Human Vaccines & Immunotherapeutics 8:11, 1564–1584; November 2012; © 2012 Landes Bioscience

Clinical trial in healthy malaria-naïve adults to evaluate the safety, tolerability, immunogenicity and efficacy of MuStDO5, a five-gene, sporozoite/hepatic stage *Plasmodium falciparum* DNA vaccine combined with escalating dose human GM-CSF DNA

Thomas L. Richie,[1,*] Yupin Charoenvit,[1] Ruobing Wang,[1,2] Judith E. Epstein,[1] Richard C. Hedstrom,[1] Sanjai Kumar,[1,2] Thomas C. Luke,[1] Daniel A. Freilich,[1] Joao C. Aguiar,[3] John B. Sacci Jr.,[1,2] Martha Sedegah,[1] Ronald A. Nosek Jr.,[4] Patricia De La Vega,[1,2] Mara P. Berzins,[1,2] Victoria F. Majam,[1,2] Esteban N. Abot,[1,2] Harini Ganeshan,[1,2] Nancy O. Richie,[1,2] Jo Glenna Banania,[1,2] Maria Fe B. Baraceros,[1,2] Tanya G. Geter,[1,2] Robin Mere,[5] Lolita Bebris,[1,2] Keith Limbach,[1,2] Bradley W. Hickey,[1] David E. Lanar,[6] Jennifer Ng,[7] Meng Shi,[6] Peter M. Hobart,[5] Jon A. Norman,[5] Lorraine A. Soisson,[8] Michael R. Hollingdale,[1,2] William O. Rogers,[1] Denise L. Doolan[1] and Stephen L. Hoffman[1,†]

[1]Naval Medical Research Center; Silver Spring, MD USA; [2]Henry M. Jackson Foundation for the Advancement of Military Medicine; Bethesda, MD USA; [3]Pan American Health Organization; Washington, DC USA; [4]Naval Medical Research Center; Bethesda, MD USA; [5]Vical; San Diego, CA USA; [6] Walter Reed Army Institute of Research; Silver Spring, MD USA; [7]C.W. Bill Young Department of Defense Marrow Donor Program; Rockville, MD USA;
[8]USAID; Washington, DC USA;

[†]Current affiliation: Sanaria Inc.; Rockville, MD USA

Keywords: malaria, *Plasmodium falciparum*, DNA vaccine, vaccine safety, clinical trials, GM-CSF, malaria challenge, controlled human malaria infection, malaria vaccine

When introduced in the 1990s, immunization with DNA plasmids was considered potentially revolutionary for vaccine development, particularly for vaccines intended to induce protective CD8 T cell responses against multiple antigens. We conducted, in 1997–1998, the first clinical trial in healthy humans of a DNA vaccine, a single plasmid encoding *Plasmodium falciparum* circumsporozoite protein (*Pf*CSP), as an initial step toward developing a multi-antigen malaria vaccine targeting the liver stages of the parasite. As the next step, we conducted in 2000–2001 a clinical trial of a five-plasmid mixture called MuStDO5 encoding pre-erythrocytic antigens *Pf*CSP, *Pf*SSP2/TRAP, *Pf*EXP1, *Pf*LSA1 and *Pf*LSA3. Thirty-two, malaria-naïve, adult volunteers were enrolled sequentially into four cohorts receiving a mixture of 500 μg of each plasmid plus escalating doses (0, 20, 100 or 500 μg) of a sixth plasmid encoding human granulocyte macrophage-colony stimulating factor (hGM-CSF). Three doses of each formulation were administered intramuscularly by needle-less jet injection at 0, 4 and 8 weeks, and each cohort had controlled human malaria infection administered by five mosquito bites 18 d later. The vaccine was safe and well-tolerated, inducing moderate antigen-specific, MHC-restricted T cell interferon-γ responses but no antibodies. Although no volunteers were protected, T cell responses were boosted post malaria challenge. This trial demonstrated the MuStDO5 DNA and hGM-CSF plasmids to be safe and modestly immunogenic for T cell responses. It also laid the foundation for priming with DNA plasmids and boosting with recombinant viruses, an approach known for nearly 15 y to enhance the immunogenicity and protective efficacy of DNA vaccines.

Introduction

Malaria is one of the most widespread and devastating infectious diseases affecting the world community, causing 216 million clinical cases and 0.665 to 1.24 million deaths in 2010.[1,2] A highly effective vaccine remains elusive, awaiting new insights into malaria biology, clearer definition of protective immune mechanisms and target antigens, and development of novel vaccine approaches or platforms. Progress to date is exemplified by the leading vaccine candidate, RTS,S, which reduced the incidence of first episodes of clinical *P. falciparum* malaria in African children by about 50% during the initial 12 mo of a

*Correspondence to: Thomas L. Richie; Email: Thomas.Richie@med.navy.mil
Submitted: 08/21/12; Accepted: 09/08/12
http://dx.doi.org/10.4161/hv.22129

Phase 3 efficacy trial.[3] While this landmark result is encouraging, significant improvements over current vaccine candidates are needed to induce the complete, sustained immunity required for campaigns to eliminate malaria and to prevent malaria in travelers, military personnel and multiple other populations in endemic countries.

RTS,S represents one approach to developing a malaria vaccine, based primarily on the induction of protective antibody responses. The vaccine contains recombinant circumsporozoite protein (CSP), the major surface antigen of the *P. falciparum* sporozoite; CSP is bound in a matrix containing hepatitis B surface antigen and adjuvant to enhance immunogenicity. This formulation induces very strong anti-sporozoite antibody responses that are thought to limit sporozoite motility and the capacity to invade hepatocytes, thereby preventing liver stage infection. While CD4+ T cells are also induced and likely serve a helper function in the induction of protective antibodies as well as in secretion of interferon-gamma (IFN-γ), RTS,S does not induce CD8+ T cells.[4]

An alternative approach to malaria vaccines aims to directly target infected hepatocytes via cell-mediated immunity (CMI). The most profound, sustained protective immunity in humans has been induced by immunization with whole sporozoites administered by mosquito bites.[5-9] In animal models, this immunity is dependent on CD8+ T cells, including when aseptic, purified, cryopreserved sporozoites are used to immunize.[10] Thus, for more than two decades there has been a significant effort to develop subunit vaccines that induce protective CD8+ T cell responses against antigens expressed in infected hepatocytes. Studies in animal models indicate that CD8+ cells can recognize CSP-derived peptides on the surface of the infected host cell in the context of MHC class I, leading to the release of toxic materials such as granzyme and perforin, which lyse the target cell, and/or to the secretion of IFNγ,[11,12] which induces the infected hepatocyte to produce NO leading to the death of the parasite. To induce CD8+ T cell immunity, vaccine developers have turned to platforms such as DNA plasmids or viral vectors that deliver the genes encoding the malaria antigens rather than the antigens themselves. After being taken up by host cells, the DNA is transcribed and translated, leading to intracellular expression of the malaria proteins. This activates the endogenous antigen presentation pathway, inducing cell-mediated immune responses including CD8+ T cells able to target developing intracellular liver stage parasites. An advantage of the DNA approach is the capacity to rapidly and efficiently produce plasmids expressing multiple proteins.

The first DNA malaria vaccine, based on *Pf*CSP was studied by our group in the mid-1990s in mice and induced sterile protection against the murine malaria *P. yoelii*.[13,14] Protection was dependent upon CD8+ T cells, as shown by the loss of protection following CD8+ T cell depletion in vivo. Cytotoxic T lymphocyte (CTL) assays showed genetically-restricted, CSP-specific killing of target cells in vitro.[14] We next showed in mice that this protection could be enhanced by priming with the *Py*CSP DNA plasmid and boosting with a recombinant poxvirus expressing *Py*CSP.[15] This was followed by a studying demonstrating that this DNA prime, recombinant virus approach also induced protection in non-human primates.[16]

More recently, poxvirus-vectored vaccines expressing CSP or other pre-erythrocytic stage malaria proteins have likewise shown promise in humans, and adenovirus-vectored vaccines in animals and in humans, in some instances generating IFNγ-secreting T cell responses that correlated with protection.[17-22] These other antigens include apical membrane antigen-1 (AMA1)[23] and thrombospondin-related adhesive protein/sporozoite surface protein-2 (TRAP/SSP2)[24,25] either expressed by sporozoites but carried into the hepatocyte on invasion or first expressed in the hepatocyte and potentially expressed on the hepatocyte surface in the context of MHC class I.

We hypothesized that by combining the DNA sequences encoding multiple antigens, protective effects may be additive or synergistic to achieve high grade immunity in a majority of vaccine recipients.[26,27] As an initial step prior to assessing the multi-antigen approach and the prime boost approach, we tested a monovalent plasmid vaccine encoding *Pf*CSP in two Phase 1 studies in humans. The *Pf*CSP vaccine did not induce CSP antibodies,[28,29] but did induce antigen-specific, cytotoxic T cell responses (CTL) to HLA-restricted synthetic peptides in 11/20 volunteers expressing HLA-A02 supertype alleles that were dependent on CD8+ T cells in depletion studies.[29,30] The next step was development of the pentavalent DNA vaccine called MuStDO5 (Multi-Stage DNA Vaccine Operation, 5 genes)[31] designed to test the multivalent approach, comprising a mixture of plasmids each encoding a different pre-erythrocytic stage *P. falciparum* antigen (3D7 strain). These were *Pf*CSP,[32] *Pf*TRAP/SSP2, *Pf* exported protein-1 (*Pf*Exp1),[33] N' and C' terminus of *Pf*liver stage antigen-1 (*Pf*LSA1)[34] and *Pf* liver stage antigen-3 (*Pf*LSA3).[35] To improve the immunogenicity of the five-plasmid mixture, a sixth plasmid encoding human granulocyte macrophage-colony stimulating factor (hGM-CSF) was added, based on enhanced protection in murine models when a plasmid encoding murine GM-CSF was combined with other plasmids encoding malaria antigens.[36] hGM-CSF recombinant protein had previously been approved by the FDA as a hematopoietic growth factor, and had been used in clinical vaccine trials as an adjuvant,[37] where it was hypothesized to act by binding to a specific cytokine α/β receptor.[38]

The clinical trial reported here aimed to establish safety, tolerability and immunogenicity of the MuStDO5 vaccine, and also to test for efficacy against malaria by allowing infected mosquitoes to bite the immunized volunteers followed by active surveillance for parasitemia (controlled human malaria infection or CHMI). We co-administered the five DNA plasmids with or without the plasmid encoding hGM-CSF, having previously demonstrated safety in preclinical studies.[39] Because this was a first-in-humans study for four of the five malaria plasmids (excepting the *Pf*CSP plasmid) and for the hGM-CSF plasmid, a four cohort, dose escalation design was used, with cohort 1 receiving 2,500 μg of the MuStDO5 cocktail (500 μg each plasmid) in phosphate buffered saline and cohorts 2, 3 and 4 receiving 2,500 μg of MuStDO5 mixed with 20 μg, 100 μg or 500 μg of the hGM-CSF plasmid, respectively. The initiation of

each cohort was staggered to allow assessment of safety prior to testing the next higher dose of hGM-CSF DNA.

Thirty-one malaria-naïve and healthy adult research volunteers received three doses of the vaccine by intramuscular jet injection (Biojector® 2000) into alternating arms and were assessed for efficacy in four separate challenges with *P. falciparum* sporozoites (CHMI) conducted 18 d after the third DNA immunization. Although no volunteers were sterilely protected, the vaccine was safe and well-tolerated and induced IFNγ responses to HLA-matched peptides derived from all five antigens.[31] IFNγ responses were boosted on exposure to parasites during challenge. This report presents safety, tolerability and antibody responses, and summarizes IFNγ ELISpot responses, which have been reported in detail elsewhere,[31] and also describes the outcome of CHMI.

Results

The primary objective of this study was to determine the safety and tolerability of MuStDO5 in combination with escalating dose hGM-CSF plasmid in healthy, malaria-naïve, adult volunteers. The secondary objectives were to measure immunogenicity and protection against sporozoite challenge.

