytes from BALB/c mice immunized with non-mutated or P2, P9/P10 mutated PyCSP DNA harvested 4 weeks after the third immunization as effector cells, stimulated *in vitro* with MHC-matched A20.2J APCs pulsed with synthetic peptides representing defined CD8+ and CD8+/CD4+ T cell epitopes. Where indicated, *in vitro* depletions of CD8+ T cells (CD8−), CD4+ T cells (CD4−) or NK cells (NK−) were carried out in splenocyte suspensions immediately prior to ELIspot (see Section 2). Data are presented as spot forming cells (SFC) per million splenocytes for $n = 3$ mice per group, pooled.

PyCSP 280–295 epitopes (Fig. 3), consistent with published data (Sedegah et al., 1994). In contrast, and as expected based on the literature, IFN-γ responses to the PyCSP 59–79 epitope were dependent on CD4+ T cells but not CD8+ T cells, and the responses to the PyCSP 57–70 epitope were dependent on CD4+ T cells and partially dependent on CD8+ T cells. Responses to the subdominant CD8+ T cell epitope PyCSP 58–67 were typically lower than responses to other epitopes, making it more difficult to ascertain significant differences in magnitude, and T cell subset dependence.

Mutation of the primary anchor completely abrogated the PyCSP-specific CD8+ T cell dependent IFN-γ responses to the immunodominant CD8+ T cell epitope (residues 280–288) and the overlapping CD8+/CD4+ epitope (residues 280–295) (Fig. 3). However, mutation had no significant effect on the CD8+ T cell dependent IFN-γ responses to the subdominant CD8+ T cell epitope (residues 58–67) or the CD4+ T cell dependent IFN-γ responses to the CD4+ T cell epitopes (residues 57–70, 59–79) (Fig. 3). These data confirm that the negative CTL or IFN-γ responses to the immunodominant 280–288/289 epitope were due to a lack of recognition of the mutated plasmid, not a lack of expression.

3.6. Effect of mutations on lymphoproliferative response to immunodominant and subdominant epitopes

As expected, mutation of the primary anchor residues of the immunodominant PyCSP CD8+ T cell epitope did not abrogate the lymphoproliferative responses to the defined CD4+ T cell epitopes (residues 57–70, 59–79) or the overlapping CD8+/CD4+ epitope (residues 280–295); responses were comparable to those induced by non-mutated PyCSP (Fig. 4A). However, there was a trend in at least one experiment to more robust CD4+ proliferative T cell responses in mice immunized with PyCSP constructs in which the primary anchor residue of the vaccine (P2) had been mutated (P2; P2,P9/P10), as compared to the non-mutated plasmids (Fig. 4B). These data are consistent with a scenario where immune responses induced by mutated PyCSP plasmids may be deviated from a CD8+ type 1 response towards a CD4+ T cell response.

3.7. Effect of mutations on PyCSP specific antibody response

Anti-PyCSP specific Ab responses were induced by all mutated PyCSP constructs (P2; P9/P10; and P2, P9/P10) as well as the non-mutated PyCSP plasmid (Fig. 5). However, in two independent experiments, Ab titers (OD units 0.5) in mice vaccinated with mutated P2, P9/P10 PyCSP ($n = 14$ tested individually, mean ± S.D. = 12,764 ± 5827) were significantly higher compared to non-mutated PyCSP ($n = 16$, mean ± SD = 6393 ± 3498) after three immunizations ($p < 0.002$). This difference was also noted with samples collected after the first and second immunizations (Fig. 5). Thus, it appears that when T cell responses to the immunodominant CD8+ T cell epitope are eliminated, there is a significant increase in Ag-specific Ab responses, These data provide additional support for our hypothesis that immune responses induced by mutated PyCSP plasmids may be deviated from a predominantly CD8+ Tc1 profile towards a CD4+ Th type that would favor Ab production.

