T cells are critical effectors of host immunity that target intracellular pathogens, such as the causative agents of HIV, tuberculosis, and malaria. The development of vaccines that induce effective cell-mediated immunity against such pathogens has proven challenging; for tuberculosis and malaria, many of the antigens targeted by protective T cells are not known. Here, we report a novel approach for screening large numbers of antigens as potential targets of T cells. Malaria provides an excellent model to test this antigen discovery platform because T cells are critical mediators of protection following immunization with live sporozoite vaccines and the specific antigen targets are unknown. We generated an adenovirus array by cloning 312 highly expressed pre-erythrocytic Plasmodium yoelii antigens into adenovirus vectors using high-throughput methodologies. The array was screened to identify antigen-specific CD8+ T cells induced by a live sporozoite vaccine regimen known to provide high levels of sterile protection mediated by CD8+ T cells. We identified 69 antigens that were targeted by CD8+ T cells induced by this vaccine regimen. The antigen that recalled the highest frequency of CD8+ T cells, PY02605, induced protective responses in mice, demonstrating proof of principle for this approach in identifying antigens for vaccine development.

INTRODUCTION

Almost all licensed vaccines are thought to mediate protection through antibody production; therefore, antigen discovery research and development has focused largely on the identification of antigens that induce protective antibodies. The availability of serum, the ease of working with antibodies, and, more recently, advances in microarray technology have facilitated these efforts. However, vaccine development for some of the most devastating infectious diseases, such as malaria, tuberculosis (TB), and HIV, has met with limited success, partially because these organisms have intracellular life cycle stages that are not targeted by antibodies, and they have developed sophisticated mechanisms to avoid clearance by host immune responses. Since T cells have been implicated in protection from these diseases, considerable efforts have been directed at developing vaccines that induce protective T cell responses. However, for infectious agents with large genomes that express many potential T cell antigens such as parasites and bacteria, many of the specific antigens that are targeted by protective CD8+ T cells are not known. Identification of the target antigens of protective T cell responses would greatly facilitate vaccine development.

Malaria killed approximately 429,000 people in 2015, most of them children, in sub-Saharan Africa. Despite decades of effort, a highly effective malaria vaccine is not available. Immunization with attenuated Plasmodium sporozoites can provide high levels of protection in mice, non-human primates, and humans. Protection is mediated by CD8+ T cells, which target a set of mostly unknown pre-erythrocytic stage antigens. Activated CD8+ T cells can kill infected hepatocytes, thereby preventing blood-stage infection, which is responsible for the clinical symptoms of the disease. However, substantial delivery issues are a considerable barrier to licensure of live sporozoite-based vaccines, and broad protection against circulating strains has not been demonstrated. An alternative approach is to identify the targets of these protective CD8+ T cell responses and formulate them into a multivalent subunit vaccine designed to induce sustained T cell immunity.

The two Plasmodium sporozoite vaccines that are associated with high levels of protection in humans are radiation-attenuated sporozoites (RSA) and live sporozoites with concomitant chloroquine treatment to kill newly emerging blood-stage parasites (SPZ+CQ).
RESULTS

Generation of an Array of Adenovectors That Express a Panel of Highly Expressed \textit{P. yoelii} Pre-erythrocytic Antigens

Pre-erythrocytic antigens, which are expressed in the sporozoite and liver stages of the \textit{Plasmodium} spp. life cycle, are particularly promising targets for malaria vaccine development, with great potential to prevent infection and transmission. The pre-erythrocytic stages of the parasitic life cycle are vulnerable to vaccine intervention because their antigens are expressed at a time when low numbers of sporozoites are transmitted by the mosquito to the human host and only a few hepatocytes become infected. We selected \textit{P. yoelii} pre-erythrocytic genes with identifiable \textit{P. falciparum} orthologs for generation of an adenovector array (Ad-array) based on their level of expression in microarray and protein mass spectrometry datasets. Gene selection was made without regard to protein function or subcellular localization. In total, 312 \textit{P. yoelii} genes were amplified from genomic DNA and cloned into E1/E3-deleted adenovirus type 5 (Ad5) vector genomes (Figure 2).

