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Identification of non-CSP antigens bearing CD8 epitopes in mice immunized with irradiated sporozoites

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Abstract

Immunization of BALB/c mice with irradiated sporozoites (IrSp) of *Plasmodium yoelii* can lead to sterile immunity. The circumsporozoite protein (CSP) plays a dominant role in protection. Nevertheless after hyper-immunization with IrSp, complete protection is obtained in CSP-transgenic BALB/c mice that are T- cell tolerant to the CSP and cannot produce antibodies [CSP-Tg/JhT(−/−)]. This protection is mediated exclusively by $CD8^+$ T cells [1]. To identify the non-CSP protective T cell antigens, we studied the properties of 34 *P. yoelii* sporozoite antigens that are predicted to be secreted and to contain strong Kd-restricted $CD8^+$ T cell epitopes. The synthetic peptides corresponding to the epitopes were used to screen for the presence of peptide-specific $CD8^+$ T cells secreting interferon-γ (IFN-γ) in splenocytes from CSP-Tg/JhT(−/−) BALB/c mice hyper immunized with IrSp. However, the numbers of IFN-γ-secreting splenocytes specific for the non-CSP antigen-derived peptides were 20 to 100 times lower than those specific for the CSP-specific peptide. When mice were immunized with recombinant adenoviruses expressing selected non-CSP antigens, the animals were not protected against challenge with *P. yoelii* sporozoites although large numbers of CD8+ specific T cells were generated.

Keywords

plasmodium; malaria vaccine; circumsporozoite protein (CSP); non-CSP antigens

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1. Introduction

Multiple immunizations of rodents, monkeys and humans with irradiated mosquitoes infected with sporozoites (IrSp) lead to sterile immunity to sporozoite challenge [2–4]. Immunization of mice with genetically-attenuated sporozoites (GaSp) is also effective [5,6]. The mechanisms of protection elicited by IrSp and the uis3/uis4 GaSp are similar [7], and involve both humoral and cellular immune responses that target the invading sporozoite and the infected hepatocyte respectively. The antibody responses are predominantly directed against the repetitive central domain of CSP [8]. The adoptive transfer of CSP-specific $CD8^+$or $CD4^+$ T cell clones, albeit in large numbers, leads to full protection against sporozoite challenge [9–11]. These observations indicated that CSP plays an important role in protection elicited by IrSp. This view was greatly strengthened by IrSp immunization of T-cell tolerant, CSP-transgenic BALB/c or C57BL/6 mice that could not make antibodies (CSP-Tg/JhT mice). After priming and boosting the transgenic mice and controls with 10^5 IrSp, protection was reversed by more than 90% in the BALB/c mice and about 50% in C57BL/6 [1,7]. Nevertheless, sterile immunity was obtained in the CSP-Tg/JhT(−/−) BALB/c mice after a second booster injection with 10^5 IrSp. The protection in the absence of CSP-specific immunity was mediated exclusively by $CD8^+$ T cells [1].

The main objective of the present study is to identify the non-CSP antigens that elicit those potent protective $CD8^+$ T cell responses in the CSP-Tg/JhT(−/−) BALB/c mice. We hoped to find new dominant or sub-dominant sporozoite antigens that might be used to prevent malaria infection in humans. The current lead malaria vaccine, GlaxoSmithKline's RTS,S, contains a portion of CSP that includes its C-terminus and the dominant repeat B cell epitope [12–13]. RTS,S has a favorable safety profile, and in many human trials, including in children and infants, consistently protected 30–50% of the vaccinees against infection. In one field study, the vaccine was capable of providing a sustained clinical benefit for up to 42 months following vaccination [14,15]. Although the results from RTS,S vaccination are impressive, there remains the goal to increase the level of efficacy of this first generation malaria vaccine candidate. One possible approach to achieve this goal is to add protective non-CSP antigens to RTS,S, or use non-CSP antigens instead of RTS,S. Unfortunately, to date few non-CSP antigens have been used in humans. The best studied antigen is ME-TRAP. Prime-boost regimens of ME-TRAP with simian adenovirus and modified vaccinia Ankara (MVA) vectors protected mice against challenge, and generated strong T cell responses in non-human primates. Nevertheless the delivery of ME-TRAP using DNA and MVA vaccines failed to protect adults in Gambia [16,17]. The IL-10 responses to Liver Stage Antigen 1 (LSA1) are associated with resistance to *P. falciparum* infection in endemic areas [18]. Nevertheless the LSA1 vaccine was not protective in humans [19]. Genetic immunization with LSA3 protected chimpanzees against falciparum malaria [20], but we found no information of its use in humans. Recently it has been reported that CelTOS is a protective antigen both in BALB/c and outbred mice [21]. CelTOS is a micronemal protein secreted by sporozoites during their migration from the skin to the liver [22]. Antibodies generated by CelTOS immunization immobilize sporozoites and inhibit infection of hepatocytes *in vitro,* suggesting that protection is at least in part antibody mediated [23].