Participant flow. The target sample size was eight vaccinees and three infectivity controls in each cohort; since no sterile protection was seen in the first three cohorts, the number of infectivity controls was expanded to four in the fourth cohort to increase power to identify delayed onset of parasitemia in vaccine recipients. 102 adult male and female volunteers age 18–50 provided informed consent and 57 of these passed screening and were determined to be eligible after 12 decided to withdraw (reason not specified by volunteer) and 33 were excluded (**Fig. 1**). Ten additional volunteers withdrew prior to the first immunization (**Fig. 1**). The 47 remaining volunteers were assigned sequentially to four cohorts assessing MuStDO5 alone or MuStDO5 plus one of the three doses of hGM-CSF-encoding plasmid (32 vaccinees and 15 non-vaccinated controls). One of the 15 non-vaccinated controls was enrolled as an extra volunteer to replace any infectivity control drop-outs, one was enrolled as a source of malaria-naïve peripheral blood mononuclear cells (PBMC) for cohort 4 (immunological control) and 13 were enrolled as infectivity controls or as both infectivity controls and immunological controls.

HLA alleles were distributed among the four cohorts based on the need to match volunteer HLA types in each cohort with a set of strongly-binding peptides identified from the five malaria antigens that were restricted by common HLA class I antigens. HLA-A02 and HLA-A03 supertypes were fairly evenly distributed among vaccinees meeting enrollment targets (see Methods) comprising, respectively, 5 and 3 volunteers in the first cohort, 3 and 6 volunteers in the second cohort, 3 and 4 volunteers in the third cohort, and 4 and 7 in the fourth cohort. Thus 15/32 (47%) and 20/32 (63%) of volunteers expressed HLA-A02 and/or HLA-A03 supertypes overall, comprising all but four (13%) of the research subjects. HLA-A01 and HLA-B07 supertypes were also represented in each cohort, while HLA-A24 and HLA-B08 supertypes were represented in some of the cohorts. A few additional class I antigens were identified; however, matched peptides were not available for these.

Cohorts 1 and 2 received their first immunizations on 03 and 20 Nov 2000, respectively, while cohorts 3 and 4 received their first immunizations on 23 Jan and 20 Feb 2001, respectively. One HLA-A02 vaccinee from cohort 3 withdrew after the first immunization (personal reason not related to adverse events) and was not replaced, reducing the sample size of vaccinees from 8 to 7 in cohort 3 (from 32 to 31 overall), and one infectivity control from cohort 1 withdrew prior to challenge (due to personal reasons not related to adverse events) and was replaced by the extra volunteer (see above). Forty-four volunteers were challenged in four separate challenges, 31 vaccine recipients and 13 infectivity controls. Six of these 44 participants missed the week 24 visit (94 d post challenge) and/or week 52 visit (290 d post challenge): 2 were lost to follow-up, 3 were military transfers, and 1 moved out of area. The final cohort composition, including demographics, is provided in **Table 1**.

Safety. Thirty-one volunteers received three immunizations and one volunteer in cohort 3 received one immunization, totaling 94 doses of MuStDO5 and 24, 22 and 24 doses of MuStDO5 combined with hGM-CSF at 20 μg, 100 μg or 500 μg, respectively. Adverse events were assessed on days 2, 7, 14 and 28 post first and second immunizations, and on days 2, 7 and 14 post third immunizations (CHMI was initiated on day 18 precluding additional observation for vaccine effects). Adverse events were classified as definitely, probably, possibly related or unrelated to vaccine administration, and also classified according to severity grade as mild, moderate or severe.

Solicited adverse events. 112 solicited local adverse events were classified as definitely related to vaccine administration (**Table 2**): injection site tenderness (69 cases), injection site pain (14 cases), injection site erythema (10 cases), injection site ecchymosis (8 cases), injection site induration (7 cases), injection site swelling (1 case), injection site pruritis (1 case), decreased arm range of motion (1 case) and ipsilateral muscle ache in the neck (1 case). Of these 112 adverse events, 104 were classified as mild (grade 1) and 8 as moderate (grade 2). In addition, there were 51 solicited systemic adverse events. None were categorized as definitely related to vaccine administration, indicating very favorable tolerability. Three were classified as probably related to vaccine administration: 2 cases of arthralgia and 1 case of general myalgia, each classified as mild (grade 1). The remaining 48 systemic adverse events were classified as possibly related to immunization: one case of vomiting was classified as moderate in severity (grade 2), while the other 47 were classified as mild in severity (grade 1), and included myalgia (3 cases), malaise (5 cases), headache (10 cases), diarrhea (3 cases), nausea (4 cases), fatigue (2 cases), arthralgia (1 case), chills (4 cases), lightheadedness/dizziness (3 cases), chest tightness (1 case), respiratory symptoms (10 cases) and fever (1 case). These possibly related systemic adverse events appeared more likely related to intercurrent respiratory and other illnesses experienced by the volunteers rather than to the vaccine. All these adverse events resolved within two or three days of vaccine administration. There were no solicited severe

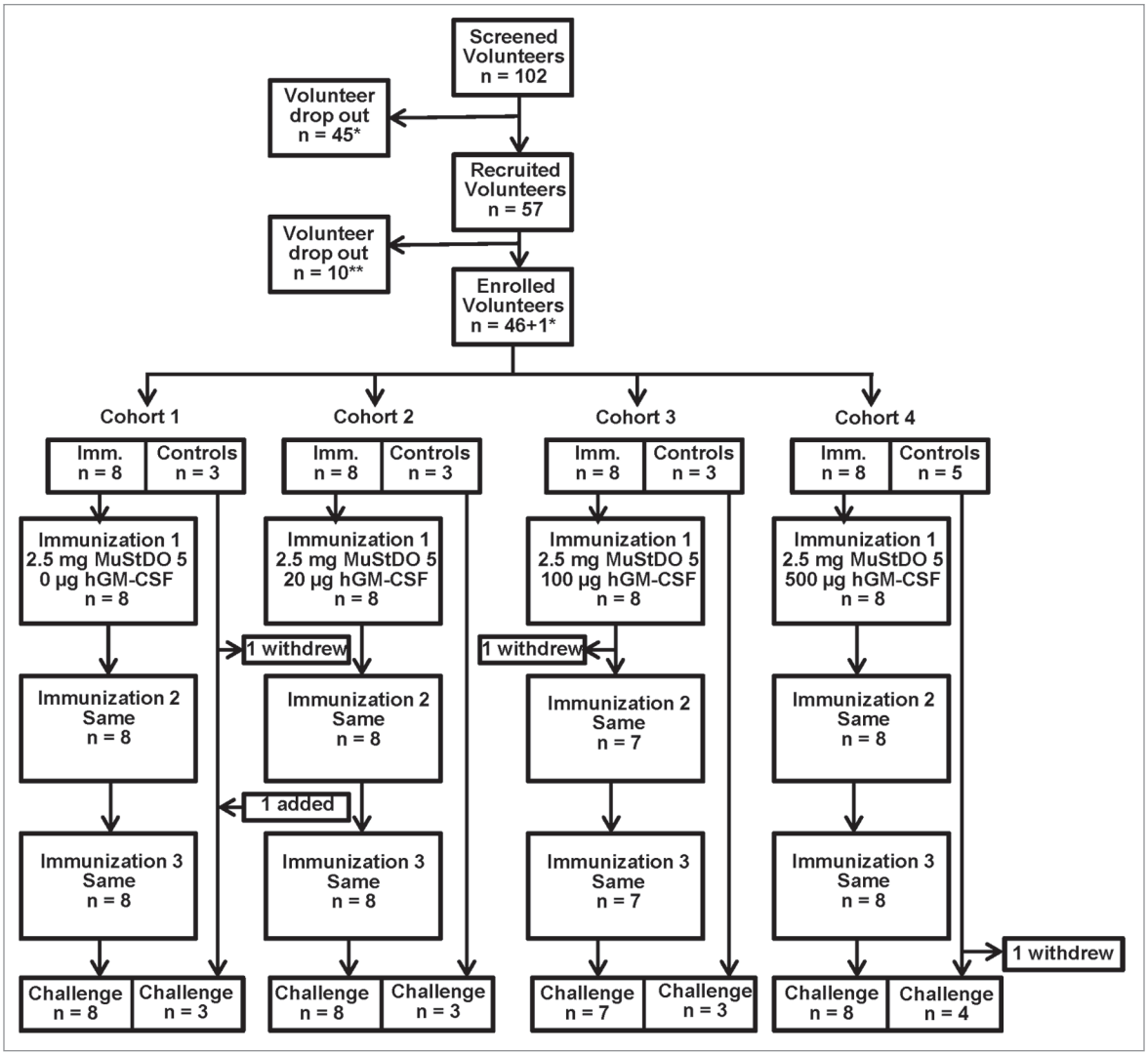

Figure 1. Flow diagram of volunteers. 102 volunteers provided informed consent and 57 passed screening. Thirty-three were excluded because: HLA type not needed (11), abnormal laboratory results (9), finding on medical history (3), finding on physical exam (3), HIV positive (1), conflicting concomitant medication (1), leaving area (1), military supervisor denied approval (1), non-compliance with appointment schedule (1), problem with method of compensation (1), enrolled in another protocol (1) and 12 withdrew of their own accord without specifying a reason. During the interval between screening and the first immunization, ten additional volunteers were dropped because: military supervisor denied approval (2), military transfer (3), pregnancy (1) or withdrew of their own accord without specifying a reason (4). Forty-six of the remaining 47 enrolled volunteers were assigned sequentially to cohorts 1–4. One volunteer in cohort 3 withdrew after the first immunization (personal reasons not related to adverse events) and was not replaced, one volunteer withdrew from the infectivity controls in cohort 1 (personal reasons not related to adverse events) and was replaced from the enrolled volunteers (denoted by an asterisk*), and one volunteer withdrew from the infectivity controls of cohort 4 prior to challenge (personal reasons not related to adverse events).

(grade 3) adverse events that were definitely, probably or possibly related to vaccine administration.

Neither the number nor the severity of adverse events varied significantly among cohorts, indicating that the expression of hGM-CSF in the muscle or any resulting systemic distribution did not affect tolerability (**Fig. 2**). In three of the cohorts, more adverse events followed the first immunizations, but this was not statistically significant in the trial as a whole.

Unsolicited adverse events. Unsolicited adverse events were recorded by asking an open-ended question "do you have any additional symptoms?" There were four clinically significant unexpected adverse events identified. Three were classified as unrelated to immunization: (1) New onset peptic ulcer disease in a control volunteer (severity grade 3); (2) Erythema multiforme in a cohort 2 volunteer, likely associated with oral herpes reactivation following malaria infection or possibly with administration of chloroquine or prochlorperazine during malaria treatment (severity grade 2); (3) Orthostatic hypotension in a cohort 1 volunteer during acute malaria infection (severity grade 2). One significant unexpected adverse event was classified as possibly related to vaccine administration: exacerbation of sleep apnea in a cohort 2 volunteer several months after malaria

Table 1. Demographic data for enrolled volunteers

	Cohort 1	Cohort 2	Cohort 3	Cohort 4	All
Vaccinees	8	8	8	8	32
Male	5	5	4	4	18
Female	3	3	4	4	14
Infectivity controls	3*	3	3	4	13
Male	2	2	1	3	8
Female	1	1	2	1	5
Immunological controls				1	1
Male				1	1
Age (mean, range)					
Vaccinees	39.9 (30–48)	29.6 (21–50)	32.9 (23–45)	37.4 (22–49)	34.9 (21–50)
Infectivity controls	24.3 (23–26)	28.0 (22–36)	43.3 (42–44)	34.5 (30–41)	32.7 (22–44)
Immunology control				42.0	42.0
Ethnicity					
American Indian/Native American	0	0	2	0	2
Asian/Pacific Islander	0	1	0	0	1
African American	3	1	3	4	11
Caucasian	7	8	5	7	27
Hispanic	0	1	1	0	2
Other	1	0	0	2	3
Totals	11	11	11	13	46

* One infectivity control withdrew prior to the challenge from cohort 1 and is not included in this table; thus the number of volunteers listed is 46, one less than the number enrolled (47).

infection associated with a 15 pound weight gain (categorized as a serious adverse event, due to accelerating the need for hospital admission for corrective surgery).