To facilitate high-throughput production of the Ad-array, we compared the efficiency of adenovector generation in multi-well plates of different sizes. The adenovector plasmid had to convert into an adenovirus vector in sufficient quantities and quality to function in the antigen screening assay. Initially, we tested conversions of two pAdFlex plasmids that expressed the \textit{P. yoelii} Hep17 antigen (AdgHep17) and the cytomegalovirus p65 antigen (AdgCMVp65). These large plasmids were transfected into 293 cells in 60-mm, 6-well, 12-well, 24-well, 48-well, and 96-well plates, and the cells were passaged to increase the adenovector titer. We observed efficient adenovector conversion in all of the wells as indicated by full cytopathic effect (CPE) at passage 2. Vector identity was verified by PCR using oligonucleotides that spanned the expression cassette (Figure 2B). Vector titers from each of the CPE wells (Table S1) demonstrated equivalent yields per infected cell. These results indicated that multiple adenovectors can be generated from pAdFlex adenovector plasmids in a parallel process in multi-well plates and that 96-well plates were suitable for the generation of the Ad-array.

The Screening System

The overall design of our antigen screening system is shown in Figure 3A. To test the elements of the screen, we first determined the MOI necessary to efficiently infect A20 cells. Cells were infected with various doses of AdGFP, an Ad5 vector expressing GFP, and the percentage of infected cells was measured 48 hr post-infection (Figure 3B). MOIs of 10, 100, or 1,000 focal forming units (ffu)/cell were required to infect approximately 2%, 10%, or 50% of the cells, respectively. To determine if adenovirus vectors could efficiently present antigen following infection of antigen presenting cells (APCs), we immunized BALB/c mice with a \textit{Py}CSP-expressing plasmid, stimulated splenocytes from these mice with APCs infected with an Ad5 vector expressing \textit{Py}CSP (Ad\textit{Py}CSP), and measured activated T cells by the enzyme linked immunosorbent spot (ELISpot) assay. We observed strong recall responses to the Ad\textit{Py}CSP-infected...
cells, even at a low MOI, comparable to those generated by pulsing APCs with a peptide containing the PyCSP immunodominant epitope (Figure 3C). Very low responses were seen in the negative controls. These results demonstrate that A20 cells (which express both major histocompatibility complex [MHC] class I and class II alleles) infected with AdPyCSP are able to present antigen to immune T cells. This process was highly efficient, as strong T cell responses were observed even at an MOI of 10, a multiplicity that resulted in transduction of approximately 2% of the target cells. Increasing the MOI resulted in substantially increased A20 cell transduction.

(Figure 3B) but only marginally increased functional activity in the ELISpot assay (Figure 3C). Thus, low-level target cell transduction is sufficient for optimal activity to detect T cell responses in the ELISpot assay. To determine whether lower-frequency T cell responses from mice immunized with sporozoite vaccines could be identified using our approach, we assayed CD8+ T cell responses specific for PyCSP from mice immunized with protective regimens of RAS and SPZ+CQ. We chose PyCSP as the test antigen because it is the most well-characterized target of T cell responses from mice immunized with these regimens.20,27 First, we assayed splenocytes from mice immunized with a highly protective three-dose regimen of RAS for the presence of PyCSP-specific T cells. We were able to recall PyCSP-specific T cells in splenocytes from these mice using AdPyCSP-infected A20 cells in both ELISpot (Figure 4A) and intracellular cytokine staining (ICS) assays (Figure 4B). Low background responses were observed in the negative controls.

It was important to assess the degree of purity of the adenovector preparation necessary for the screen because if unpurified adenovectors were suitable, this would greatly simplify generation of the Ad-array. Accordingly, we compared highly purified AdPyCSP (purified over three successive CsCl gradients) with cell lysates containing unpurified recombinant adenovector. PyCSP-specific CD8+ T cell
responses were detected with both purified and unpurified vectors using ELISpot (Figure 4A) and ICS (Figure 4B) assays. Our results indicated that vector purification is not required to identify antigens that recall CD8+ T cell responses in mice immunized with RAS.

Ad-array vectors contain 25bp-long attB sequences flanking the transgene (Figure 2B), which are remnants of the recombinase cloning reaction. We compared Ad-array vectors directly with our vaccine adenovectors, which do not carry the flanking attB sequences. Our results indicate that the attB sequences did not inhibit the capacity to recall T cell responses in mice (Figure 4C), indicating that Ad-array vectors are suitable for screening.