In addition to looking for non-CSP protective antigens elicited by hyper-immunization of BALB/c mice with IrSp, we performed some experiments to evaluate the protective potential of selected non-CSP malaria antigens. For this purpose we used adenovirus vectors that are known to induce potent $CD8^+$ T cell-mediated protective immunity and also antibody responses against pathogens [24–26].

2. Materials and methods

2.1. Selection of non-CSP antigens and epitopes

We preferentially selected non-CSP antigens with predicted signal sequences from sporozoite and liver stage libraries [27,28]. This is because, in order for the antigens to be recognized by effectors T cells, the antigens have to be secreted into the cytoplasm of the infected liver cells [or of neighboring cells] and then processed and presented on the plasma membrane in association of MHC class I [1]. Because the liver stages develop inside a parasitophorous vacuole (PV) and need to traverse the PV membrane to enter the cytoplasm, a higher priority was given to antigens bearing Pexel motifs [29,30]. The effectiveness of T cell recognition should increase when large numbers of peptides derived from non-CSP antigens are present on the surface of the target cells. Thus we selected preferentially highly transcribed *P. yoelii* genes [27,28] assuming that the corresponding protein would also be highly represented in the parasite. We made sure that all of the selected genes have *P. falciparum* orthologs. A total of 34 non-CSP candidate antigens were selected, including a previously reported protective antigen CelTOS, also named antigen 2 [21,23,31]. From each candidate we chose CD8+ T cell epitopes based on three independent computer algorithms that predict binding affinity to MHC class I molecule:

http://www-bimas.cit.nih.gov/cgi-bin/molbio/ken_parker_comboform (NIH)

http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm (SYFPEITHI)

http://tools.immuneepitope.org/analyze/html/mhc_binding.html (IEDB)

All selected molecules, epitopes and predicted binding to K^d are shown in the supplementary Table 1.

2.2. Mice

Six- to eight-week-old female BALB/c (H2-K^d) mice were purchased from Taconic (Germantown, NY). BALB/c mice transgenic for CS that were also unable to make antibodies [CSP-Tg/JhT(−/−)] were bred and maintained at the NYU School of Medicine. All mice were housed and maintained at NYU School of Medicine Animal Facility in a pathogen free mouse facility according to institutional guidelines. All animal studies were approved by the institution's Institutional Animal Care and Use Committee.

2.3. Mosquitoes and IrSp immunization

Anopheles stephensi mosquitoes were reared at 27°C and 80% humidity under a 12/12 h light/dark cycle, and adults were fed on 10% sucrose solution. *P. yoelii* -infected mosquitoes were maintained at 25°C. Live salivary gland sporozoites were obtained 14 days after the infective meal. The mosquitoes were rinsed in 70% ethanol, washed in DMEM, and the salivary glands removed. The glands were gently ground in a tissue homogenizer, centrifuged at 800 rpm for 4 min to remove mosquito debris, and sporozoites were counted in a haemocytometer and kept on ice until use. For challenge, 10^4 sporozoites were inoculated into mice intravenously. Prior to immunization, the sporozoites were irradiated (15,000 rads). Mice were immunized intravenously three times with 10^5 IrSp at 2 weeks intervals.

2.4. ELISPOT assay

Multiscreen plates (Millipore, Bedford MA) were coated with 10 μg/ml of anti-mouse IFN-γ (BD Pharmingen). After overnight incubation, the wells were washed twice with PBS and then twice with wash Medium (DMEM containing 10% FCS). After blocking the wells with wash medium for 6 hours at 37°C, splenocytes obtained from immunized mice and synthetic

peptides corresponding to the CD8+ T cell epitopes were added to the wells. After incubation at 37°C and 5% CO_2, for 24–28 h, the plates were extensively first washed with PBS, and then with PBS containing 0.05% of Tween 20 (PBST). The wells were then incubated with a solution of 2μg/mL of biotinylated anti-mouse IFN-γ (BD Pharmingen) overnight. Finally, the plates were incubated with peroxidase-labeled streptavidin (Kirkegaard and Perry Laboratories, KPL, Gaithesburg, MD), and the spots were developed by adding AEC substrate set (BD bioscience) [32] according to the manufacture's instruction.

2.5. Recombinant adenovirus

The general strategy for the construction of recombinant adenoviruses expressing non-CS pre-erythrocytic antigens is presented in Fig. 3. Selected non-CSP candidate antigens were codon optimized for the expression into mammalian cells. Signal peptide and transmembrane domain were removed from the codon optimized gene and c-Myc-tag was placed at the end of the gene. Partial or full length of the gene was cloned into modified pShuttle-CMV5 vector (Stratagene) at *Kpn* I and *Hind* III site. The GFP expression cassette was introduced into the pShuttle-CMV5 vector (pShuttle-CMV5-GFP) to produce the fluorescent virus. The rest of the procedure for the generation of recombinant adenovirus was performed as described in AdEasy™ Adenoviral Vector System (Stratagene).