Laboratory tests. Laboratory tests were assessed on days 0, 2, 7, 14 and 28 relative to each of the three immunizations and also at the end of the study (week 52). These were complete blood count (CBC), urinalysis, and a chemistry panel including creatine phosphokinase (CPK), alkaline phosphatase, bilirubin, lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen and creatinine. There were no clinically significant abnormalities in any of these tests classified as definitely, probably or possibly related to vaccine administration except for CPK elevations (see below), and there were no trends observed when cohort means were assessed other than a trend of decreasing hemoglobin in both vaccinees and controls that was temporally related to the large blood draws required for lymphocyte collections and a trend of decreasing bilirubin during the first half of the study that was probably also related to blood drawing, in both cases with cohort means returning to baseline during follow-up. There were seven findings of elevated CPK. All seven coincided with the onset of a vigorous exercise program by the volunteer, and other than minor myalgia in some instances, there were no signs or symptoms of these elevations. Two CPK elevations were in control volunteers so could not be linked to the vaccine. Two CPK elevations were in volunteers receiving 20 g hGM-CSF, one noted 14 d after the first immunization (9,671 IU/L, normal range ≤ 203) with a normal CPK 7 d after the first immunization and at all-time points after the second and third immunizations, and the second noted 111 d after the third immunization (20,077 IU/L) with a normal CPK 48 d after the third immunization and at all prior time points. There was one elevated CPK in a volunteer receiving 100 μg of GM-CSF, with an elevation 28 d after the third immunization (15,894 IU/L) with a normal CPK 14 d after the third immunization and at all prior time points. Finally, there were two events in volunteers receiving 500 μg hGM-CSF, one with an elevation noted 14 d after the second immunization (5,954 IU/L) with a normal CPK 7 d after the second immunization and at all prior and subsequent time points, and the second noted in another volunteer 7 d after the second immunization (2,697 IU/L) with a normal CPK 2 d after the second immunization and at all prior and subsequent time points. Due to the roughly equal distribution of CPK elevations among vaccine recipients and controls, to the apparently random timing with respected to immunizations (following first, second or third immunizations), rapid normalization and lack of recurrence on re-challenge with vaccine, and to the proximity of each elevation to newly-initiated vigorous, military style exercise programs, these CPK elevations were classified as possibly related to immunization.

Due to concerns that DNA vaccines might induce autoimmune responses,[40] we measured anti-double stranded DNA antibodies at 0, 4, 8, 12, 24 and 52 weeks and found no abnormalities (**Fig. 3A**). Based on similar concerns, we measured

Table 2. Number of volunteers experiencing solicited local and systemic adverse signs and symptoms (days 0–28 post immunizations 1 and 2, and days 0–14 post immunization 3)

	Cohort 1 n = 8			Cohort 2 n = 8			Cohort 3 n = 8 →7			Cohort 4 n = 8			
Sign or Symptom	**DNA1**	**DNA2**	**DNA3**	**DNA1**	**DNA2**	**DNA3**	**DNA1**	**DNA2**	**DNA3**	**DNA1**	**DNA2**	**DNA3**	**Total**
LOCAL	**n = 8**	**n = 8**	**n = 8**	**n = 8**	**n = 8**	**n = 8**	**n = 8**	**n = 7**	**n = 7**	**n = 8**	**n = 8**	**n = 8**	
Tenderness	6(75%)	5(62%)	4(50%)	7(88%)	5(63%)	4(50%)	6(75%)	6(86%)	5(71%)	8(100%)	7(88%)	6(75%)	69(62%)
Pain	2(25%)	2(25%)	1(13%)	2(25%)	1(13%)		1(13%)		2(29%)	2(25%)	1(13%)		14(13%)
Ecchymosis	2(25%)	3(38%)	2(25%)									1(13%)	8(7%)
Induration	2(25%)*	1(13%)*	1(13%)*							1(13%)*	1(13%)*	1(13%)*	7(6%)
Erythema	1(13%)	2(25%)	1(13%)	1(13%)			1(13%)		1(14%)	1(13%)	1(13%)	1(13%)	10(9%)
Swelling										1(13%)*			1(1%)
Pruritis								1(14%)					1(1%)
Decreased arm range	1(13%)												1(1%)
Ipsilateral neck muscle ache									1(14%)				1(1%)
Total Local AEs	**14**	**13**	**9**	**10**	**6**	**4**	**8**	**7**	**9**	**13**	**10**	**9**	**112**
Rate (AEs/ immuniz)	1.75	1.63	1.13	1.25	0.75	0.50	1.00	1.00	1.29	1.63	1.25	1.13	
SYSTEMIC													
Myalgia				1(13%)		1(13%)	**1(13%)** 1(13%)						4(8%)
Malaise			1(13%)				3(38%)	1(14%)					5(10%)
Headache	1(13%)				1(13%)	1(13%)	3(38%)	1(14%)	2(25%)	1(13%)			10(20%)
Diarrhea	1(13%)	1(13%)					1(13%)						3(6%)
Nausea					1(13%)		1(13%)	1(14%)				1(13%)	4(8%)
Vomiting								1(14%)					1(2%)
Fatigue								2(29%)					2(4%)
Arthralgia							**1(13%)** 1(13%)	**1(14%)**					3(6%)
Chills	1(13%)						3(38%)						4(8%)
Lightheaded		1(13%)			1(13%)		1(13%)						3(6%)
Chest tightness				1(13%)									1(2%)
Respiratory symptoms							4(50%)	4(57%)			2(25%)		10(20%)
Fever							1(13%)						1(2%)
Total Systemic AEs	**3**	**2**	**1**	**2**	**3**	**2**	**21**	**11**	**2**	**1**	**2**	**1**	**51**
Rate (AEs/ immuniz)	0.38	0.25	0.13	0.25	0.38	0.25	2.63	1.57	0.29	0.13	0.25	0.13	
Total All AEs	**17**	**15**	**10**	**12**	**9**	**6**	**29**	**18**	**11**	**14**	**12**	**10**	**163**

(Upper half of table) 112 local adverse events were reported after DNA immunizations, all occurring and resolving within the first 0–7 d. All were classified as directly related to immunization. Of these, 104 were classified as mild (grade 1), and 8 were classified as moderate (Grade 2, denoted by an asterisk*). (**Lower half of table**) 51 systemic adverse events were reported days 0–28 after immunizations 1 and 2 and days 0–18 after immunization 3; of these, none were classified as directly related to immunization and three were classified as probably related to immunization (bold). The rest (48) were classified as possibly related to immunization, including 47 assessed as mild (grade 1), and one case of vomiting assessed as moderate (grade 2) in severity. Because the 48 possibly-related adverse events all appeared more likely associated with intercurrent illnesses than immunization (e.g., cluster of respiratory symptoms in cohort 3 during the first two immunizations, which took place during the months of January and February, respectively), the systemic adverse event rates listed in the table are likely overestimates of the true rate of vaccine-related systemic adverse events.

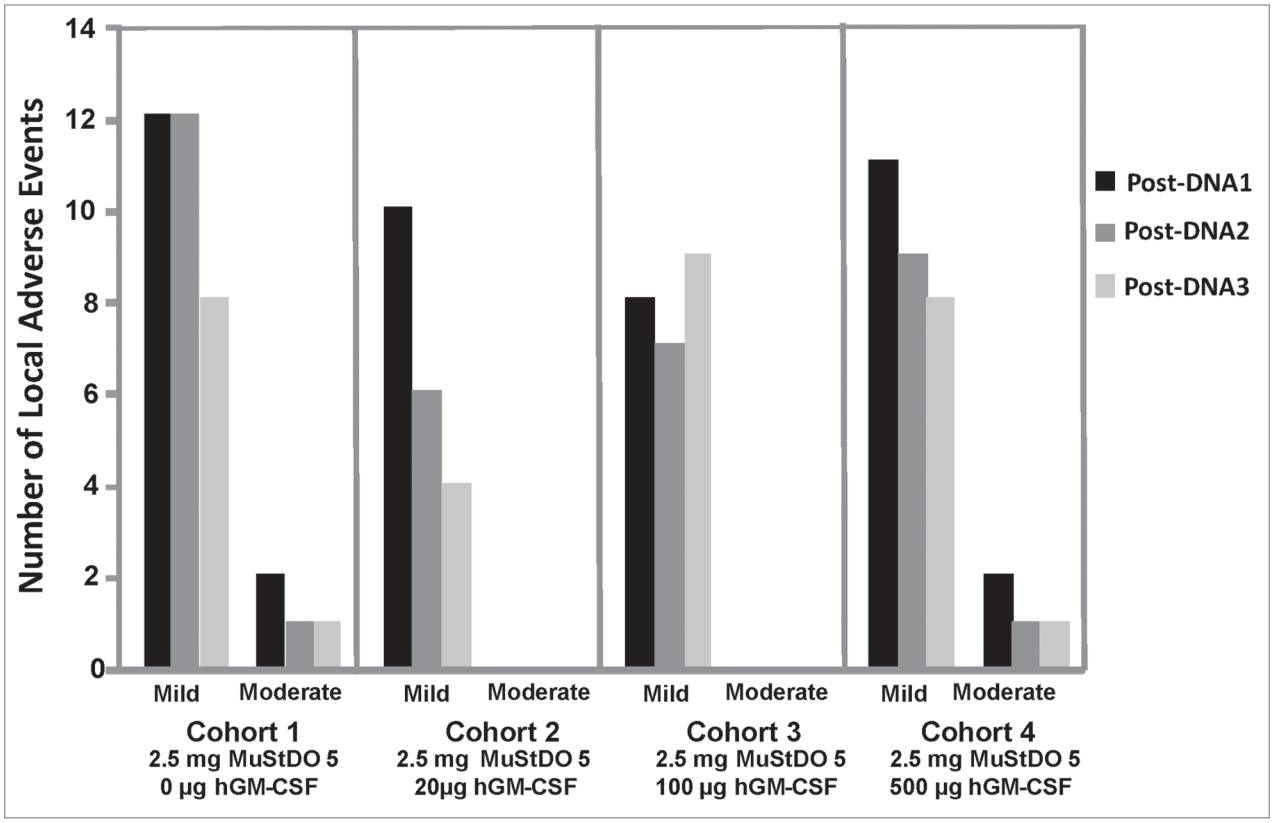

Figure 2. Frequency of Mild and Moderate Adverse Events in Cohorts 1, 2, 3 and 4 after each immunization. Local adverse events are shown after each DNA immunization, and are grouped according to mild (grade 1) or moderate (grade 2) severity. Increasing doses of hGM-CSF-encoding plasmid did not affect tolerability.

anti-human GM-CSF antibodies at the same time points in an assay developed by Vical, Inc., and again found no abnormalities (**Fig. 3B**). Hemoglobin A1C was measured at screening and at week 15 without recording any abnormal values or rising trends within cohorts (data not shown).

Overall, the vaccine was very well-tolerated by all recipients, with nearly all definitely-related reactogenicity limited to the site of immunization, and resolving within two days of vaccine administration.

Immunogenicity. *ELISA.* Antibody responses to four of the antigens (*Pf*CSP, *Pf*SSP2/TRAP, *Pf*EXP1, *Pf*LSA1) were measured pre-immunization, two weeks after the third immunization (four days prior to challenge), six weeks after the third immunization (25 d post challenge) and 8 weeks after the third immunization (38 d post challenge). No ELISA was performed for *Pf*LSA3. There were no significant increases in antibody titers to any of the four vaccine antigens by ELISA following MuStDO5 immunization (**Fig.** 4). Antibody titers to the four tested antigens by ELISA were significantly boosted by exposure to malaria parasites during CHMI ($p < 0.05$ after correcting for multiple comparisons for all antigens and all cohorts except LSA1 for cohort 2, where $p = 0.09$, and for cohort 4, where $p = 0.07$). However, the degree of boosting was not significantly different from that seen in the infectivity controls (**Fig. 4A**). The addition of the hGM-CSF plasmid to the vaccine did not appear to influence the extent of boosting of antibodies on exposure to malaria, as seen by comparing boosting among the four cohorts (**Fig. 4B**).

IFA assays. IFA assays to sporozoites and blood stage parasites were performed at the same time points as ELISAs. There were no significant increases in antibody responses to whole sporozoites or blood stage parasites for any of the volunteers comparing pre- and post-immunization sera (**Fig. 5**). Cohort 1 was also assessed for antibodies to liver stage parasites at the same time points, and, as for sporozoites and blood stages, there were no significant increases in antibody responses (**Fig. 5**).