Mice immunized with a two-dose regimen of 200, 2,000, and 20,000 SPZ+CQ were completely protected from P. yoelii sporozoite challenge (Figure 4D). Figure 4E shows that PyCSP-specific T cells were induced by immunizing mice with a highly protective 2,000 SPZ+CQ regimen. Splenocytes from immunized mice had a high background of activated CD8+ T cells. When incubated with A20 cells infected with the negative control vectors AdNull and AdGFP, 0.8%–0.9% of the CD8+ T cells were activated. A20 cells infected with AdPyCSP recalled PyCSP-specific T cell responses that were more frequent than the negative controls. Statistically significant results were observed with MOIs of 10 and 100 ffu/cell. These data suggested that it would be possible to utilize our Ad-array technology to identify new antigen targets of protective T cell responses following immunization of mice with SPZ+CQ.

Identification of the Antigen Targets of CD8+ T Cells Induced following Vaccination with Protective Regimens of SPZ+CQ
To generate protective T cells for the identification of antigens, we used the 2,000 SPZ+CQ regimen and harvested splenocytes 2 weeks after the last sporozoite immunization. The full array was screened simultaneously, in triplicate, against these freshly isolated splenocytes by ICS to identify pre-erythrocytic stage antigens able to recall IFNγ-expressing CD8+ T cells. A20 cells infected with 100 ffu/cell AdgPyCSP were included as a positive control. Negative controls included uninfected A20 cells and A20 cells infected with 100 ffu/cell of AdNull and AdGFP vectors. The mean of the negative controls was 1% IFNγ-expressing CD8+ T cells (Figure 5). We defined antigens with responses greater than 2 SD of the mean of the negative controls (>1.2% CD8+ IFNγ+ cells) as positive hits in our screen. By this definition, 69 of the antigens in the array were positive and were targeted by CD8+ T cells induced in mice immunized with SPZ+CQ (Figure 5; Table 1). Thirteen of these antigens recalled
higher-frequency CD8+ T cell responses than PyCSP. The antigen that recalled the highest response was PY02605. We also analyzed CD4+ T cell responses and tumor necrosis factor (TNF)-α and interleukin (IL)-2 cytokines by ICS. CD4+ T cell responses were not observed in this system. CD8+ TNF-α-expressing T cells were observed and tended to mirror the CD8+ IFNγ responses. Very low levels of IL-2-expressing cells were observed (data not shown).

PY02605 Is a Protective Antigen

Since the SPZ+CQ regimen induces protective T cell responses directed against antigens expressed in the pre-erythrocytic stages of the parasite life cycle,26,27,32 we hypothesized that a subset of antigens identified in the SPZ+CQ screen would induce protective immune responses when delivered using a potent vaccine regimen designed to optimize CD8+ T cell responses. Accordingly, we compared antigens that ranked at the two extremes of our screening strategy, PY02605 and PY05837, for their capacity to protect mice against a sporozoite challenge. PY02605 recalled the highest frequency of CD8+ T cells and PY05837 did not recall antigen-specific T cell responses in our screen. We tested the protective capacity of these antigens using a DNA prime-Ad5 boost regimen in BALB/c mice.33 Mice were immunized with 100 μg of DNA vector expressing the specific antigen and then boosted 6 weeks later with 1 × 10^10 particle units (PUs) of an Ad5 vector expressing the same antigen. Two weeks after the Ad5 boost, mice were challenged with P. yoelii sporozoites and protection was monitored by microscopic examination of Giemsa-stained blood smears. 43% of the PY02605 immunized mice were sterilely protected (6 of 14 mice), indicating that PY02605 can provide protection in mice (Table 2). Only 1 mouse from the group of 14 immunized with the PY05837 antigen was negative for blood-stage parasitemia following challenge, suggesting that this antigen is not protective. The positive control group, which was immunized with PyCSP-expressing DNA and Ad5 vectors, protected 100% of the mice. The negative control group, immunized with DNA and Ad5 vectors that did not express any transgene (Null) did not protect any mice (Table 2). These data indicate that our antigen discovery system is capable of identifying protective antigens.
PF3D7_0932900 Is Immunogenic in Mice