2.6. Western blot analysis

For the detection of antigen expressed by recombinant adenoviruses, Western blot analysis was performed. Briefly, AD-293 cells (Stratagene) were infected with a recombinant adenovirus expressing each of the antigens and then resuspended in SDS sample buffer and boiled for 5 min to make cell extracts. The cell extracts were run on SDS-PAGE and transferred to Sequi-Blot™ polyvinylidene difluoride membranes (Bio-Rad). After the membrane was incubated with a mouse anti-human c-Myc monoclonal antibody (BD Pharmingen), bound antibody was detected with horse radish peroxidase (HRP)-coupled anti-mouse IgG. Finally, blot was developed using Novex® ECL HRP chemiluminescent substrate reagent kit (Invitrogen).

2.7. Recombinant adenovirus immunization, and challenge

BALB/c mice were immunized i.m. with 10^{10} virus particle of the recombinant adenovirus expressing the *plasmodial* sequence. We made recombinant adenoviruses of P36p (PY01340) [33,34], TRAP-like protein (PY01499) [35,36], Krueppel-like Protein (PY00839), and to a group of proteins that were highly immunogenic in human volunteers vaccinated and protected against sporozoite challenge after immunization with falciparum IrSp, ie, Ag2 or CelTOS (AY427752.1), Ag5 (PY00410) and Ag17 (PY00455) [31]. Two weeks after immunization, splenocytes were isolated and IFN-γ ELISPOT was performed. Another group of immunized mice was challenged by the i.v. administration of 1×10^4 live *P. yoelii* sporozoites and the level of protection measured as described below.

2.8. Evaluation of protection by RT-PCR

The amounts of *Plasmodial* rRNA in the livers of immunized mice were measured 42 hours after sporozoite challenge. The liver was washed twice in PBS and homogenized in 10ml Trizol (Invitrogen). 200μl of the homogenized liver was transferred into fresh tubes containing 600μl trizol. RNA was isolated according to the manufacturer's instructions. 400 ng of total RNA was reverse-transcribed from each of the samples using RT-PCR kit (PE Applied Biosystems) with random hexamers. The real time PCR was performed as described earlier [37].

2.9. Statistical Analysis

All results are presented as mean values ± SD. Student's *t* tests were used to determine the statistical significance.

3. Results

3.1. T Cell Responses to non-CSP antigens

Groups of wt BALB/c mice and [CSP-Tg/JhT(−/−)] mice with the BALB/c background were immunized with three doses of 10^5 *P. yoelii* IrSp with intervals of 2 weeks. The level of $CD8^+$ T cell responses were evaluated by IFN-γ ELISPOT assay, in which splenocytes from immunized mice were incubated with peptides corresponding to the $CD8^+$ T cell epitopes of different antigens. The CSP-Tg/JhT(−/−) mice were used to exclude the possibility that the high level of immune responses to CSP generated by the IrSp immunization would interfere with the generation of T cells specific to non-CSP antigens in wt mice. A total of 34 non-CSP candidate antigens were studied (Table 1). The results are shown for wt BALB/c mice in Fig. 1 and for the [CSP-Tg/JhT(−/−)] mice in Fig. 2. The immunodominant $CD8^+$ T cell epitope - SYVPSAEQI (H-$2K^d$ restricted)-derived from *P. yoelii* CSP (AAA29558.1) was used as positive control. As shown, the level of $CD8^+$ T cell responses to non-CSP antigens were 20 to 100 times lower than that to CSP (Fig. 1). Although unlikely it is possible that larger number of $CD8^+$ T cells were present in the liver or elsewhere. As expected, the response to the $CD8^+$ T cell epitope of CSP in [CSP-Tg/JhT(−/−)] mice was negligible, because T cells to CSP were tolerant in these mice. The overall responses to non-CSP antigens in the transgenic mice were still very low (Fig. 2).

3.2 Immunization with recombinant adenoviruses and protection against sporozoite challenge

Of the antigens listed in Figs 1 and 2, we selected 6 for expression by a recombinant adenoviral vector. Our aim was to obtain preliminary information about their immunogenicity, in particular their ability to elicit CD8+ T cells and protection against challenge with sporozoites. Three of the chosen antigens P36p (Py52) - PY01340, Krueppel-like protein - PY00839, and TRAP-like protein - PY01499 are highly transcribed during the sporozoite stage [27,28]. The other three antigens namely Ag2 (PyCelTOS), Ag 5 (Hypothetical Protein), and Ag 17 (Hypothetical Protein) were immunogenic in human volunteers vaccinated with falciparum IrSp. [31]. Immunization of BALB/c mice and outbred mice with adjuvanted recombinant CelTOS led to sterile protection in a sizable proportion of the animals [21].]. We performed Western blot assays using the extracts of cells infected with each of recombinant adenoviruses and confirmed the expression of c-Myc tagged antigens, as a transgene (Fig. 4).