3.8. Effect of mutations on cytokine secretion in serum samples

Multiplexed cytokine assays showed no difference between mutated and non-mutated groups for the Th1 type cytokines IFN-γ, TNF-α, IL-2, IL-12, IL-18, IL-1α, and IL-1β. However, increased responses in constructs with a mutated P2 anchor residue as compared to constructs with the native residue at P2 were noted for the following Th2 type cytokines [median (25%, 75%)]: IL-4, 63 (49, 93) versus 48 (34, 65), $p = 0.028$

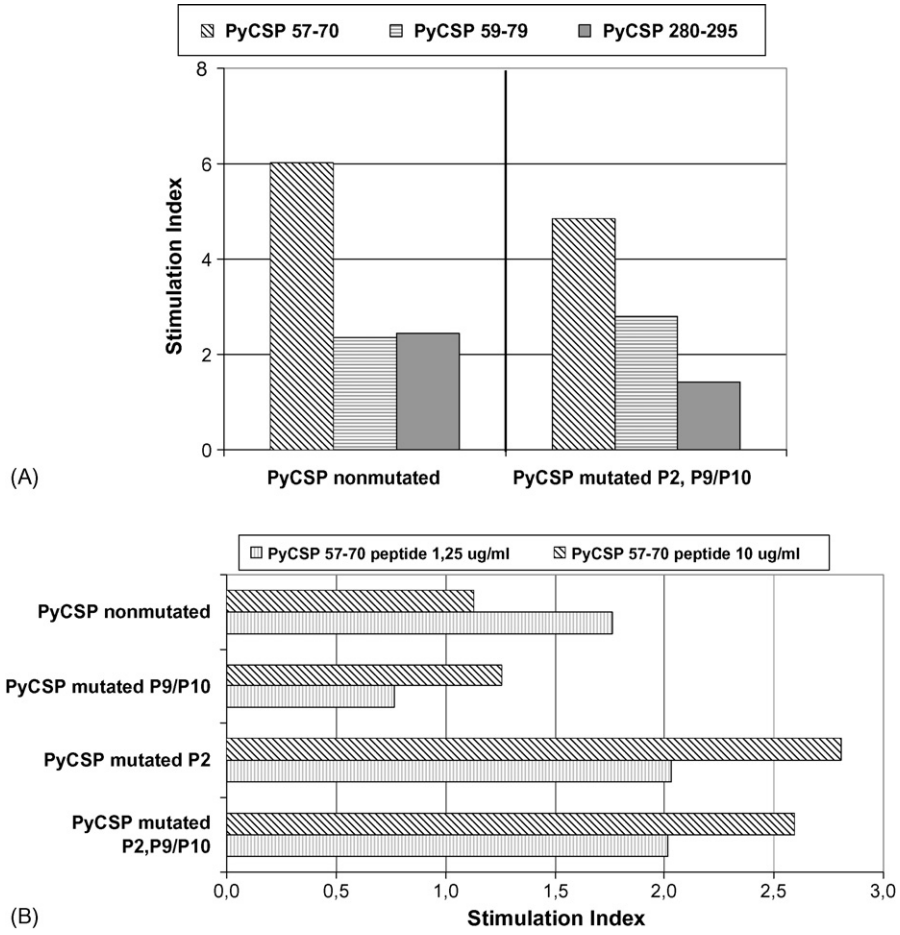

Fig. 4. Lymphoproliferative responses to immunodominant and subdominant CD4+ or CD4+/CD8+T cell epitopes induced by immunization with non-mutated and mutated plasmid DNA. Splenocytes from BALB/c mice immunized with non-mutated PyCSP or mutated P2, P9/P10 or P2/P1/P10 PyCSP plasmids harvested at 4 weeks after the third immunization ($n = 3$ per group, pooled), and stimulated *in vitro* with synthetic peptides representing defined immunodominant CD4+ T cell epitope 57–70, CD4+/CD8+ T cell epitope 280–295, or the subdominant CD4+ T cell epitope 59–79. Data is presented at a peptide concentration of 10 μg/ml (A) and cell concentration of 125,000 cells per well. Similar results were obtained at other cell and peptide concentrations (not shown).