To begin evaluation of a selected pre-erythrocytic antigen as a vaccine candidate, we cloned the *P. falciparum* ortholog of PY02605 into a highly immunogenic and low seroprevalent gorilla adenovector (GC46) \(^\text{34,35}\) and tested immunogenicity in mice. PF3D7_0932900 was highly immunogenic, inducing robust antigen-specific CD8+ T cell responses, PY02065, is a 173 amino acid conserved protein with unknown function that is upregulated throughout the liver stage of the *P. yoelii* life cycle. Terminal protection by the SPZ+CQ regimen relative to RAS was observed using this regimen, identifying several pre-erythrocytic antigens that are targeted by T cell responses induced following immunization with *P. yoelii* and *P. berghei* RAS or SPZ+CQ; however, these antigens have not demonstrated protective efficacy when tested in challenge experiments. \(^\text{21–23,38–41}\)

Advantages of the Ad-array technology over other T cell antigen discovery efforts are that adenoviruses efficiently infect many cell types including APCs \(^\text{37}\) and that, following infection, full-length proteins are expressed and multiple undefined epitopes are presented, similar to what occurs during a natural infection. The Ad-array technology was as effective as the immunodominant PyCSP peptide in detecting PyCSP-specific CD8+ T cell responses. Moreover, this technology was highly sensitive, detecting low-level PyCSP-specific CD8+ T cell responses following immunization with RAS and SPZ+CQ. Other T cell antigen discovery efforts utilized in silico algorithms to predict epitopes with high MHC binding affinity and then generated libraries of peptides corresponding to the selected epitopes. \(^\text{21–23,38–41}\)

Although these methods are improving, they are expensive and limited in their capacity to predict protective T cell epitopes. \(^\text{42,43}\) In the mouse malaria models, epitope prediction algorithms have supported the identification of several pre-erythrocytic antigens that are targeted by T cell responses induced following immunization with *P. yoelii* and *P. berghei* RAS or SPZ+CQ; however, these antigens have not demonstrated protective efficacy when tested in challenge experiments. \(^\text{30–40}\)

We focused on identifying T cell responses specific for pre-erythrocytic antigens following immunization with a highly protective SPZ+CQ vaccine regimen because of the potential for increased diversity of antigen responses associated with this regimen relative to RAS. \(^\text{44,45}\) Using this regimen, we identified a set of 69 pre-erythrocytic stage antigens that were targeted by vaccine-induced CD8+ T cells. Twentieth of these antigens recalled more robust responses than PyCSP and were expressed in the liver stage of the *P. yoelii* life cycle (Table S2). \(^\text{39}\) The antigen that recalled the most frequent CD8+ T cell responses, PY02065, is a 173 amino acid conserved Plasmodium protein with unknown function that is upregulated throughout the liver stage of the *P. yoelii* life cycle. \(^\text{29}\) Since protection induced by the SPZ+CQ regimen is dependent on CD8+ T cells, we hypothesized that a subset of the 69 antigen-specific CD8+ T cells that we identified may contribute to the robust protection observed with this regimen. We tested this hypothesis by evaluating the protective efficacy induced by a DNA prime-Ad5 boost regimen expressing the antigen that recalled the most frequent CD8+ T cells, PY02065. This robust T cell vaccine regimen was partially protective, inducing sterile protection (absence of blood-stage infection) in 6 of 14 mice following *P. yoelii* sporozoite challenge. This finding strongly suggests that our antigen discovery system is effective at identifying the targets...
of protective T cell responses to malarial infection, although additional antigens that recalled CD8+ T cells in our screen will need to be tested to further confirm our hypothesis.