After constructing recombinant adenoviruses expressing those six antigens, we compared their immunogenicity and protective efficacy with that of a recombinant adenovirus bearing CSP. The immunogenicity was only evaluated by an IFN-γ ELISPOT assay using selected $CD8^+$ T cell epitopes in two separate experiments (Fig. 5). Notably, the splenocytes of mice immunized with adenovirus expressing P36p, Ag2 (CelTOS) and Ag5 produced similar or even higher numbers of IFN-γ ELISPOT, when compared to splenocytes of mice immunized with adenovirus-expressing CSP. To test the protective capacity of each antigen, mice immunized with recombinant adenoviruses were challenged with 10^4 live sporozoites of *P. yoelii,* 2 weeks after the immunization. Two days after the challenge, the livers were collected from the challenged mice and the amount of parasite loads was determined by a real-time RT-PCR [37,38]. We performed three independent experiments shown in Fig. 6. Because the infectivity of sporozoites varies from week to week, the amounts of the parasite load in the liver also varies. Nevertheless, it is apparent that the parasite load in the livers of

the group of mice immunized with adenovirus-expressing CSP was significantly ($p<0.01$) lower compared to that of mice immunized with adenovirus-expressing non-CSP antigens. Thus only mice immunized with adenovirus-expressing CSP were protected (Fig. 6).

4. Discussion

More than forty years have passed since the demonstration that immunization of mice with irradiated sporozoites (IrSP) induces sterile protection against a sporozoite challenge [2]. This was followed by the discovery of the circumsporozoite protein (CSP), its role in the parasite infectivity and efforts to develop CSP-based vaccines [39]. CSP makes up the bulk of the proteins at the sporozoite surface [40], is the main target of antibody responses [8] and displays two non-restricted CD4+ T-cell epitopes. Although CSP is an immunodominant protective antigen [1], two studies using distinct approaches showed that sterile immunity against malaria infection can be obtained independently of CSP. One study used CSP-transgenic mice that cannot make antibodies and are T-cell tolerant to CSP. After triple immunization with a high dose of IrSp the transgenic mice were fully protected, and the protection was mediated by CD8+ T cells. [1]. In the other approach, mice were immunized with *P. berghei* IrSp, and were fully protected from challenge with recombinant *P. berghei* parasites bearing the CSP of *P. falciparum*. Since there was no cross-reactivity between the two CSPs, the authors concluded that sterile immunity to the recombinant parasite was not CSP-dependent [41].

The main objective of our studies was to characterize the non-CSP antigens that can elicit powerful protective CD8+ T cell responses. When approaching this question it is important to consider the multiple complexities that have to be overcome for the T-cell mediated elimination of the infected liver cells (exoerythrocytic forms or EEFs). One of them is the scarcity of EEFs among non-infected liver cells. If the challenge after immunization is with 10^4 sporozoites, the ratio of EEFs to normal liver cells will be only about 1/10,000 even if the efficiency of infection is 100%. Therefore very large numbers of CD8+ T cells are needed to find and destroy all of the EEFs [42,43]. To complicate the task of the T cells, the main lymphokine that inhibits the liver stages, interferon γ, is only effective during the very early development of the parasite [44]. Thus not only the T cells targets are scarce but the time window for the EEF destruction is limited.

None of the selected 34 P. *yoelii* non-CSP antigens (Table 1) came even close to the immunogenicity of CSP in BALB/c mice. The explanation is that CSP is very abundant in salivary gland sporozoites [39], has a very potent Kd-restricted epitope, and is released while the parasites traverse cells while traveling from the site of mosquito bite to the liver [45,46]. The released CSP can be picked up by dendritic cells where it remains for weeks for antigen presentation [47]. Even after sporozoites invade hepatocytes and the parasite is enclosed in a vacuole, CSP traverses the vacuole membrane and enters the hepatocyte cytoplasm where it enters the antigen processing pathway. The traversal of the membrane of the vacuole is facilitated by the presence in CSP of two functional Pexel motifs [48]. Thus CSP is not only poised to generate powerful CD8 responses, but is an excellent target for CD8+ T cells. As shown in Figures 1 and 2 the number of interferon-γ ELISPOTs generated by the non-CSP antigens was 20 to 100 times lower than those obtained with CSP. Thus we failed to discover any single antigen that accounts for the sterile immunity observed in the CSP-Tg/JhT(−/−)] BALB/c mice hyper-immunized with IrSp. A tentative conclusion is that the observed protection reflects the sum of activities of many minorprotective antigens within the IrSp[49].