Exposure to malaria parasites during challenge induced significant increases in antibody titers to sporozoites, liver stage and blood stage parasites ($p = 0.004$ for each, after correction for multiple comparisons). The fold increase recorded was similar in vaccine recipients and in controls.

IFN-γ ELISpot assay. ELISpot assays were conducted using fresh PBMCs collected pre-immunization (day 0), 2 weeks after the second immunization and 2 weeks after the third immunization, by stimulating with individual peptides. After the trial was completed, additional ELISpot assays were performed with blinded, frozen PBMCs from pre-immunization (day 0), pre-challenge (approximately 1 week pre-challenge), 4 weeks

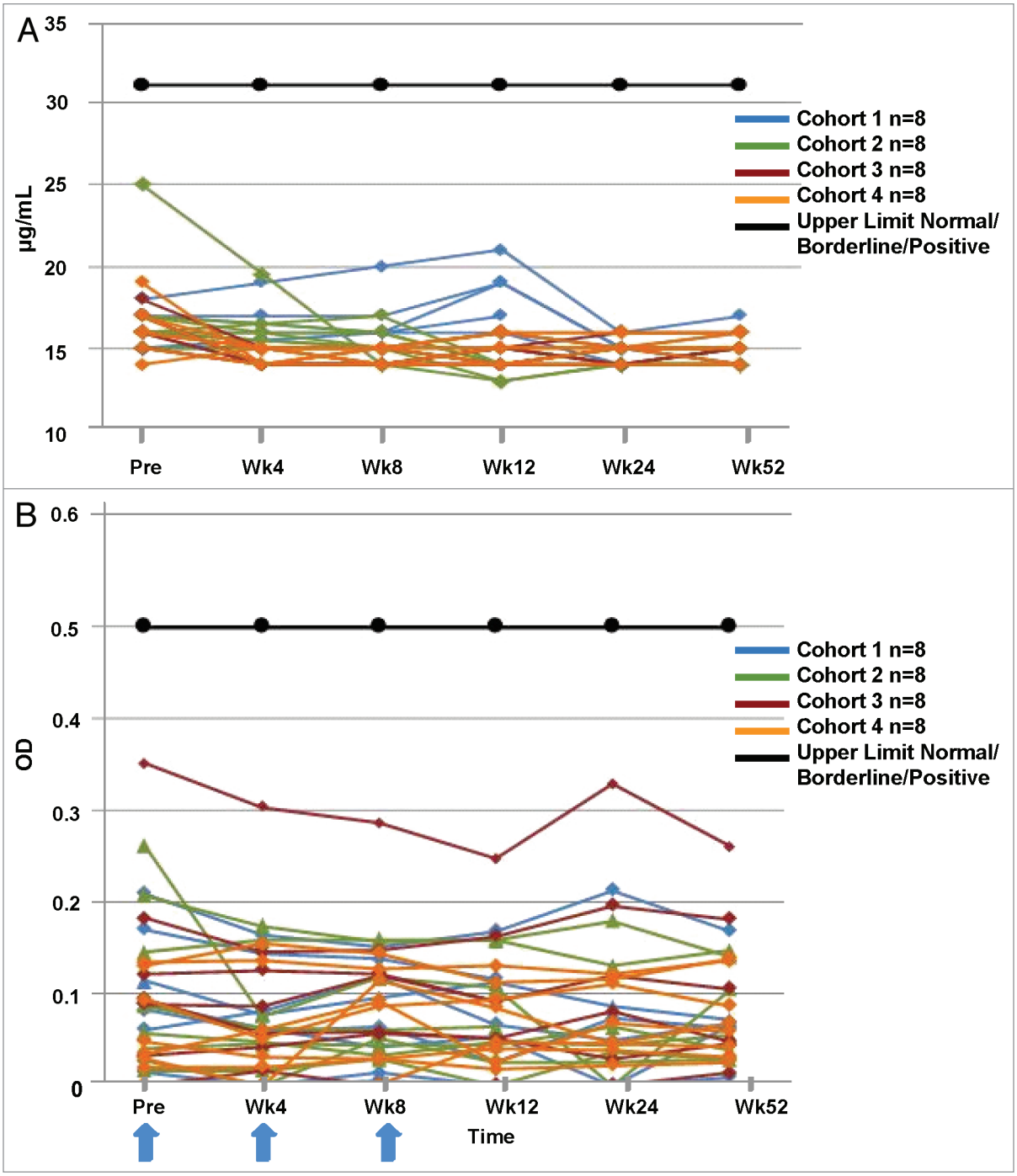

Figure 3. Anti-dsDNA and anti-hGM-CSF antibody responses following DNA immunization. Anti-dsDNA levels (IU/mL) (**A**) and anti-hGM-CSF levels (optical density) (**B**) were measured at pre-immunization, at 4, 8, 12, 24 and 52 weeks. Cohorts 1, 2, 3 and 4 are color-coded. The upper black line in each panel represents the division between normal and borderline positive values for these tests. There are no data for the four-week time point for anti-dsDNA for cohorts 1 and 2 (data points used in the graph are averages of the values at 0 and 8 weeks). Anti-dsDNA levels were measured by the National Naval Medical Center Clinical Laboratory using fresh sera, and the anti-hGM-CSF antibodies were measured by Vical Inc., using frozen sera.

and 9 weeks after challenge, with all time points from a given volunteer assessed simultaneously, to confirm the data from fresh assays.

Detailed ELISpot results have been previously published[31] and are summarized here. Following immunization, positive, antigen-specific IFN-γ responses were detected by fresh ELISpot assay in 15/31 volunteers to multiple class I and/or class II restricted T cell epitopes representing all 5 antigens. Responses to CSP occurred in 14/31, to EXP-1 and LSA-1 in 12/31, to LSA-3 in 7/31 and to TRAP/SSP2 in 4/31 volunteers. Nine of 15 responders produced IFN-γ to both class I and II (DR-binding) peptides. Overall, positive responses were detected to 38.2% (13/34) of the class

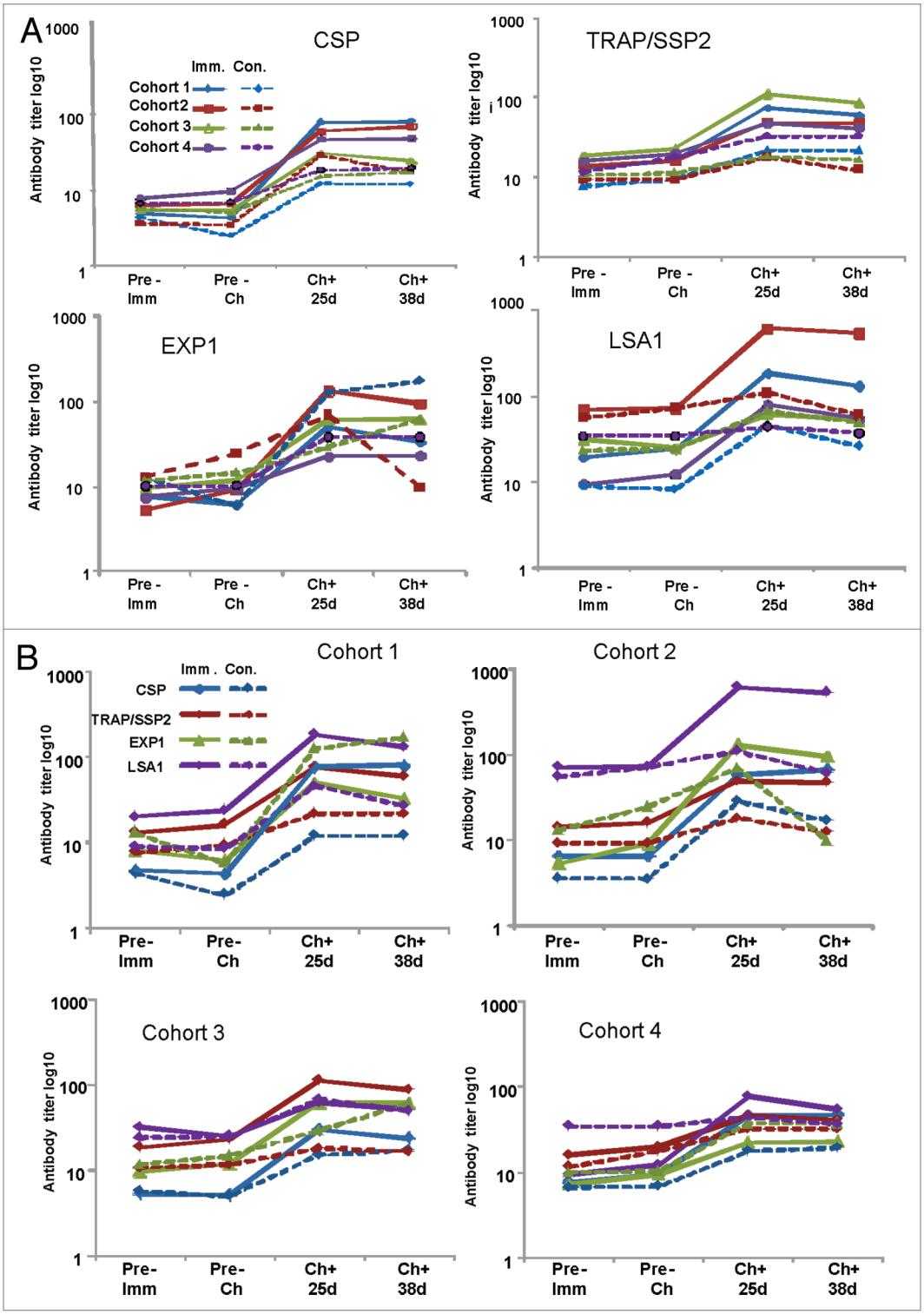

Figure 4. ELISA antibody responses by antigen and by cohort. (**A**) The four graphs provide geometric mean antibody titers to CSP, TRAP/SSP2, EXP1 and LSA1, respectively, as measured by ELISA pre-immunization (Pre-Imm), two weeks after the third DNA immunization/pre-challenge (Pre-Ch), and 25 d and 38 d after challenge (Ch+25, Ch+38). Cohorts are color-coded, with solid lines representing immunized volunteers, dotted lines representing infectivity control volunteers. (**B**) The four graphs provide geometric means of antibody titers to CSP, TRAP/SSP2, EXP1 and LSA1 for each cohort, respectively. Antigens are color-coded, with solid lines representing immunized volunteers, dotted lines representing infectivity control volunteers.

I-restricted peptides (3 for CSP, 1 for SSP2/TRAP, 2 for EXP1, 5 for LSA1 and 2 for LSA3) and to 94.1% (16/17) of the class II-restricted peptides (4 for CSP, 3 for SSP2/TRAP, 2 for EXP1, 5 for LSA1 and 2 for LSA3). Inclusion of hGM-CSF plasmid, rather than improving responses, appeared to inhibit IFN-γ responses to class I peptides in cohorts 2, 3 and 4 compared with cohort 1, while responses to class II peptides appeared unaffected by the cytokine.

Exposure to malaria following CHMI significantly boosted both class I and class II responses, with the number of responding volunteers increasing from 15/31 volunteers prior to challenge to 23/31 volunteers following challenge, with the magnitude of individual responses increasing from 2- to 143-fold.[31] The magnitude of boosting in vaccinees appeared greater than in controls. The inhibitory trend shown by hGM-CSF for class I responses prior to challenge (3/23 volunteers positive for IFN-γ responses to class I in cohorts 2–4 vs. 3/8 in cohort 1, p = 0.15) was enhanced post challenge (4/23 volunteers positive for IFN-γ responses to class I in cohorts 2–4 vs. 5/8 in cohort 1, p = 0.015), while responses to class II peptides remaining unaffected.