Our malaria vaccine development efforts focus on improving DNA-Ad5-CA, a first-generation vaccine candidate that induced an encouraging 27% sterile protection in human volunteers challenged with controlled human malaria infection (CHMI). DNA-Ad5-CA is a DNA prime-Ad5 boost regimen that expresses two *P. falciparum* antigens: PfCSP and apical membrane antigen 1 (PfAMA1). CD8+ T cell responses specific for PfAMA1 were associated with protection, and 2 of the 4 protected volunteers had the highest frequency of CD8+ T cell responses specific for PfCSP. Our goal is to increase vaccine efficacy to meet the preferred product characteristic target of 75% efficacy set forth by the World Health Organization (WHO). Since overall efficacy of DNA-Ad5-CA was

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<td>GV0137</td>
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nt, nucleotide.
individual exons were designed to have the same melting temperature (Tm) at 66°C. Primer Design, Gene Amplification, and Plasmid Cloning

MATERIALS AND METHODS

We included genes encoding proteins ≥130 amino acids in length because larger proteins have a greater probability of containing T cell epitopes. The DNA sequences of the selected genes used for amplification were obtained from the PlasmoDB database. All of the single exon genes were amplified directly from P. yoelii 17XNL genomic DNA using a pair of gel purified primers: the forward (fwd) primer contains the Kozak consensus CACC upstream of the starting codon ATG, the reverse (rev) primer retains the native stop codon at the end of the oligonucleotide, and both of the primers were designed to have the same melting temperature (Tm) at 66°C. PCR amplification was performed using high-fidelity platinum Taq polymerase (Invitrogen, Carlsbad, CA) or Hot Star polymerase (QIAGEN, Gaithersburg, MD). For multi-exon genes, we amplified individual exons first, and then assembled individual exons into an intact gene product using the cDNA synthesis PCR. All PCR-amplified gene products were cloned into a shuttle vector, pCR8/GW/TOPO (Invitrogen, Carlsbad, CA). The correct orientation of the clones was confirmed by DNA sequencing using M13 fwd and M13 rev oligonucleotides. The pAdFlex plasmids pACEI(3511rfC)E3(10X) and pACE1(t.rfC.MCS)E3(10X) contain the full-length Ad5 genome with deletions in the E1 and E3 regions. Within the E1 region, we inserted attB1 and attB2 elements for lambda recombination and a CMV promoter and poly(A) sequences for antigen expression. The genes in the shuttle vector were moved into these destination gateway-converted aden plasmids with high efficiency using the Gateway LR reaction (Invitrogen, Carlsbad, CA), in 96-well plate format to generate an array of pAdFlex plasmids expressing pre-erythrocytic genes from P. yoelii (pAdPy1-360).

Ad-Array Viral Vector Construction

The Ad-array is an array of adenovirus vectors expressing P. yoelii antigen genes constructed from an array of pAdFlex plasmids. Briefly, the recombinant Ad5 genomes containing an antigen gene expression cassette in the E1 region were liberated from pAdFlex plasmids by digestion with Pac I or I-Ceu restriction endonuclease, which cleaves adjacent to the inverted terminal repeats (ITRs) of the Ad5 genome. This vector DNA (60 ng) was transfected into monolayer 293 cells seeded at 70,000 cells per well in 96-well plates using 0.7 μL polyfect transfection reagent (QIAGEN, Gaithersburg, MD). Three days later, transfected cells were frozen and thawed three times to lyse the cells, releasing infectious recombinant virus, and 18 μL of these lysates was used to infect fresh 293 cells in another 96-well plate. Cell lysates were serially passaged in this manner every 3 days until CPE was observed. The identity of the adenovector was determined using primers Ad5s278 (CGCGGGAAAACTGAATAAGA) and Ad5a3598 (GCTGCTGCAAACAGATACA) as indicated in Figure 2B. Vector yields were determined by using the ffu assay on a subset of approximately 10% of the array to ensure that sufficient quantities of vector were being produced.

Production of Adenovirus Vectors for Protection Studies in Mice

To generate sufficient vector for mouse protection experiments, residual CPE lysates were expanded in four 10-cm plates and the cell lysates were harvested 3 days later. The expanded cell lysates were analyzed to determine infectious vector particle concentration, were used to seed a production run in suspension 293-ORF6 cells in shaker flasks using serum-free media. Three days after infection, the recombinant vectors were released from infected cells by three cycles of freeze-thawing, treated with benzonase (EMD Millipore, Billerica, MA), purified by banding on two successive CsCl gradients, dialyzed into final formulation buffer and stored at −80°C. Physical PUs were determined by absorbance at 260 nm following disruption of the capsid with SDS.