We also generated recombinant adenovirus expressing a few non-CSP antigens. Among them are antigen 2 (CelTOS, [23]) and 5 [31] that were specifically recognized by CD4+

and/or CD8 + T cells of at least 50% of humans volunteers immunized with IrSp of *P. falciparum*. The number of IFN-γ ELISPOTs generated by peptides from CelTOS, antigens 5 and P36p (pointed out by arrows in figures 1 and 2) was similar or even greater that those obtained by the dominant Kd epitope of CSP (Figure 5). Unexpectedly, after challenge of the immunized mice with live *P. yoelii* sporozoites, only CSP was protective. A plausible explanation for this paradoxical result is that the non-CSP antigens did not enter the class I pathway of antigen presentation in the EEFs or, more likely, because they are much less abundant than CSP. In other words, the non-CSP antigens were very immunogenic but were poor targets to the CD8+ T cells. Of course, other methods of immunization that elicit good antibody responses, or effector CD4+ T cells may lead to protective immunity. For example, the injectionin mice of recombinant CelTOS *P. falciparum* or *P. berghei* protein in Montanide adjuvant, or a plasmid encoding *P. berghei* CelTOS, induced sterile immunity after challenge with *P. berghei* sporozoites [21,23]. The serum of the immune mice immobilized *P. berghei* sporozoites, suggesting that the protection was at least in part antibody mediated.

In summary, we failed in our attempt to discover powerful protective non-CS antigens in our *P. yoelii* model system. Nevertheless, as recently reported [50], it might be worthwhile to investigate whether a mixture of antigens would lead to protection. In fact, one of the protective antigens in reference 50 (PY03424) was among those that elicited a higher numbers of ELISPOTS in our studies (Figures1 and 2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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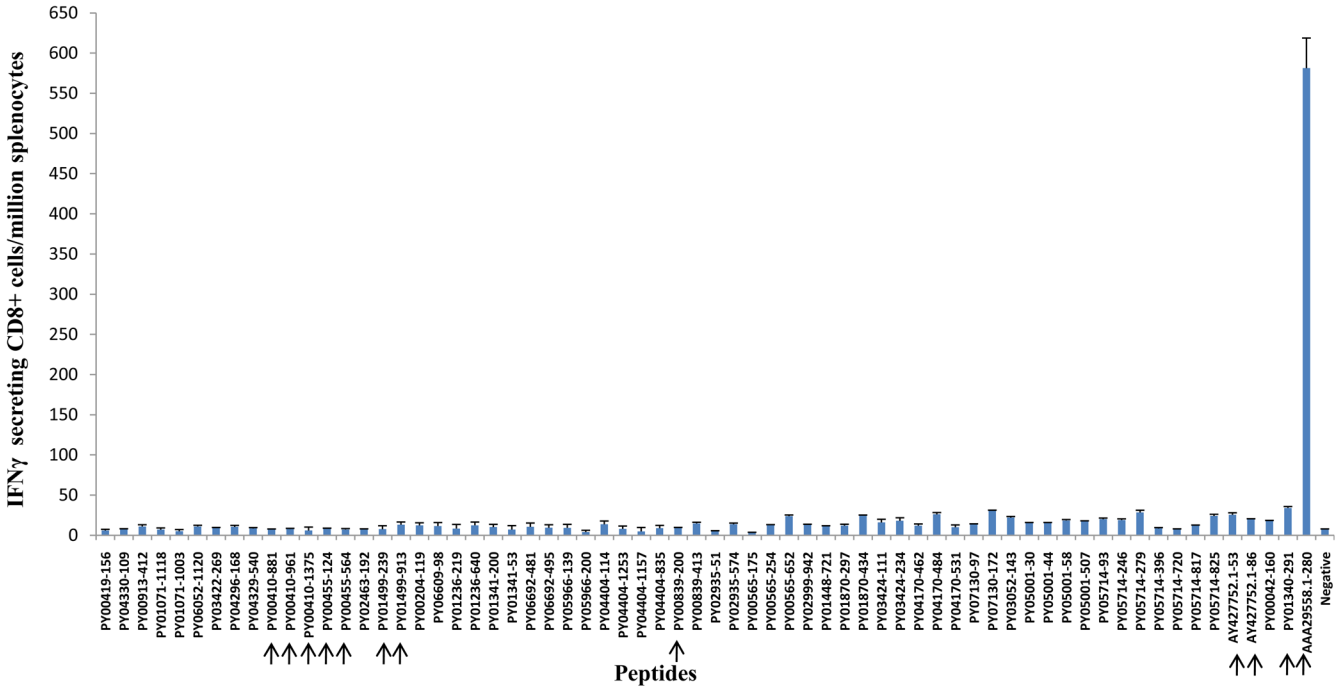

Fig 1.