(Mann–Whitney *U*-test); IL-6, 188 (75, 325) versus 67 (37, 183), $p = 0.028$; IL-10, 85 (32, 133) versus 58 (17, 94), $p = 0.140$. There was no effect on IL-13. Data are consistent with a shift from a Th1 profile towards Th2 responses when the P2 residue is mutated.

3.9. Effect of mutations on sterile protective immunity

Consistent with previous studies, 48.1% sterile protection was obtained in BALB/c mice following immunization with a plasmid DNA construct encoding the non-mutated PyCSP (Table 4). Mutation at P9 and P10 representing the C-terminus primary anchor residues had no apparent effect on protection, since 52.0% sterile protection was obtained in mice immunized with the mutated P9/P10 construct ($p = 1.0$, non-mutated versus P9/P10 mutated) and there was no observable delay in the prepatent period as compared with the non-mutated construct (data not presented). In contrast, data demonstrated that the N-terminus primary anchor residue at P2 was important for protective efficacy since mutation of the P2 position, with or without mutation of P9/P10 positions, decreased the protection to a level of 25.9–33.3% ($p = 0.16$). These data support the importance of the immunodominant CD8+ T cell epitope 280–288 in PyCSP DNA vaccine induced protection (Sedegah et al., 1994), and the importance of the N-terminal but not the C-terminal primary anchors.

Interestingly, however, significant sterile protection relative to the controls was obtained in mice immunized with plasmid DNA mutated at either position P2 only, or positions P2 and P9/P10 (Table 4). Although protective immunity was markedly reduced by mutation of the primary P2 anchor of the 280–288/289 epitope, it was not completely abrogated despite that this epitope is the only immunodominant CTL epitope identified on the PyCSP, and that CTL specific for this epitope can adoptively transfer protection. Thus, mice immunized with a DNA vaccine that elicits CD8+ T cell responses specific for the immunodominant CD8+ T cell epitope, and mice immunized with a vaccine that cannot produce CD8+ T cells against that epitope, are both significantly protected against *Plasmodium* sporozoite challenge. However, the immunity elicited in the absence of CD8+ T cell responses to the immunodominant epitope is not as robust as in the presence of effective CD8+ IFN-γ effector responses directed against the immunodominant CD8+ T cell epitope, since the level of protective efficacy and

Table 4
Sterile protection against sporozoite challenge induced by immunization with plasmid DNA constructs

Immunogen	Sequence	Number of protected/challenged	%Protection	*p*-Value (relative to non-mutated)	*p*-Value (relative to control)
Negative control	–	0/25[a]	0		–
PyCSP native (non-mutated)	S**Y**VPSAEQI**L**	13/27	48.1	–	0.0001
PyCSP mutated P2[b]	S**A**VPSAEQI	7/27	25.9	0.16	0.0062
PyCSP mutated P9/P10	SYVPSAEQ**AA**	13/25	52.0	0.78	<0.0001
PyCSP mutated P2, P9/P10	S**A**VPSAEQ**AA**	8/24	33.3	0.28	0.0017
P2 mutations		(7/27 + 8/24) 15/51	29.4	0.10	0.0025
Any mutations		(7/27 + 13/25 + 8/24) 28/76	36.8	0.30	0.0004

[a] All naive mice (9 mice) and all control mice immunized with vector DNA containing an unrelated insert (16 mice) became infected.
[b] Site-directed mutagenesis was carried out using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene), as described by the manufacturer.

overall immune responses are lower. Overall, these data indicate that immune effector mechanisms distinct from those directed against the CD8+ 280–288 epitope are capable of conferring sterile protective immunity, albeit not as robust as in the presence of CD8+ T cell responses, and are consistent with a scenario of immune deviation as indicated above.