Efficacy. *Controlled human malaria infection.* Thirty-one vaccinated and 13 unvaccinated volunteers underwent CHMI as previously described[41] (8, 8, 7 and 8 vaccinees and 3, 3, 3, and 4 controls in the four cohorts, respectively). The days to parasitemia in cohorts 1–4 are shown in **Figure 6** as Kaplan-Meier survival curves. The mean days to parasitemia were similar in the four cohorts: 10.7, 10.0, 11.1 and 11.1 d, respectively. No volunteer was completely protected, and there were no significant differences in the mean days to parasitemia for vaccinees relative to controls in any of the cohorts, although a trend suggesting delay in vaccine recipients was seen in cohort 2 that was not statistically significant (p = 0.08).(**Fig 7**)

Because the proposed mechanism of protection for MuStDO5 is genetically restricted cell-mediated immunity, we repeated the efficacy analysis limited to the volunteers with the two most common class 1 antigens among vaccine recipients, HLA-A02 or HLA-A03, to increase the homogeneity of the study subjects. This excluded 7 of 31 vaccine recipients, and 8 of 13 infectivity controls. Examining the remaining 24 vaccinees and 5 controls, the addition of GM-CSF tended to prolong the days to parasitemia (10.4, 10.7, 11.3 and 11.2 d for the four cohorts, respectively), but this was not statistically significant (p = 0.52).

Discussion

The identification of a vaccine approach that successfully targets liver stage malaria is a critical step in the development of a highly protective vaccine. The number of hepatic parasites resulting from an infectious mosquito bite is relatively small, probably no more than 100,[42] and development in the liver is non-pathogenic. Their elimination prior to transformation into the highly pathogenic blood stages would entirely prevent illness. Moreover, the roughly five-day span of liver stage development in *P. falciparum* appears long enough for cytotoxic or IFN-γ-secreting effector cells to work, as demonstrated by the induction of sterile immunity to *P. yoelii* in BALB/c mice[13,14] by immunization with *Py*CSP,[14] *Py*SSP2[43] and *Py*Hep17 (PfEXP1),[33,48] where the duration of liver stage development is just over two days[13,44] or by the induction of sterile immunity to *P. knowlesi* in non-human primates (NHP) where the duration of liver stage development is just over five days and thus similar to *P. falciparum*.[16,45-47] In both the mouse[14] and NHP[96] models, protection induced with gene-based vaccines was dependent on CD8+ T cells that eliminated infected hepatocytes by direct cytotoxicity or through an IFN-γ-mediated mechanism. Thus the development of protective gene-based platforms protecting humans against *P. falciparum* should be feasible.

With this trial, we took the first steps to clinically develop the multi-antigen approach, combining the DNA encoding five pre-erythrocytic stage antigens into a plasmid mixture (MuStDO5) and assessing intramuscular administration via jet injection in 32 (dropping to 31) healthy, malaria-naïve adults, aiming to induce cell-mediated protection. The rationale for multiple antigens was based on the genetic restriction of adaptive cell-mediated immunity: since each malaria antigen provides only limited numbers of strongly-binding class I epitopes for the diverse MHC antigens of the human population, the combination of multiple protective malaria proteins is likely required to achieve broad population coverage.[48] In prior non-clinical studies, we have shown that multivalent DNA vaccines comprising plasmid mixtures can be immunogenic in mice and NHP without significant interference,[16,45,49,73] although plasmids encoding certain malaria proteins could theoretically suppress the immunogenicity of other plasmids if included in the mixture,[50] as shown in vitro.[51]

The first objective of the clinical trial was to explore safety and tolerability. The trial demonstrated that the MuStDO5 vaccine was both safe and well tolerated and caused no severe or serious side effects. Adverse events were mostly mild, mostly local to the injection site, and rapidly resolving. Injection of DNA plasmids did not induce anti-ds-DNA antibody responses or anti-hGM-CSF responses, allaying concerns that DNA immunization might lead to auto-immunity.[52,53] Several immunized volunteers developed acute elevations of the muscle enzyme CPK, but these events showed no consistent timing with respect to immunizations, occurred with equal frequency in unimmunized control volunteers, and, in each case, followed the initiation of military style physical training. Moreover, the degree of CPK elevation was consistent with that previously reported for civilians or military service members engaging in intense exercise programs,[54,55] so it did not appear that the vaccine had rendered the study subjects more sensitive to exercise-induced muscle damage. None of the individuals with elevated CPK in our study had associated changes in blood creatinine levels (data not shown), indicating that there was no renal insult. Overall, MuStDO5 administration was safe, consistent with findings from other DNA vaccine trials in malaria,[29] tuberculosis[56] and HIV.[57]

The second objective was to assess immunogenicity. The MuStDO5 vaccine successfully induced IFN-γ responses to each of the five antigens; positive responses were detected to 38.2% (13/34) of the class I-restricted peptides and to 94.1% (16/17) of the class II-restricted peptides tested.31 About half the volunteers responded to class I or class II peptides from at least one antigen, although only 6/31 (19%) demonstrated class I responses.

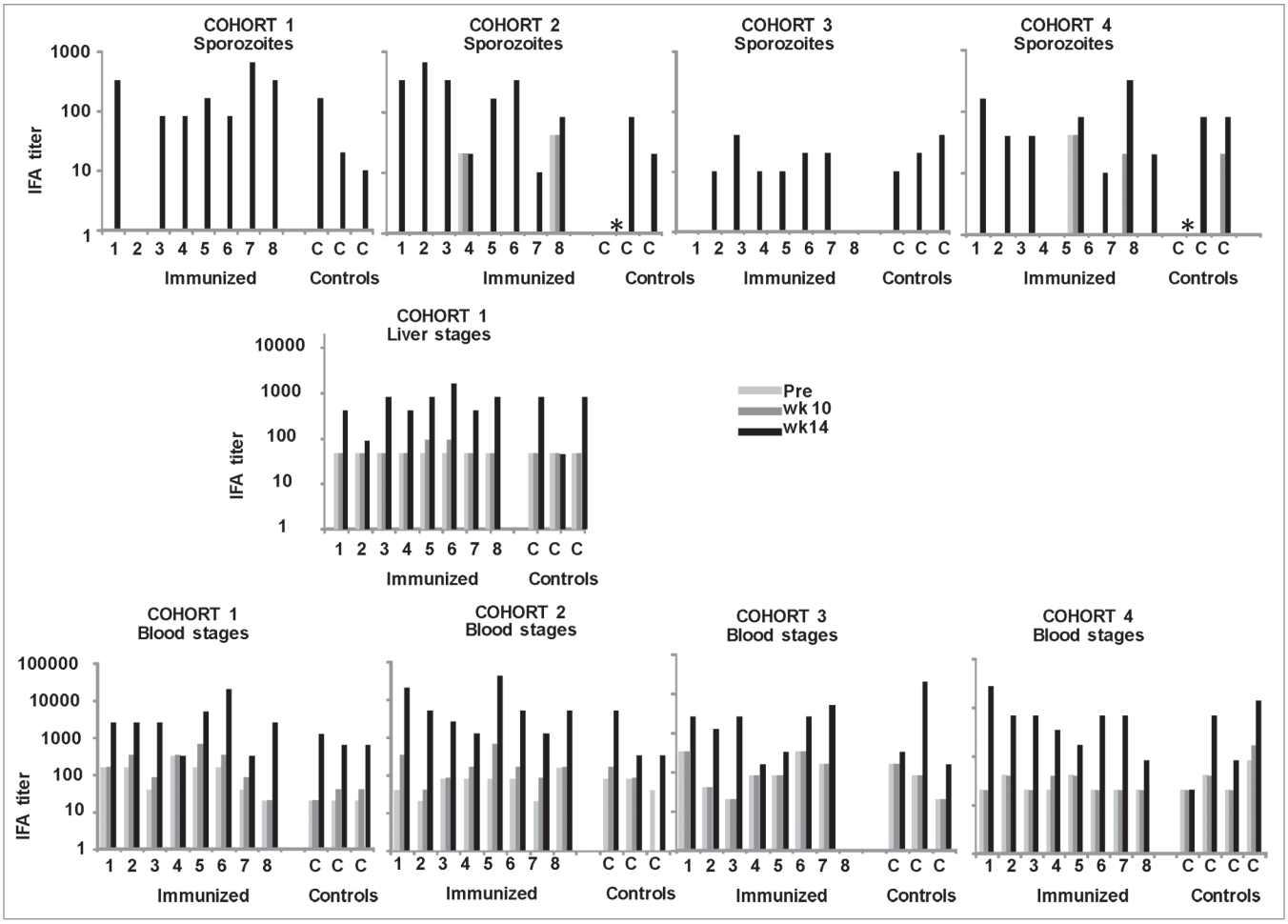

Figure 5. Immunofluorescent antibody responses to sporozoites, liver and blood stages. The figures show IFA titers against sporozoites, liver stages and blood stages, measured at pre-immunization (Pre), week 10 (pre-challenge) and week 14 (25 d after challenge). Time points are color-coded in different shades of black and gray. Cohorts 1, 2, 3 and 4 were tested against sporozoites and blood stages and cohort 1 against liver stages. Infectivity controls were measured at the same time points. *Volunteer not tested.

The responses were boosted by exposure to malaria challenge, a potentially useful attribute for any vaccine used in endemic areas where frequent exposure to the parasite could boost the protection induced by such a vaccine. The results also indicated that responses to CSP were not inhibited by inclusion in the five-plasmid cocktail when historically compared with the CSP plasmid administered alone in earlier studies.[31] However, cellular responses were generally of low magnitude, ranging from 12 to 96 net spot forming cells (sfcs)/106 PBMCs (geometric mean, 23.4), and likely much higher responses are needed to protect. This could be achieved by a number of improvements, such as codon-optimization to enhance expression in mammalian cells,[58] delivery via electroporation to improve cellular uptake or use of an adjuvant superior to GM-CSF.[59]

Antibody responses were also measured in this trial. As has been found with some other DNA vaccines including our earlier clinical studies with the monovalent PfCSP-encoding plasmid, these responses were negligible, with no significant pre-/post-immunization differences recorded. Like cellular responses, antibody responses, particularly against PfCSP, were boosted on challenge indicating a potential benefit of DNA vaccination for residents of endemic areas where frequent malaria exposure could boost protective responses and accelerate the development of naturally acquired immunity. However, non-immunized controls were equally boosted by exposure to CHMI. As with cellular responses, the low antibody titers indicated the need for improved DNA vaccine technologies such as the use of superior adjuvants, or the use of improved regimens such as DNA priming followed by viral vector boosting.

The third objective was to assess protection. The challenge results showed no protection, neither sterile protection nor significant delay in the onset of parasitemia. A post-hoc analysis of volunteers with A02 and/or A03 class I supertype alleles in cohorts 1–4 showed the same negative results.

A fourth objective was to determine if immune responses and protection could be enhanced by co-administration of plasmid expressing a human cytokine. A sixth plasmid encoding hGM-CSF was added to MuStDO5 using a dose-escalation design, based on murine studies demonstrating a doubling of protection from 28% to 58% when a DNA plasmid encoding murine