T- Cell Responses to non-CSP antigens in BALB/c mice induced by immunization with irradiated sporozoites. Mice were immunized 3 times with 10^5 IrSp with interval of two weeks. The frequency of epitope-specific $CD8^+$ T cells in spleens was assessed 10 days after the last immunization by IFN-γ ELISPOT using peptides representing $CD8^+$ T cell epitope. Results are expressed as the mean±SD of epitope–specific T cells. A total of 34 non-CSP candidates (65 $CD8^+$ epitopes) were studied. The arrows point to candidates used to generate recombinant adenoviruses.

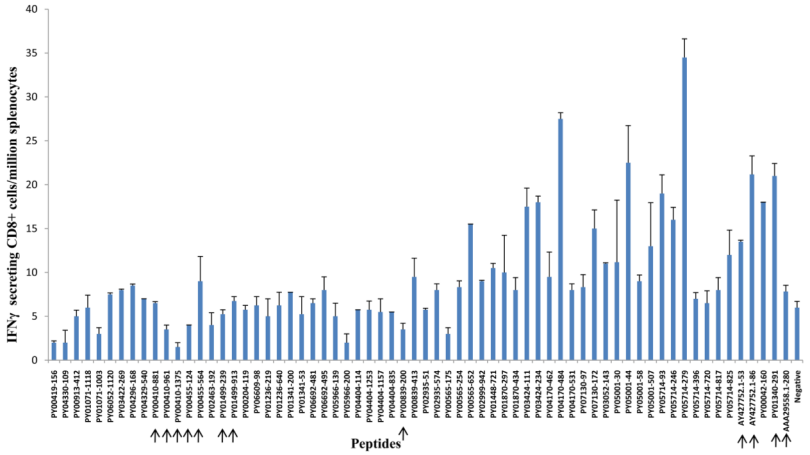

Fig 2.

T- Cell Responses to non-CSP antigens in [CSP-Tg/JhT(−/−)] BALB/c mice. The experiments were done precisely as described in the legend of fig. 1.

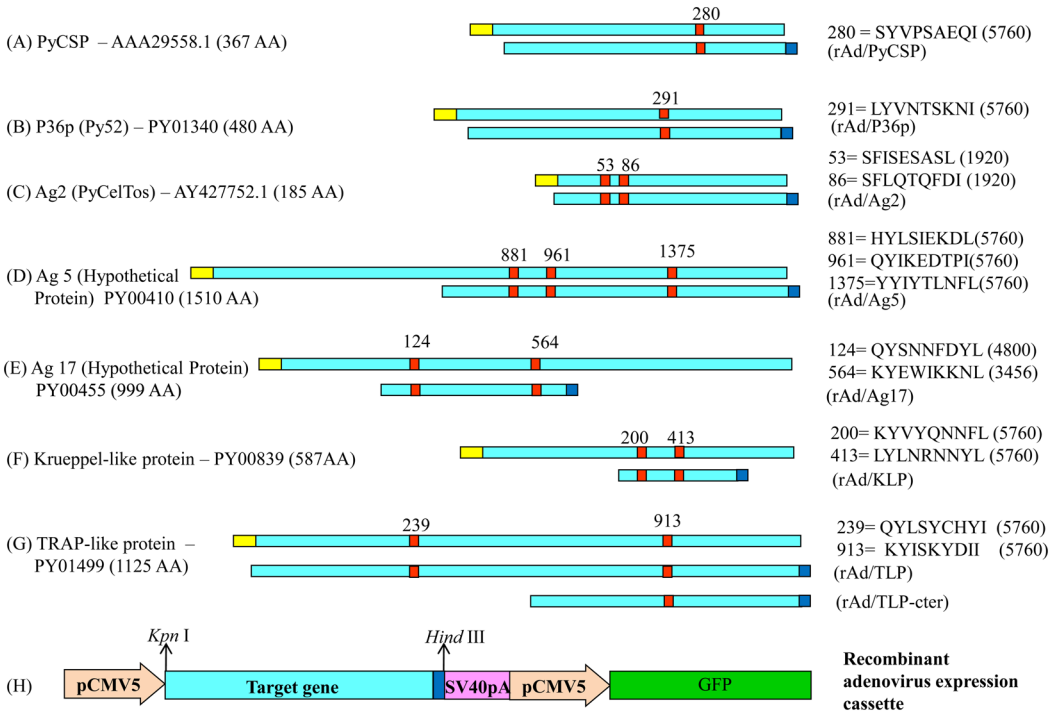

Fig 3.