Mice, Parasites, and Cells

The study protocols were reviewed and approved by the Walter Reed Army Institute of Research/Naval Medical Research Center (NMRC) and GenVec Institutional Animal Care and Use Committees in compliance with all applicable federal regulations governing the protection of animals in research. Female (6- to 8-week-old) BALB/c mice were purchased from Harlan Laboratories (http://www.envigo.com) or Charles River Laboratories (Wilmington, MA). CD1 outbred mice (5–6 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). P. yoelii 17XNL non-lethal strain, clone 1.1) parasites were maintained by alternating passage in Anopheles stephensi mosquitoes and female CD1 mice. HEK293 cells, a

Table 2. Immunization with PY02605 Partially Protects BALB/c Mice from P. yoelii Sporozoite Challenge

<table>
<thead>
<tr>
<th>Vaccine Antigen</th>
<th>Protected/Total</th>
<th>Protection (%)</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null</td>
<td>0/14</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>PyCSP</td>
<td>14/14</td>
<td>100</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PY02605</td>
<td>6/14</td>
<td>43</td>
<td>0.016</td>
</tr>
<tr>
<td>PY05837</td>
<td>1/14</td>
<td>7</td>
<td>1</td>
</tr>
</tbody>
</table>

*Fisher’s exact test (two-sided) versus null. Alpha < 0.05.
human embryonic kidney cell line transformed by sheared adenovirus type 5 DNA, were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in DMEM supplemented with 10% calf serum. The A20.2J (ATCC clone HB-98) B cell line derived from BALB/c mice, which expresses both class I and class II MHC genes, was purchased from ATCC and maintained in RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS) and 1% glutamine.

Immunization Regimens

RAS were generated by exposing salivary gland \( P. yoelii \) sporozoites to 10,000 rads. To obtain splenocytes for testing recall responses to \( PyCSP \), female BALB/c mice were immunized, via tail vein injection, with three doses of RAS (10,000, 5,000, and 5,000) at 3-week intervals.

For SPZ+CQ immunizations, female BALB/c mice were immunized with two administrations (1 month apart) of live infectious \( P. yoelii \) sporozoites. Various doses of sporozoites were tested (group 1 = 20,000, group 2 = 2,000, group 3 = 200, group 4 = 0). Infected mice received a 0.1 mL intraperitoneal injection of a solution of chloroquine hydrochloride (Sigma-Aldrich, St. Louis, MO) 8 mg/mL diluted in PBS, to kill newly emerging blood-stage parasites, starting on the same day as sporozoite immunizations and continuing for 10 consecutive days following each sporozoite immunization. For both SPZ+CQ and RAS immunizations, most of these mice were euthanized 2 weeks after the last dose to obtain splenocytes for T cell analysis. However, a subset of the mice was infected with \( P. yoelii \) sporozoites to insure that 100% sterile protection had been achieved, as described below.

For plasmid DNA immunizations, BALB/c mice were immunized with 100 \( \mu \)g DNA vector, \( pPyCSP \), in a 0.1 mL volume by intramuscular injection, as described previously. Two weeks later, mice were euthanized to obtain splenocytes for analysis. For DNA-Ad5 immunizations, BALB/c mice were immunized with 100 \( \mu \)g of DNA vector, \( pcdNA3.2-Dest \) (Invitrogen, Carlsbad, CA) in a 0.1 mL volume by intramuscular immunization. Six weeks later, these mice were boosted with 1 \( \times 10^{10} \) PU of Ad5 vector in a 0.1 mL volume. For both DNA and Ad5 administrations, we performed bilateral injections into the tibialis anterior muscles with a 0.3-mL syringe and a 29G1/2 needle (Becton Dickinson Co., Franklin Lakes, NJ).

For GC46.PF3D7._0932900 immunizations, BALB/c mice were immunized with a single dose of 1 \( \times 10^{9} \) PU of GC46.PF3D7._0932900 or a control adenovector that does not express a transgene, GC46.Null. Three weeks post-immunization, mice were euthanized and GC46.PF3D7._0932900-specific CD8\(^+\) (A) and CD4\(^+\) (B) T cell responses were measured by ICS and flow cytometry following 4-hr stimulation using pooled overlapping 15-mer peptides.