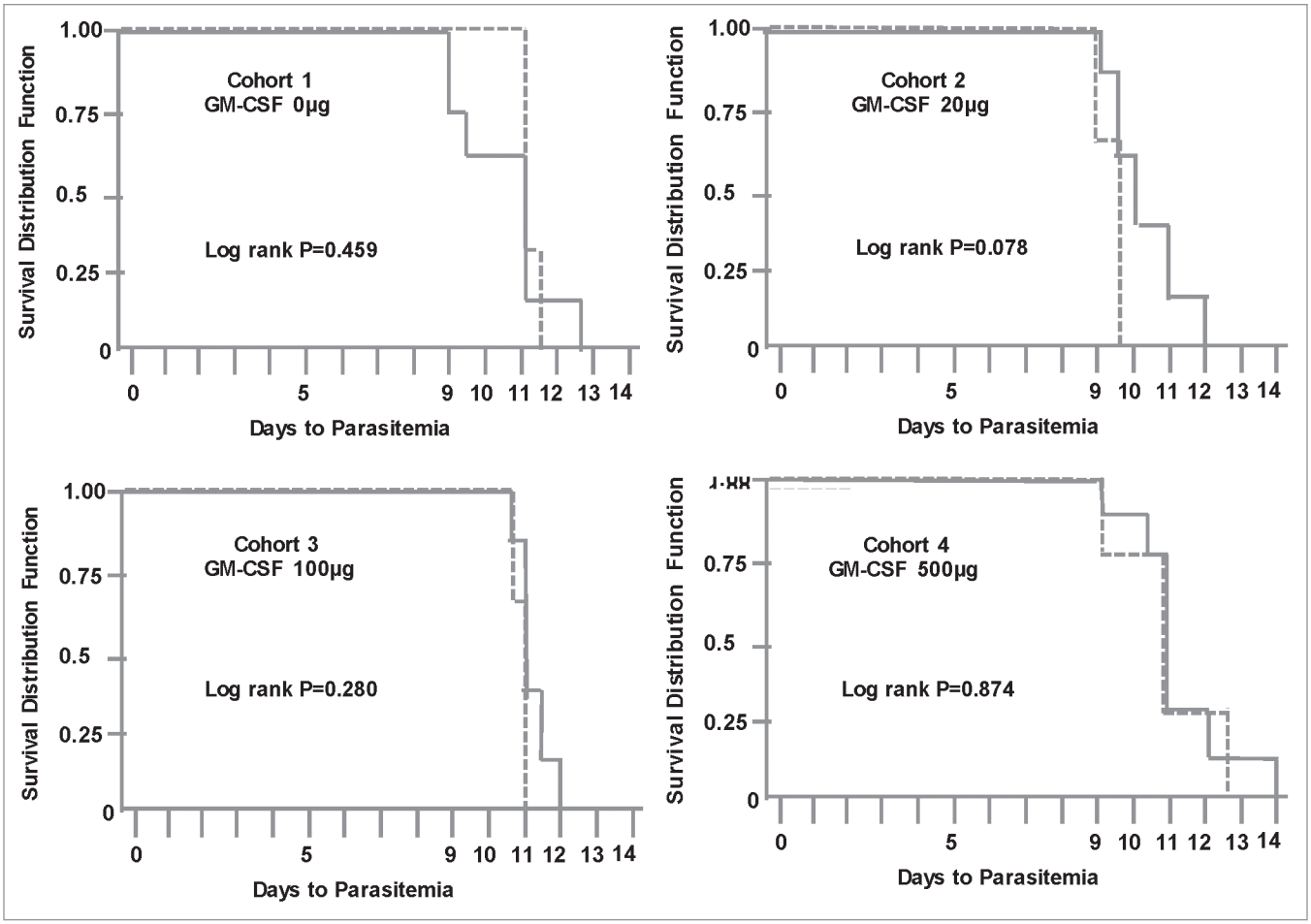

Figure 6. Vaccine efficacy by Kaplan-Meier Plot. The figure present parasitemia-free survival curves (Kaplan-Meier) for immunized (solid lines) and infectivity controls (dotted lines) for the four cohorts, respectively, based on microscopic examination of peripheral blood smears. Log rank test was used to test the statistical significance between the two groups. There was no significant difference in time to parasitemia between immunized volunteers and control volunteers.

GM-CSF was added to a protective DNA plasmid encoding *P. yoelii* CSP.[36] In the murine model, GM-CSF DNA induced a 30-fold increase in antigen-specific antibodies, a 5-fold increase in antigen-specific IFN-γ spot forming cells and a significant ($p < 0.05$) increase in protection using a DNA/poxvirus prime/boost regimen.[60]

Most prior experience with GM-CSF has been with the recombinant protein, approved for use in stem cell and bone marrow transplant patients to reconstitute the myeloid series (sargramostim) or as part of an immunotherapy regimen for prostate cancer (sipuleucel-T). Recombinant GM-CSF enhances phagocytosis of *P. falciparum* blood stage parasites by human neutrophils,[61] has been shown to protect against murine malaria in combination with the synthetic peptide met-enkephalin[62] and has been used to enhance immunogenicity of plasmid malaria vaccines in chimpanzees.[63]

The DNA encoding GM-CSF has previously been used as an adjuvant to recruit dendritic cells to the site of an immune response[64] and to enhance the immunogenicity and protection in murine and NHP malaria models including in neonatal mice.[36,60,65-71] GM-CSF DNA has also been shown to enhance the avidity of elicited immunoglobulin G for SIV envelope glycoproteins and to enhance antibody-dependent cellular cytotoxicity in association with significant improved efficacy against SIV challenge.[72] In other models however, GM-CSF DNA has been reported to both augment and suppress cellular or humoral responses, according to the antigen, mouse strain and whether or not it is combined with other cytokines.[73-76] Human studies of GM-CSF DNA have been restricted to cancer patients, where hGM-CSF-encoding plasmid has been well-tolerated[77,78] and may have shown some benefit.[79] This is the first study where it was used in healthy humans to enhance a prophylactic vaccine against an infectious agent.

The finding that GM-CSF offered no apparent benefit, and indeed may have diminished immunogenicity for class I responses following immunization and following boosting on exposure to malaria challenge,[31] was unexpected, contrasting with our findings in animal malaria models[36,80] as well as those of Li et al.[70] One potential explanation could be non-optimal expression levels. When we studied the injection of murine GM-CSF DNA in mice, it was not possible to detect systemic levels of the protein.[39] While a likely advantage with regard to tolerability, this may

indicate that at the site of induction for the immune response, which could be at the injection site, regional lymph nodes or more distant splenic or hepatic sites, GM-CSF levels were too low or transient to impact the induction of the immune response. On the other hand, the trend toward a delay in the onset of parasitemia in cohort 2 that was not accentuated with higher hGM-CSF doses in cohorts 3 and 4 raises that possibility that tissue levels of hGM-CSF could also have been too high, and that lower doses might have been more effective.

As second explanation for our results could be that GM-CSF was expressed either too early or too late relative to the malaria antigens and that staggered administration would have been more effective (Hartikka J., unpublished data). For example, GM-CSF might trigger the early maturation of dendritic cells, without allowing the opportunity for appropriate antigen uptake and presentation. However, the injection of the plasmid encoding hGM-CSF into humans either prior to or following the injection of MuStDO5 would be impractical, even if it improved the quality of immune response.[81] In addition, plasmids encoding other cytokines have significantly enhanced immunogenicity when co-administered with a DNA vaccine, including IL-15,[82,83] IL-12 and IL-28B,[84] high mobility group box 1[85] and RANTES,[86,87] and may be preferable to GM-CSF. Alternatively, an adjuvant that acts through an immunostimulatory mechanism could bypass the need to include single cytokines as co-treatments.

In summary, the MuStDO5 vaccine trial represented a first step in the development of the DNA vaccine approach to inducing protective cell-mediated immunity targeting multiple pre-erythrocytic stage antigens. The vaccine was safe and well-tolerated and induced IFN-γ responses to all five malaria proteins, supporting the multi-antigen strategy, but it was insufficiently immunogenic, and GM-CSF did not enhance immunogenicity or protection, contrary to expectations based on animal models. Key approaches to improving DNA vaccines, already under study at the time the MuStDO5 trial was performed, include codon optimization and other enhancements to the plasmid, use of more compatible plasmid mixtures to eliminate interference, electroporated administration, use of more concentrated formulations to allow delivery of higher plasmid doses, co-administration with alternative cytokine plasmids, immunostimulatory adjuvants or boosting with recombinant viral vectors such as adenovectors. In follow-on studies of DNA vaccines for the prevention of malaria, we have used codon optimized plasmids, higher doses of DNA (1 mg per plasmid), and boosting with recombinant adenovirus vectors; these three changes, planned at the time that the MuStDO5 trial was performed, have resulted in improved immunogenicity and protection, to be reported in future publications.

Methods

Human subjects research. *Ethical review.* This study was conducted in accordance with federal regulations regarding the protection of human participants in research including 32 CFR 219 (The Common Rule), The Nuremberg Code, The Belmont Report and all pertinent regulations of the Department of Defense, the Department of the Navy, the Department of the Army, the Bureau of Medicine and Surgery of the US Navy and the internal policies for human subject protections and the standards for the responsible conduct of research of the Naval Medical Research Center (NMRC) and US Army Medical Research and Materiel Command (USAMRMC). NMRC holds a Federal Wide Assurance (FWA 00000152) from the Office for Human Research Protections (OHRP). All NMRC key personnel contributing to or performing human research efforts were certified as having completed mandatory human research ethics education curricula and training under the direction of the NMRC Office of Research Administration (ORA) and Human Subjects Protections Program (HSPP). Written informed consent was obtained from all subjects before screening and enrollment in this study. The trial was performed under US Food and Drug Administration Center for Biologics Evaluation and Research (CBER) Investigational New Drug Application BB-IND 8687, sponsored by Vical, Inc.

Human subjects recruitment. Volunteers were pre-screened by telephone under a separate screening protocol and if meeting minimal enrolment criteria (age 18–50, good health, availability over the time course of the trial, no plans for pregnancy/lactation) were invited to the NMRC Clinical Trials Center for screening. After obtaining informed consent, volunteers provided a medical history, received a physical examination and laboratory testing for complete blood count (CBC), serum biochemistries (blood urea nitrogen, creatinine, AST, ALT, CPK, LDH, alkaline phosphatase, bilirubin), hemoglobin A1C and serologic tests to characterize their anti-dsDNA, anti-hepatitis B surface antigen, anti-hepatitis B core antigen, anti-hepatitis C and anti-HIV serologies. Volunteers were excluded if they had a history of malaria, travel to a malaria-endemic area within the previous 12 mo, had plans for pregnancy or fathering a child, had evidence of active hepatitis or HIV infection, were anti dsDNA positive, had a history of splenectomy or were positive on *P. falciparum* sporozoite immunofluorescence test (IFAT). Volunteers were selected according to HLA type, since T cell responses were measured by stimulating PBMC with a limited repertoire of HLA class I and II alleles for which strongly-binding peptides had been identified from the five antigens. Distribution goals were as follows: HLA-A02 supertype (15 class I peptides available from CSP, SSP2/TRAP, EXP1, LSA1 and LSA3) – at least 3 vaccinees and 1 immunological control per cohort; HLA-A03/11 supertype (7 class I peptides available from CSP, SSP2/TRAP, EXP1 and LSA1) at least 2 vaccinees and 1 immunological control per cohort; HLA-A01, HLA-B07 and HLA-B08 supertypes (12 additional class I peptides) to fill remaining slots. Class II typing was not performed and did not influence cohort assignment (a set of 17 DR-binding peptides from the five antigens was used for testing in all volunteers).

Group assignment. Volunteers were sequentially allocated to the staggered vaccine cohorts as they met eligibility requirements, filling each cohort in turn, with the only restriction being to meet the target HLA A02 and HLA A03 super types numbers described above. Thus, if HLA targets for a cohort had not been met, eligible volunteers lacking the sought-for allelic family were moved to the next cohort.

Blinding. This was an open label clinical trial where the researchers and volunteers knew which treatment was being administered (there was only one vaccine treatment in each cohort, as immunological and/or infectivity controls received no injections). However, all laboratory assessments were blinded, including the microscopists who undertook diagnosis of malaria infection during CHMI.

Sample size and statistical analysis. This was the first safety and immunogenicity study in humans of a multivalent DNA malaria vaccine with or without a recombinant plasmid expressing hGM-CSF; for this reason, the sample size of each study group was limited by safety considerations and was not calculated with regard to power for demonstrating a specified level of efficacy. Vaccine efficacy was represented by Kaplan-Meier plots. Log rank tests were used to compare time to parasitemia between control and immunized volunteers for each cohort, and time to parasitemia in A02 and A03 volunteers comparing between cohorts. After normalizing time to parasitemia data for the four cohorts for A02 and A03 volunteers to a common mean, the Mann-Whitney U test was used to compare vaccine recipients and controls. The sign rank test was used to see whether the differences in ELISA and IFA between pre- and post-immunization, and between pre- and post-challenge were equal to 0. The Wilcoxon test was used to evaluate whether the boosting effect for antibodies (difference in immune measures between pre- and post-challenge) was different between control and immunized volunteers for each cohort. ELISA and IFA data were log transformed prior to the analysis. The Bonferroni method was used to adjust P-values for multiple comparisons. Two-sided $p < 0.05$ was considered significant in all tests.