Construction of recombinant adenoviruses. (A–G) Schematic representation of the candidates used to generate recombinant adenoviruses. (H) pShuttle-CMV5-antigen-GFP plasmid. Symbols: yellow box, signal sequence; Aqua box, target gene; red box, peptide; blue box, c-Myc tag.

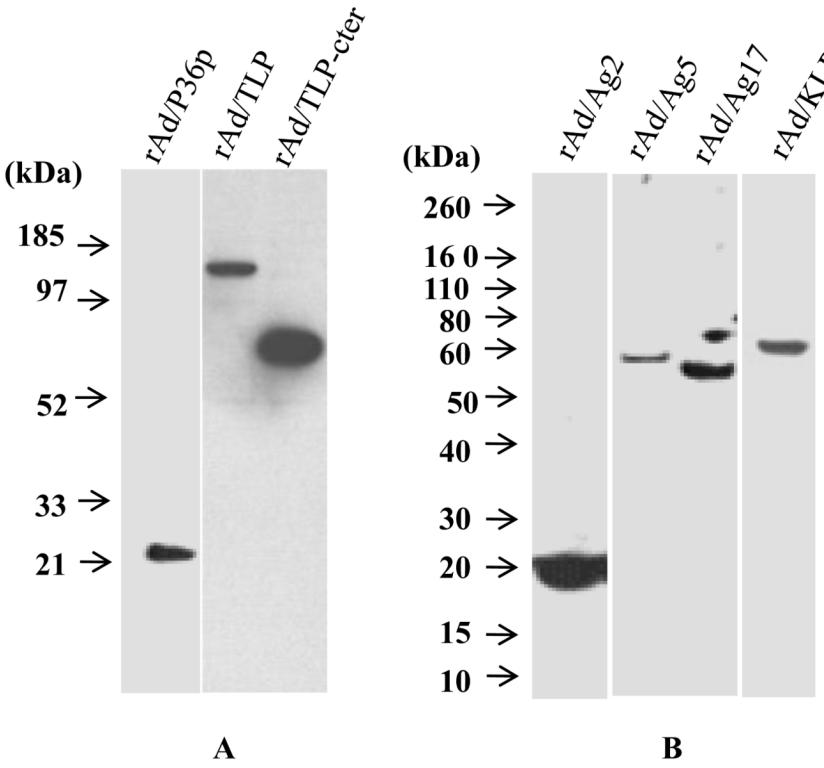

Fig 4.

Western blot analysis of the expression of recombinant adenovirus c-Myc tagged antigen in transfected AD-293 cells. Total cell lysates were prepared and the presence of recombinant protein was detected using the c-Myc monoclonal antibody.

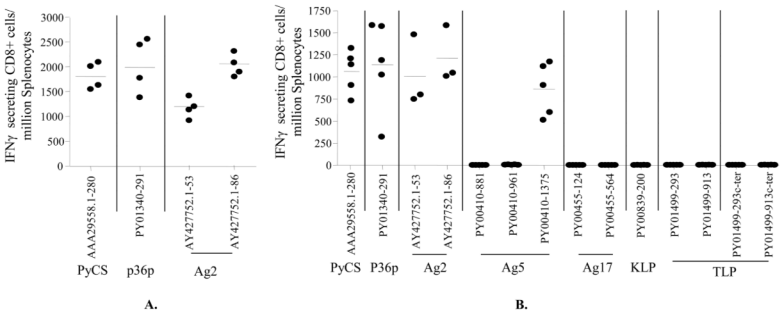

Fig 5.

CD8$^+$ T- cell responses induced by recombinant adenoviruses expressing non-CSP antigens. Groups of naive BALB/c mice (4 mice per group in A and 5 mice per group in B except for Ag2, which consists of 3 mice per group) were immunized i.m. with 10^{10} virus particle of the recombinant adenovirus expressing the *Plasmodial* sequence. Two weeks later, splenocytes were isolated and the number of IFN-γ secreting, epitope specific CD8$^+$ T- cells was determined by an ELISPOT assay. The horizontal bars represent median values. In all experiments, the results represent one of two experiments that showed similar results.

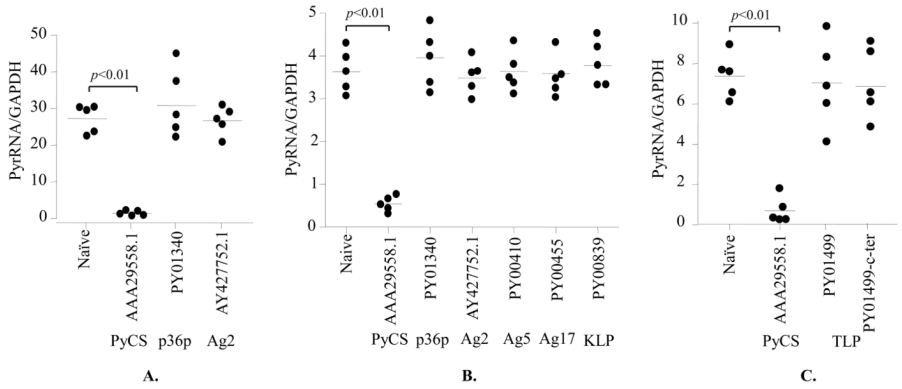

Fig 6.