Protection Studies

Protection studies were performed as previously described. Mice were challenged intravenously in the tail vein with 200 \( P. yoelii \) sporozoites using a 1-mL syringe and 26G1/2 needle. Sporozoites were hand dissected from infected mosquito salivary glands and diluted for challenge in M199 medium containing 5% normal mouse serum (Gemini Bio-Products, West Sacramento, CA). The development of parasitemia was monitored over the next 2 weeks by microscopic examination of Giemsa-stained blood smears. Mice were considered protected if no parasites were observed in any sample at day 6, day 9, or day 14 post-challenge.

Infection of A20 Cells

A20.J2 cells (ATCC, Manassas, VA), also referred to as A20 cells, are a B cell line derived from BALB/c mice that expresses both class I and class II major histocompatibility complex genes. A20 cells were grown in 15 mL fresh RPMI-1640 media plus 20% FBS and 1% L-glutamine in 24-well plates at a density of 5.0 \( \times 10^5 \) cells/mL, they were used to seed 12-well plates at a density of 1.2–1.8 \( \times 10^6 \) cells/mL, they were used to seed 12-well plates at a density of 5.0 \( \times 10^5 \) cells/well. The following day, the cells were infected with AdGFP, an adenovirus vector that expresses GFP, for 2 hr in a volume of 200 \( \mu \)L. After infection, cells were washed with PBS, overlaid with 1 mL fresh media, and incubated at 37°C in a 5% CO\(_2\) incubator. When the cells reached a density of 1.2–1.8 \( \times 10^6 \) cells/mL, they were used to seed 12-well plates at a density of 5.0 \( \times 10^5 \) cells/well. The following day, the cells were infected with AdGFP, an adenovirus vector that expresses GFP, for 2 hr in a volume of 200 \( \mu \)L. After infection, cells were washed with PBS, overlaid with 1 mL fresh media, and incubated at 37°C and 5% CO\(_2\) for 48 hr. The percentage of the GFP\(^+\) cells was analyzed by flow cytometry. For the array screening, 6 \( \times 10^5 \) A20.J2 cells were infected with 150 \( \mu \)L CPE lysate from each of the Ad-array vectors in a volume of 350 \( \mu \)L in 24-well plates for 2 hr. After infection, 0.25 mL fresh media were added to each well and the infected cells were incubated at 37°C and 5% CO\(_2\) for 24 hr before plating in the stimulations described below.

ICS and Flow Cytometry Analysis

Stimulation by Ad5-Infected A20 Cells

Splenocytes harvested from SPZ+CQ or RAS-immunized animals were stimulated by co-culture with infected/irradiated A20 cells in
96-well plates. Spleens were gently crushed using the flat end of a 3-cc or 10-cc syringe plunger, and the cell suspension was passed through a 70-μm filter. The splenocytes were washed with ice-cold 2% FBS/10 mM HEPES/1x Hank’s balanced salt solution (HBSS), suspended in RPMI, counted, diluted to 1 × 10⁵ cells/mL in RPMI medium, and plated at 1 × 10⁶ cells/well in 96-well plates. At 24 hr after infection, A20 cells were irradiated in a Pantak X-Rad 320 irradiator at 16,660 rads. After irradiation, the viable cell concentration was adjusted to 1.5 × 10⁶ cells per mL and 1.5 × 10⁶ infected cells were transferred to each well of U-bottom 96-well plates preloaded with 1 × 10⁶ splenocytes from vaccinated or naive mice, in triplicate, and incubated for 5 hr at 37°C. BD Golgi Plug (BD Bioscience, San Jose, CA) was added 1 hr into the incubation to block cytokine release. Cells were centrifuged at 1,200 rpm for 5 min, the supernatant was flicked, and the cell pellets were resuspended by gentle vortexing. Live and dead cells were first stained with LIVE/DEAD Fixable Aqua stain kit (BD Biosciences, San Diego, CA), then the cells were blocked with FC Block (BD Biosciences, San Diego, CA). After blocking, cell surface markers were stained with the following antibodies (fluorochromes): CD4-eFluor-450 (clone RM4-5; eBioscience, San Diego, CA) and CD8a-PerCP-Cy5.5 (clone 53-6-7; BD Biosciences, San Diego, CA). Following fixation/permeabilization steps, the samples were stained intracellulary with the following antibodies (fluoro-chromes): IFN-γ-PE (clone XMG1.2), TNF-α APC (clone MP6-XT22), and IL-2-FITC (clone JES6-5H4; BD Biosciences, San Diego, CA). The frequency of CD4+ and CD8+ T cells, as well as antigen-specific IFNγ, TNF-α, and IL-2 intracellular cytokine-positive T cells was determined in an 8-color upgraded FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) with a 96-well Automated Micro-sampling System (AMS) (Cytex, Fremont, CA). Data were analyzed using FlowJo software (Treestar, Inc., Ashland, OR).