Assessment of safety. Vaccine recipients were observed for 30 min after each immunization and returned on days 2, 7, 14 and 28 for follow-up. Solicited adverse events were recorded by direct questioning on days 0, 2, 7, 10, 14 and 28 following the first and second immunizations and on days 0, 2, 7, 10 and 14 following the third immunization, and by a diary card filled out days 1 through 7 after each immunization to record oral temperature. Solicited adverse events were malaise, fatigue, fever (subjective), chills, rigors, headache, dizziness, myalgia, arthralgia, cough, nausea, vomiting, abdominal pain and dark urine while unsolicited adverse events were identified by the open-ended question, "Have you experienced any other symptoms?" The injection sites were observed for tenderness, swelling, warmth, induration and erythema. Associated axillary lymphadenopathy and limitation of arm movement were also assessed by examination. Safety labs were measured on D0, 2, 7, 14 and 28, and included complete blood count (CBC), urine analysis and blood chemistries (same as for screening, see above). These safety labs were repeated again on day 49 after the last vaccination. An autoimmunity panel (anti-dsDNA ELISA, anti-hGM-CSF) was measured on day 28 after each vaccination and in some volunteers at 24 and 52 weeks after the last vaccination. Hemoglobin A1C, first performed at screening, was repeated at 7 weeks after the last immunization. Monitoring for serious adverse events was conducted throughout the trial (hospitalization, persistent or significant disability, life threatening events such that failure to intervene could result in hospitalization or death).

Local adverse events were graded according to specific criteria (NCI Common Toxicity Criteria Version 2.0).[88] Systemic adverse events were graded as mild (no interference with the activities of daily living, no intervention required), moderate (minimal intervention required to increase comfort and to carry out the daily activities) or severe (preventing daily activities). Each adverse event was classified as definitely (temporal relationship, no alternate etiology), probably (temporal relationship and alternative etiology apparent but less likely), possibly (temporal relationship but alternative etiology more likely) related or unrelated (no temporal relationship or definitive alternative diagnosis) to vaccine administration, and was made according to the principal investigator's medical judgment in coordination with other physicians evaluating the adverse event.

Assessment of efficacy. Volunteers were challenged with malaria 18 d after their third DNA immunization by bites of *Anopheles stephensi* mosquitoes infected with the *P. falciparum* NF54 strain (from which the 3D7 clone is derived)[89] in a secure room in the joint Walter Reed Army Institute of Research (WRAIR)/NMRC insectary. The infected mosquitoes were produced by NMRC at the Biological Research Institute, Rockville, MD and transported to WRAIR/NMRC the morning of the challenge. For each volunteer, five mosquitoes were allowed to feed for five minutes, after which they were dissected to determine if they had taken a blood meal and had a minimum 2+ salivary gland infection for sporozoites.[41] If required, additional mosquitoes were allowed to feed until five infected mosquitoes with a 2+ salivary score had fed on each volunteer. Starting on day 7 after challenge, volunteers were housed at the Navy Lodge on the campus of the National Naval Medical Center in Bethesda, Maryland. Each volunteer had a daily Giemsa-stained thick blood film examined for the presence of asexual malaria parasites by a certified expert microscopist, and confirmed by a second reader. The identity of immunized and non-immunized volunteers was not known by the microscopists reading the malaria smears. Symptomatic, undiagnosed volunteers had additional smears performed at the discretion of the study doctor, not to exceed one smear every 8–12 h. Volunteers who developed malaria were treated with a standard oral dose of chloroquine phosphate (total 1500 mg base given in divided doses: 600 mg initially followed by 300 mg given 6, 24 and 48 h later) under direct supervision with back-up drugs available in case of intolerance. Vomiting was treated with prochlorperazine if indicated to assist with retention of chloroquine doses.

Vaccines. *Vaccine composition.* MuStDO5 contained 5 DNA plasmids encoding genes for *P. falciparum* proteins expressed during the sporozoite and liver stages.[26,27,49] VCL-2510 (*Pf*CSP), VCL-2519 (*Pf*SSP2), VCL-2523 (*Pf*EXP1), VCL-2551 (N'C-term *Pf*LSA1) and VCL-2556 (*Pf*LSA3). Sequences were based on the 3D7 strain of *P. falciparum*. All five transgenes were manufactured by Vical, Inc. The clinical testing of plasmid VCL-2510 containing the full-length gene of *P. falciparum* CSP has been previously reported.[28,29,31,90] The MuStDO5 vaccine was extensively tested for safety in mice and rabbits[39] and was found to be safe and well tolerated without any evidence of inducing autoimmunity. Diagrams of the five plasmids are provided in **Figure 8**. Each is a closed circular DNA plasmid produced under

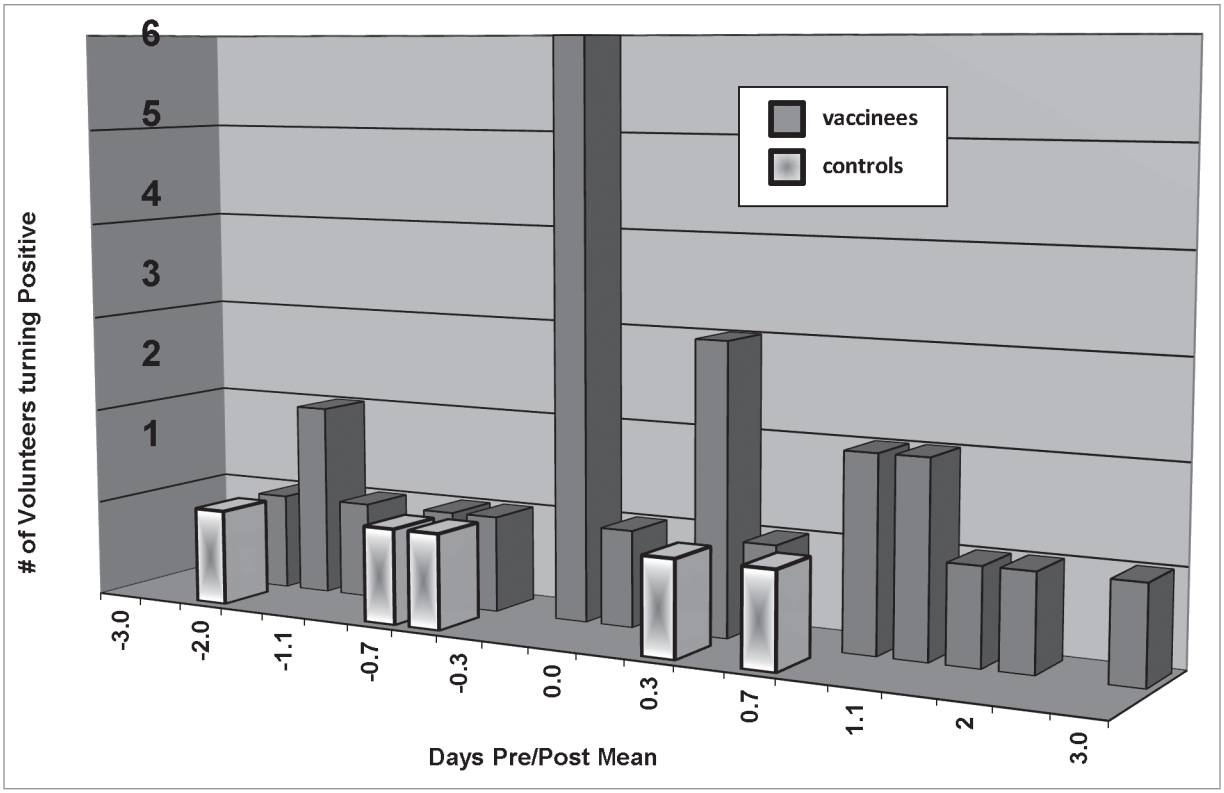

Figure 7. Days to parasitemia of combined HLA A02 and A03 volunteers. Days to parasitemia are shown in HLA A02 and A03 volunteers, comparing vaccinees (gray bars) and infectivity controls (white bars). Since the mean day of onset of parasitemia was different for each of the four challenges, days prior to or following the group-specific mean day of onset were calculated for each volunteer in order to standardize. Data were then combined on a common timeline with mean day of onset for each cohort set to zero. There were no statistically significant differences in time to parasitemia between cohorts (p = 0.52, Wilcoxon) or between immunized volunteers and infectivity controls (p = 0.32, Mann-Whitney).

cGMP from bacterial cells grown in kanamycin selective media. Expression is controlled by the promoter/ enhancer of the human cytomegalovirus immediate early (CMV IE) gene. Each plasmid contains two open reading frame sequences: one encodes the kanamycin resistance protein which is expressed in bacterial cells and the other encodes a human tissue plasminogen activator protein (hTPA) leader / malaria fusion protein which is expressed in mammalian cells.

Vaccine formulation. The MuStDO5 vaccine was vialed at 3 mg/mL (600 μg/mL each plasmid) in phosphate buffered saline (PBS) containing 10 mM sodium phosphate in 0.9% sodium chloride solution, 1.5 mL total per vial. To prepare the vaccine for administration, 0.3 mL was added to the MuStDO5 vial from one of four other vials containing, respectively, 0, 0.12, 0.6 or 3 mg/mL PBS of a sixth plasmid, VCL-1723 (hGM-CSF), creating final concentrations of each malaria plasmid of 500 μg/mL, and a final concentration of hGM-CSF plasmid of 0, 20, 100 or 500 μg/mL (total plasmid concentration of 2.5, 2.52, 2.6 or 3 mg per mL of solution). Vaccine was withdrawn from each vial immediately after mixing, each mL containing 2.5 mg MuStDO5 combined with 0, 20, 100 or 500 μg of hGM-CSF, for administration to cohorts 1–4 respectively.

Vaccine administration. Four cohorts (each n = 8 vaccinees) were immunized with 2.5 mg of the 5 plasmid mixture together with GM-CSF at 0 μg (cohort 1), 20 μg (cohort 2), 100 μg (cohort 3) or 500 μg (cohort 4). The vaccine was injected into the deltoid muscle by needleless jet injection (Biojector® 2000, Bioject, Inc.). Pressure was applied to each injection site for one minute immediately following actuation of the device to limit hematoma formation. Three injections were given at 0, 4 and 8 weeks, alternating arms between injections.

Laboratory testing. *HLA typing.* Class I intermediate resolution molecular typing was performed by Georgetown University C. W. Bill Young/Department of Defense Marrow Donor Program. Molecular HLA class I typing was performed using the Sequence Specific Oligonucleotide Probe (SSOP) method. Volunteers were assigned HLA types (HLA A and HLA B) at the intermediate resolution with their respective HLA allele codes based on the code list at: (www.bioinformatics.nmdp.org/Allele_Code_Lists/Index.html).

Microscopy. Two slides were made for each blood sample. Ten μl of blood was smeared onto each of two 1-cm × 2-cm rectangles on each slide. The slides were dried on a 37°C heat block for 5–10 min. Slides were stained with fresh Giemsa stain (4% solution of stain in phosphate buffered saline pH 7.0–7.2) for 45–60 min, rinsed with water and allowed to dry. The slides were viewed under oil immersion at a total magnification of 1,000×. For asymptomatic individuals, 360 fields were read in 5 vertical passes. For symptomatic individuals, 1,080 fields were read in approximately 15 vertical passes. A volunteer was determined