3.10. Effect of mutations on liver stage parasite burden in vivo

Consistent with the sterile protection reported above, significant partial protection as measured by a reduction in liver-stage parasite burden by TaqMan™ RT-PCR was noted in BALB/c mice immunized with the non-mutated PyCSP DNA vaccine (geometric mean [95% CI] *P. yoelii* 18S rRNA/mouse GAPDH, non-mutated 0.0013 [0.0008, 0.0028] versus control plasmid 0.0087 [0.0040, 0.0191], $p = 0.0009$, calculated by Student's *t*-test on log transformed values). As expected, this was significantly abrogated by *in vivo* depletion of effector CD8+ T cells prior to challenge (0.0257 [0.0117, 0.0566], $p < 0.0001$). Also consistent with the results of studies using blood-stage parasitemia as the read-out of protection, and the importance of the immunodominant CD8+ T cell epitope, mutation at positions P2, P9/P10 in the PyCSP 280–288 epitope significantly abrogated the PyCSP DNA vaccine-induced protection (0.0170 [0.0044, 0.0669], $p = 0.0039$).

Consistent with the trend for deviation of the effector immune response from a CD8+ Tc1 profile to a CD4+ Th profile with mutation of the primary anchor residues described above, a different pattern of CD4+/CD8+ T cell dependence was noted in mice immunized with the mutated compared to the non-mutated PyCSP constructs. Specifically, there was a significant difference in the effect of CD8+ depletion in mice immunized with the mutated versus non-mutated plasmid (0.0076 [0, 0.0191], $p = 0.0124$); partial protection after *in vivo* depletion of CD8+ T cells in the mutated group was not significantly different from that achieved in the undepleted non-mutated group ($p = 0.1972$). In contrast, *in vivo* depletion of either CD4+ T cells or IFN-γ significantly abrogated protection as compared to the levels achieved by immunization with non-mutated plasmid (0.0234 [0, 0.0489], $p = 0.0004$ and 0.0298 [0, 0.0631], $p < 0.0001$, respectively). Overall, data demonstrate that IFN-γ dependent protective immunity directed at the liver stage can be induced by vaccination with plasmid DNA constructs where the encoded immunodominant CD8+ T cell epitope is mutated to abrogate binding to the class I MHC, but that this protection is significantly reduced as compared to immunization with non-mutated constructs, and is mediated by CD4+ T cells rather than CD8+ T cells.

4. Discussion

We have previously demonstrated that the protective immunity induced by immunization with plasmid DNA encoding PyCSP is absolutely dependent on CD8+ T cells and IFN-γ but not CD4+ T cells (Doolan et al., 1996a; Sedegah et al., 1994). Other studies have identified a single immunodominant CD8+ T cell epitope on PyCSP, at residues 280–288 (Weiss et al., 1990). Herein, we evaluated the effect of selectively mutating this single immunodominant CD8+ T cell epitope on the immunogenicity and protective immunity induced by PyCSP DNA vaccines in mice. This approach allowed us to elucidate the role of immunodominant versus subdominant CD8+ T cell epitopes in protection against *P. yoelii* sporozoite challenge induced by DNA vaccination, and to further dissect the relative contribution of CD4+ versus CD8+ T cells in the effector mechanisms of pre-erythrocytic stage immunity.

Our data shows that the immunodominant PyCSP 280–288 epitope is absolutely required for optimal protection against malaria induced by immunization with PyCSP DNA. Furthermore, recognition of the non-mutated and P9/P10 mutated plasmid and 10-mer peptide shows that the P2 N-terminus primary anchor residue, but not P9 or P10 C-terminus primary anchor residues, of this epitope is critical for CD8+ T cell recognition. CTL specific for this 9-mer epitope can adoptively transfer protection, and site-directed mutagenesis of the epitope abrogates the CTL response (Weiss et al., 1988). Interestingly, the *P. yoelii* and the *P. berghei* CTL epitopes have identical residues at anchor positions: PyCSP (280–289) (S**Y**VPSAEQI**L**) and PbCSP (249–260) (NDDS**Y**IPSAEKI). It has been shown that a CD8+ CTL clone induced by immunization with *P. yoelii* irradiated sporozoites recognizes both epitopes and protects mice against both *P. yoelii* and *P. berghei* sporozoite challenge upon adoptive transfer (Weiss et al., 1992). This provides further evidence for the importance of the PyCSP 280–288 epitope and the conservation of the anchor amino acids in the