ICS Flow Cytometry Analysis

Stimulation by PF3D7_0932900 Peptide Pools

Splenocytes from GC46.PF3D7_0932900 immunized mice were harvested and plated at 2 × 10⁶ cells per well in a 96-well v-bottom plate. Cells were stimulated for 4 hr in the presence of 20 μg/mL brefeldin A (Sigma-Aldrich, St. Louis, MO) with either 15-mer peptides for the PF3D7_0932900 antigen at 2 μg/mL, overlapping by 10 amino acids (Mimotopes, Victoria, Australia), or 1% DMSO as a negative control. Subsequently, cells were stained with the LIVE/DEAD Fixable Blue Dead Cell Stain Kit, for UV excitation (Invitrogen, Grand Island, NY), surface stained with CD14 phycoerythrin (PE) (clone Sa14-2; Life Technologies, Grand Island, NY), CD19 Brilliant Violet 650 (clone 6D5; Biolegend, San Diego, CA), CD3 Alexa 700 (clone 17A2; Biolegend, San Diego, CA), CCR7 PerCP-Cy5.5 (clone 4B12; eBioscience, San Diego, CA), CD44 Pacific Blue (clone IM7; Biolegend, San Diego, CA), and CD62L Brilliant Violet 786 (clone MEL-14; BD Biosciences, San Diego, CA) and permeabilized using Cytofix/Cytoperm reagent (BD Biosciences, San Diego, CA). Cells were then intracellularly stained with CD4 Brilliant Violet 605 (clone RM4-5; Biolegend, San Diego, CA), together with CD8 Horizon V500 (clone 53-6-7), TNF Cy7PE (clone MP6-XT22), IFNγ allophycocyanin (clone XMG1.2), and IL-2 FITC (clone JES6-5H4; BD Biosciences, San Diego, CA). Samples were acquired using an LSR Fortessa (Becton Dickinson Immunocytometry Systems, San Jose, CA) and data were analyzed using FlowJo version 9.4.11 (TreeStar Inc., Ashland, OR). To identify antigen-specific responses, cells were gated on forward scatter (threshold), exclusion of aggregates, and subsequently to include single, viable cells, CD14+, CD3+, CD19+, CD3+, lymphocytes, and either CD4+ or CD8+ populations.

ELISpot Assay

RAS splenocytes were stimulated in vitro with Ad5-transduced or PyCSP peptide-pulsed APCs and the number of P. yoelii antigen-specific IFNγ-secreting spot-forming cells was evaluated after a 36-hr culture period. Splenocytes were tested at 400,000, 200,000, and 100,000 per well, while APCs were tested at 100,000 per well. Assays were performed in triplicate and the number of IFNγ-secreting cells, recognized as spot-forming cells (SFCs), was counted using an automated ELISpot Reader manufactured by AutoImmun Diagnostika (AID) GmbH (Strassberg, Germany). Data were presented as the number of IFNγ-secreting SFCs per million spleen cells.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and can be found with this article online at http://dx.doi.org/10.1016/j.omtm.2017.08.003.

AUTHOR CONTRIBUTIONS


CONFLICTS OF INTEREST

D.E.B. is employed at GenVec, Inc. J.T.B., P.C., B.A.M., G.E., and C.A.L. were employed at GenVec, Inc. at the time this work was performed.

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or employee of the U.S. Government as part of that person's official duties. P.C., G.E., B.A.M., C.R.K., D.E.B., and J.T.B. are/were employees of GenVec Inc., a for-profit corporation. This work was supported by the NIH (grants 1R43AI084269-01 and 1R43AI100467-01 to J.T.B.), the Department of Defense (grant A043-189-0667 to J.T.B.), and the NMRC Military Infectious Disease Research Program (work unit number 602236N.04427.B27.A0242).

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