The parasite burdens in the livers of immunized and naive mice, as determined by real-time PCR. Groups of BALB/c mice (5 mice per group) were immunized i.m. with 10^{10} virus particle of the recombinant adenovirus expressing the *Plasmodial* sequence and challenged two weeks after the immunizations by i.v. injection of 10^4 *P. yoelii* live sporozoites. The protection against a sporozoite challenge is shown as the inhibition of liver infection. The horizontal bars represent median values. In all experiments, the results represent one of two similar experiments.

Table 1

All the antigens and peptides used in this study.

P. yoelii gene ID	Annotation	Peptide position	Peptide sequence
*PY00419	Hypothetical Protein	156	IYLLAGGFI
*PY04330	PfATPase3	109	FYGNKKNRI
*PY00913	CAAT-box DNA binding protein subunit B	412	KYNNNLNEI
*PY01071	Multidomain scavenger receptor protein PbSR precursor	1118	NYYTPDTSI
		1003	KYILTEHEI
PY06052	Erythrocyte membrane protein PFEMP3	1120	KYIPTNKEI
PY03422	Hypothetical Protein	269	FYLFENTRI
*PY04296	Protein disulfide isomerase precursor	168	KYYNMINDL
PY04329	PfSUB-1	540	TYKQVVSIL
PY00410	Ag5	881	HYLSIEKDL
		961	QYIKEDTPI
		1375	YYIYTLNFL
PY00455	Ag17	124	QYSNNFDYL
		564	KYEWIKKNL
*PY02463	Hypothetical Protein	192	FYMPSYTEI
PY01499	TRAP-like protein	239	QYLSYCHYI
		913	KYISKYDII
*PY00204	Hypothetical Protein	119	DYQEQYPEI
*PY06609	Hypothetical Protein	98	NYIDKKNII
*PY01236	Hypothetical Protein	219	SYLYLLNKL
		640	AYLITVISI
PY01341	Pbs36	200	AYPGDVVGI
		53	DYNKTIKLL
*PY06692	Eukaryotic aspartyl protease, putative	481	KYVFGKLVI
		495	EYMIVNDDL
*PY05966	Hypothetical Protein	139	LYKKHKDKI
		200	IYDKFGEKI
*PY04404	Ser/Thr protein phosphatase, putative	114	IYTHIFNEL
		1253	TYDEMKYSL
		1157	EYEENEENI
		835	GYFSAHDNL
PY00839	Krueppel-like protein	200	KYVYQNNFL
		413	LYLNRNNYL
*PY02935	Hypothetical Protein	511	KYLKLHEYI
		574	RYLKERNEI
*PY00565	ClpB protein	175	AYVEAEMLL

***P. yoelii* gene ID**	**Annotation**	**Peptide position**	**Peptide sequence**
		254	EYISIEHLL
		652	MYVDNIRAI
PY02999	Guanylyl cyclase enzyme-related	942	IYLFTITFI
PY01448	Hypothetical Protein	721	SYMNLIPHI
PY01870	ATP-dependent RNA Helicase	297	IYLSDAHEI
		434	RYGHLGLAI
PY03424	Falstatin	111	NFSDNNEEI
		234	GYIWALLGV
PY04170	Lecithin:cholesterol acyltransferase, putative	462	GYIDGKDIL
		484	KYEVLKSHI
		531	KYINIVMHI
PY07130	Hypothetical Protein	97	IYEKDKTPL
		172	IYKESISLL
PY03052	Sporozoite surface protein 2 precursor	143	MYRPDAIQL
*PY05001	Heat shock protein	30	IYIWCGFGL
		44	IYMCKYVFL
		58	TYFSFSYFL
		507	TYQDNQPAV
PY05714	Hypothetical Protein	93	KYFFIFRSI
		246	IYDYIITFI
		279	KYIFKKEQL
		396	NYRKNNNDI
		720	SYNKLKKEI
		817	IYAPYISLI
		825	IYISYIVAI
AY427752.1	Ag2 or CelTOS	53	SFISESASL
		86	SFLQTQFDI
PY00042	Unknown protein	160	FYIFVAIFL
*PY01340	Py52 (P36p)	291	LYVNTSKNI
*AAA29558.1	Circumsporozoite protein	280	SYVPSAEQI

*PEXEL/VTS motif present.