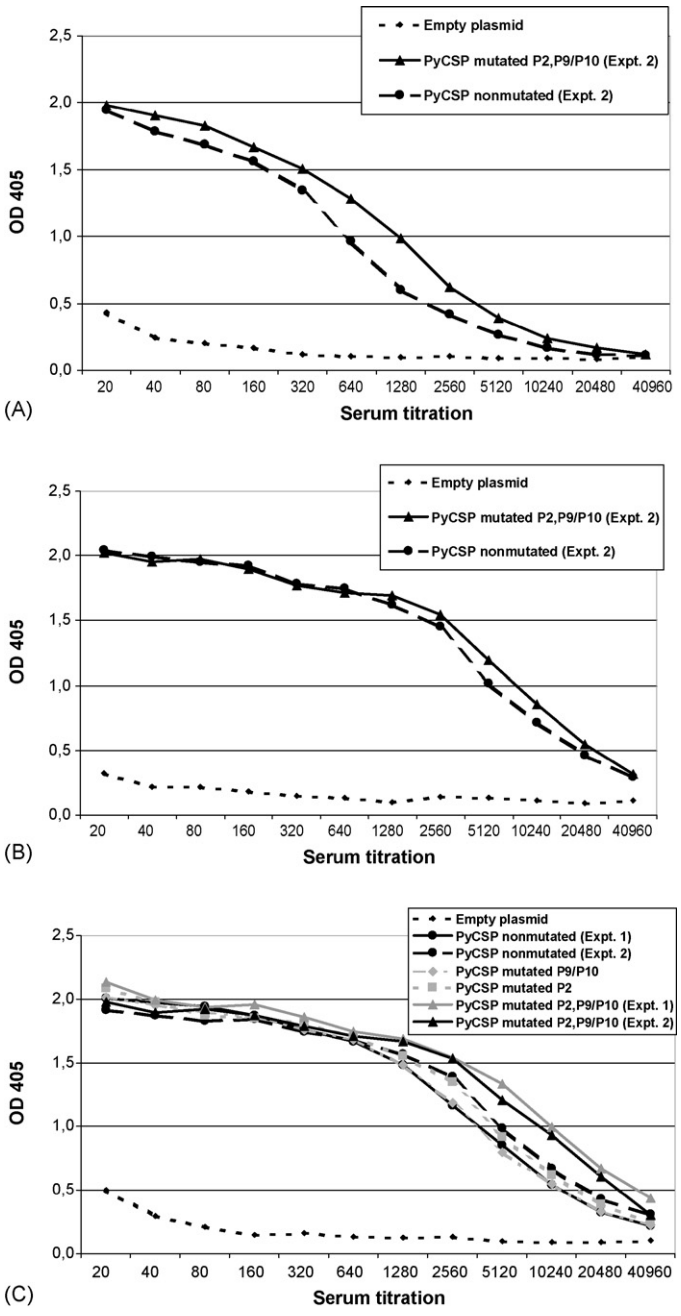

Fig. 5. Antibody responses induced by immunization with non-mutated and mutated PyCSP plasmids, detected by ELISA against recombinant full length PyCSP. Data show results with pooled sera ($n = 10$) collected 12 days after (A) first immunization, (B) second immunization, or (C) third immunization, including results from two separate experiments (Expt. 1 and Expt. 2) and all vaccine constructs.

protective immunity induced by immunization with PyCSP and irradiated sporozoite vaccines in mice.

Consistent with data from the protection studies, *in vivo* and *in vitro* immunogenicity data showed that IFN-γ responses induced by PyCSP DNA vaccination were directed predominantly to the single immunodominant CD8+ T cell epitope, that these IFN-γ immune responses were dependent on CD8+ T cells but not CD4+ or NK cells, and that the P2 (but not P9/P10) primary anchor residue is critical. Mutation of this residue completely abrogated the CD8+ PyCSP-specific CTL activity and CD8+ T cell dependent IFN-γ and TNF-α responses to the immunodominant CD8+ T cell epitope (residues 280–288) and the overlapping CD8+/CD4+ epitope (residues 280–295). The lack of detectable response to the intact protein in the absence of a specific response to the 280–288/289 epitope suggests that there was no induction of CTL specific for any other epitopes on the PyCSP Ag, including the subdominant CD8+ T cell epitope located at residues 58–67 or CD4+ T cell epitopes. Cytokine and lymphoproliferative T cell responses to other epitopes and Ab responses to the intact protein exclude the possibility that negative CD8+ T cell-mediated responses with the mutant plasmid were due to a failure in Ag processing and recognition, or a lack of expression.

Nonetheless, mutation of the P2 anchor residue of the 280–289 epitope, or the P2, P9/P10 anchors associated with MHC binding inhibited but did not completely abrogate protective immunity against *P. yoelii* in mice, despite that this epitope is the only immunodominant K_d restricted CTL epitope identified on the PyCSP. These data suggest that when the immunodominant CD8+ T cell response is eliminated, liver-stage protection is mediated by effector cells specific for a T cell epitope(s) distinct from the immunodominant CD8+ T cell epitope and/or by a different effector mechanism.

In a given protein Ag, there is a hierarchy of T cell epitopes, originally defined by Sercarz and co-workers (Gammon et al., 1987). Those epitopes eliciting the greatest immune responses following protein or peptide immunization are termed immunodominant. Those epitopes stimulating weaker and more variable responses following protein immunization, with strong responses being induced by peptide immunization, are termed subdominant. Subdominant epitopes are generated by natural Ag processing, and T cells elicited by peptide immunization can recognize intact protein. Other epitopes that are not recognized following immunization with the intact protein but that are nevertheless immunogenic and to which responses are induced by peptide immunization are termed cryptic. Cryptic epitopes are capable of binding the MHC but are not naturally processed by the immune system, or are processed at subthreshold levels. T cells specific for these cryptic epitopes are present in the normal T cell repertoire, and may become visible to the immune system under certain conditions.

In this context, it is possible that abrogation of the protective immunity directed against the immunodominant CTL epitope reveals protective effector cells directed against an epitope previously presented subdominantly or cryptically. Our data demonstrate that the suboptimal protection induced by immunization with mutated PyCSP DNA is mediated by effector cells specific for T cell epitopes distinct from the immunodominant CD8+ T cell epitope, including but not restricted to the previously defined CD4+ T cell epitopes (residues 57–70, 59–79). Immunogenicity data showed that mutation of the primary anchor residues had no significant effect on the CD8+ T cell dependent IFN-γ responses to the subdominant CD8+ T cell epitope (residues 58–67) but did enhance the CD4+ T cell dependent lymphoproliferative responses to the CD4+ T cell epitopes (residues 57–70, 59–79), and the Ab responses to recombinant PyCSP.

Liver-stage protection and *in vivo* depletion studies showed that the partial protection induced by immunization with mutated plasmid was mediated by CD4+ T cells, rather than by CD8+ T cells. This indicates that, in this system, the effector function of Ag-specific CD4+ T cells (involving secretion of IFN-γ) is relatively more important than CD8+ effector T cells directed against an epitope presented subdominantly.

We interpret our data as consistent with the scenario of immune deviation (Parish, 1996) where, when the immunodominant CD8+ T cell epitope is rendered non-functional, effector responses are deviated from a CD8+ IFN-γ dependent profile to a CD4+ T cell IFN-γ dependent helper profile with enhanced CD4+ lymphoproliferative responses and enhanced Ab responses. Results of IFN-γ ELIspot, intracellular cytokine staining, lymphoproliferation and Ab immunogenicity assays, *in vivo* liver-stage and blood-stage protection assays, as well as *in vitro* and *in vivo* depletions of effector CD4+ and CD8+ T cells are consistent with this hypothesis. In particular, the significantly higher Ab titers are indicative of the induction of a more robust CD4+ Th effector function by immunization with DNA plasmids in which the primary anchor residues involved in binding to the class I MHC molecules have been mutated.

Overall, data suggested that the protective immune responses induced by DNA vaccination are directed predominantly against immunodominant CD8+ T cell epitopes, and that although responses can be induced against other epitopes including CD4+ T cell epitopes, these responses are poor and not adequate for optimal protection against parasite challenge.

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