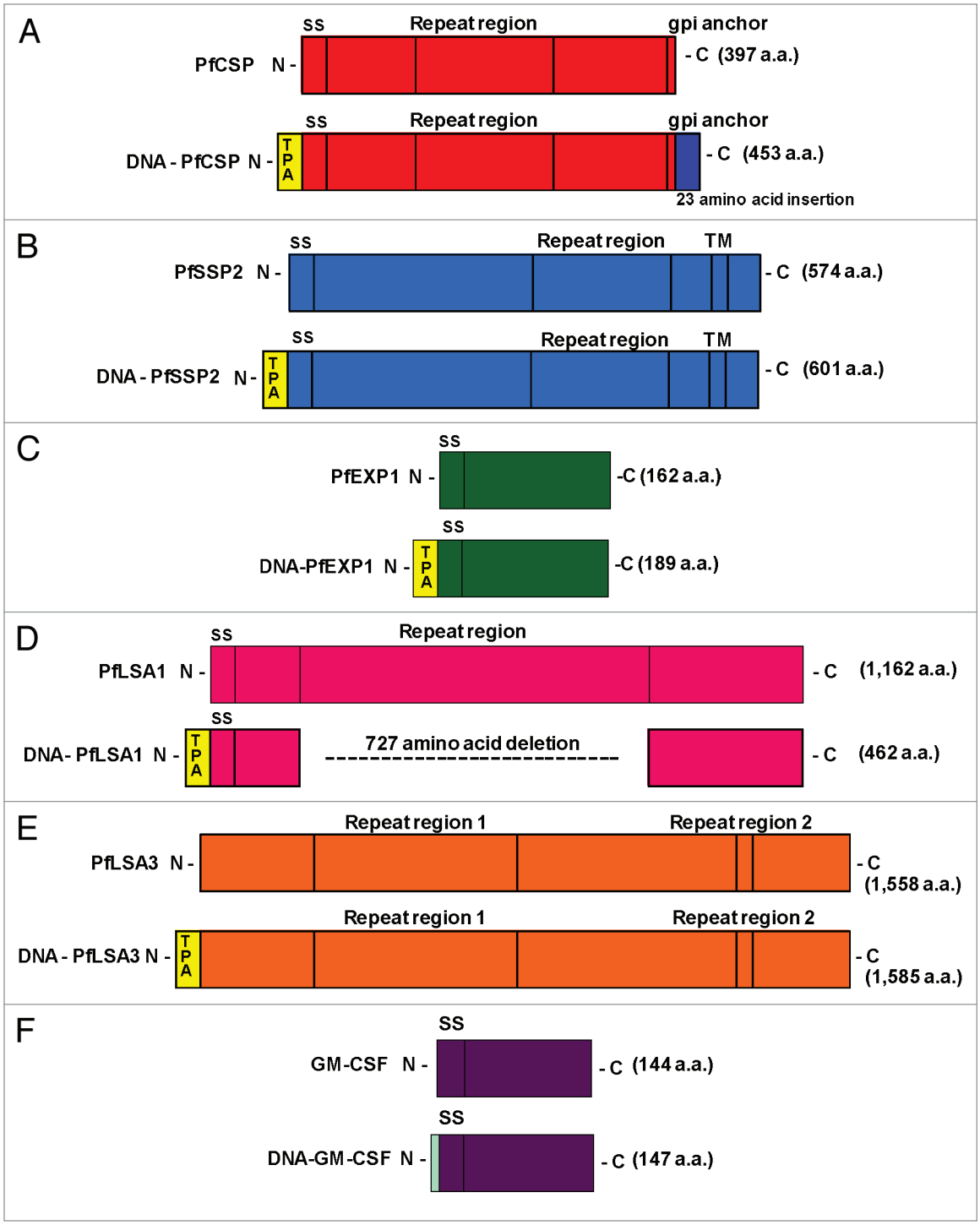

Figure 8. For figure legend, see page 1580.

to be parasitemic when two parasites were found and confirmed by an expert microscopist during the fixed searching routine. The physician on duty was then informed by the microscopist of the volunteer's status and also observed the confirmed parasites. Parasitemia for asymptomatic research subjects was calculated from the number of parasites observed in 5 passes (0.45 μL ^{3}), providing a limit of detection of 4 to 5 parasites/μL, and for symptomatic research subjects was calculated from the number of parasites observed in 15 passes (1.35 μL ^{3}), providing a limit of detection of 1 to 2 parasites/μL. The expert microscopists had extensive experience in malaria microscopy and passed a proficiency test on parasite detection.

Antibody assays. Blood was collected in preservative free heparin, centrifuged, and plasma separated and frozen at -70°C until tested. All time-points for a given volunteer were blinded and assayed together.

Figure 8 (See previous page). Schematic diagram of the DNA gene products. Each panel presents the native protein (above) and the protein expressed by the DNA (below) for the five vaccine antigens and for hGM-CSF. The amino acid length is provided in parentheses to the right of the diagram. Identical colors indicate identical sequences. SS = native signal sequence. TPA = human tissue plasminogen activator leader sequence (increases expression in mammalian cells) (yellow boxes). (**A**) The PfCSP DNA vaccine includes the full length native sequence CSP gene. The locations of the endogenous PfCSP signal sequence, the PfCSP repeat region, the PfCSP glycosylphosphatidylinositol (gpi) anchor and a 23 amino acid C-terminus insertion derived from the transcriptional terminator of the bovine growth hormone gene (dark blue box) are shown. (**B**) The PfSSP2 DNA vaccine includes the full length native sequence SSP2 gene. The locations of the endogenous PfSSP2 signal sequence, the PfSSP2 repeat region and the PfSSP2 transmembrane region are shown. (**C**) The PfEXP1 DNA vaccine includes the full length native sequence EXP1 gene. The location of the endogenous PfEXP1 signal sequence is shown. (**D**) The PfLSA1 DNA vaccine includes the full N- and C- termini of the native sequence LSA1 gene minus the repeat region. The locations of the endogenous PfLSA1 signal sequence and the PfLSA1 repeat region are shown. The 727 amino acid deletion encompassing the PfLSA1 repeat region is represented by dashes. (**E**) The PfLSA3 DNA vaccine includes the full length native sequence LSA3 gene. The locations of the PfLSA3 repeat regions are shown. (**F**) The hGM-CSF DNA plasmid includes the full length native sequence human GM-CSF gene. A 3 amino acid insertion generated during the cloning of the GM-CSF plasmid is located at the N-terminal end and is represented by a light blue box (the first few amino acids of the native GM-CSF protein, MWLQSLLLL, were replaced by MALWILQSLLLL in the DNA construct).

Immunofluorescence antibody (IFA) using sporozoites. Serum antibody levels were assessed by IFA against air-dried sporozoites. NF54 strain sporozoites from infected mosquitoes were suspended in 3% BSA at a concentration of 10^6 sporozoites per mL. An aliquot of 10 μL containing 10^4 sporozoites was delivered into each well of the antigen slide. The antigen slides were allowed to air dry at room temperature and were kept at -70°C until used. An amount of 20 μL of a 2-fold serial dilution of test or control serum in PBS containing 2% BSA was added to each well of the antigen slides. The slides were incubated for one hour at 37°C, washed 3 times in PBS, 5 min each wash. Each well was incubated for 30 min at 37°C with 20 μL of a 1:50 dilution of FITC-labeled goat anti-human IgG (H^+L) (Kirkegarard and Perry). The slides were washed again, mounted in a Vectashield mounting medium (Vector Laboratories, Inc.) and examined under an Olympus UV microscope. Positive control was a serum sample from a volunteer immunized with radiation-attenuated *P. falciparum* sporozoites. Seroconversion was defined as a 4-fold rise, as compared with the pre-immune plasma, against *P. falciparum* parasites.

Immunofluorescence antibody (IFA) using blood stage parasites. Serum antibody levels were assessed as previously described[91] using the NF54 strain of *P. falciparum*. Slides were examined under an Olympus UV microscope and end-point titers were determined as the last dilution above the background that fluorescent parasites were observed. Background fluorescence was established for each group by the pre-immunization serum (pre-bleed).

Immunofluorescence antibody (IFA) using liver stage parasites. Slides containing cryosectioned *P. falciparum* infected chimp liver[92] were stored at -80°C wrapped in foil in a plastic bag. Prior to IFA, tissue section slides were removed from the freezer and placed in a dessicator for equilibration to room temperature. Diluted antiserum was then applied to the sections (in a volume sufficient to cover the tissue) and the slides were processed as previously described.[49] Briefly, the slides were incubated for 30 min. at 37°C in a humidity chamber. Liver section slides were placed in a staining dish and washed 3 times for 5 min with PBS. A fluorescein-conjugated IgG (Kirkegaard and Perry) was used as the secondary antibody. The secondary antibody was diluted 1:40 into PBS containing 0.02% Evan's blue. The Evan's blue was added to act as a counterstain and to suppress any autofluorescence in the tissue. The diluted secondary antibody was added and the slides placed in a humidity chamber, in the dark, and incubated at 37°C for 30 min. Tissue sections were then washed and the slides mounted, using Vectashield mounting medium (Vector Labs). The stained slides were screened with a Nikon Eclipse E600 epifluorescent microscope.

ELISA. Antibodies were measured by the enzyme-linked immunosorbent assay (ELISA) using recombinant proteins (0.5 μg/mL for CSP, 1.0 μg/mL for SSP2/TRAP, 2.0 μg/mL for EXP1, 4.0 μg/mL for LSA1) as previously described,[93,94] Mean + SD of the OD readings of quadruplicate assays were recorded. Samples were considered positive if the mean OD value of the plasma sample post-immunization was greater than the mean OD plus 2 standard deviations of the plasma sample pre-immunization.

Recombinant proteins for ELISA. P. falciparum CSP, SSP2/TRAP and LSA1 were produced as recombinant proteins in *E. coli* with a HIS_6-tag added at the C-terminus to facilitate purification on a Ni-NTA agarose column. These recombinant proteins encoded the following amino acids of their respective molecules from the 3D7 strain of parasite: *Pf*CSP aa L_{19}-N_{405}; *Pf*SSP2/TRAP aa D_{48}-K_{394}; and *Pf*LSA1 aa E_{1628}-L_{1909}. Recombinant *Pf*EXP1 encoding for the full length gene from the K1 strain of parasite was expressed in *E. coli* and was a gift from Hoffmann-La Roche, Basel, Switzerland. The further details for the plasmid construction, recombinant expression and purification of these proteins are available elsewhere.[73]

Peptides. Peptides were chosen by searching the amino acid sequences (*P. falciparum* strain 3D7) of the five proteins for motifs that predict binding to the common HLA class I alleles in the study population. Peptides predicted to bind were synthesized and tested in binding assays to determine those with high affinity.[95] A total of 51 synthetic peptides were selected and studied in this trial including 34 MHC class I-restricted CD8+ T cell epitopes at 8–10 amino acids in length and 17 class II-restricted CD4+ T cell epitopes at 20–23 amino acids in length. All peptides were purchased from AnaSpec at > 90% purity. Of the 34 defined class I-restricted epitopes, 9 were derived from *Pf*CSP, 6 from *Pf*SSP2/TRAP, 4 from *Pf*EXP1, 9 from *Pf*LSA1, and 6 from *Pf*LSA3, restricted by 7 different HLA-A and -B supertype families.[90] Of 17 HLA-DR-restricted epitopes, 4 were derived from *Pf*CSP, 3 from *Pf*SSP2/TRAP, 2 from *Pf*EXP1, 5 from *Pf*LSA1, and 3 from *Pf*LSA3. As previously described[30,31] peptides containing a known HLA-A0201-restricted epitope from influenza matrix protein (residues 58–66, GILGFVFTL) and

a known HLA-A0301-restricted epitope from influenza nuclear protein (residues 265–273, ILRGSVAHK) were used as positive controls, and a peptide containing a known HLA-A0201-restriction from HIV gag protein (residues 77–85, SLYNTVATL) was used as a negative control.

Ex vivo IFN-γ ELISpot assay. The *P. falciparum* antigen-specific IFN-γ-producing cells were quantified by ELISpot assay after 36 h in vitro stimulation in the presence of 10 μg/ml of each peptide (fresh assays, HLA class I-matched peptides tested individually) or 5 μg/ml of each peptide (frozen assays, HLA class I-matched peptides and DR peptides tested in antigen-specific pools) as described previously.[90] Responses were expressed as sfc/10^6 PBMCs, and were considered significant if: (1) the mean number of cells in wells with experimental peptide was significantly greater ($p < 0.05$, student's T test) than in wells with control peptide; (2) the net sfcs/well (mean sfcs in experimental peptide wells minus mean sfcs in control peptide wells) was ≥ 5 sfcs/well; and (3) stimulation index (the ratio of mean sfcs in experimental peptide wells to mean sfcs in control peptide wells) was greater than 2.0. IFN-γ responses were considered to be significantly enhanced by challenge if the number of IFN-γ sfcs/10^6 PBMCs was at least 2 times higher than that before the challenge.

Anti-human GM-CSF. An ELISA protocol specific for the detection of human anti-human GM-CSF immunoglobulin G (anti-hGM-CSF IgG) was developed at Vical Inc. A lyophilized plasma specimen was obtained from the National Institute of Biological Standards and Control (NIBSC) and was used as a positive control for the assay. Normal human sera obtained from Vital Products were used as negative control specimens. A net specific signal was obtained by subtracting non-specific binding in uncoated wells from the signal of wells coated with recombinant human GM-CSF (hGM-CSF) antigen.

Recombinant human GM-CSF (Fitzgerald) was coated on 96-well ½ area plates (Costar cat #3690) at a concentration of 1 μg/mL in carbonate buffer and incubated overnight at 4°C. Blank wells coated with carbonate buffer without antigen were also prepared to test non-specific binding of the samples. The plates were then washed with PBS+ 0.05% Tween-20 (PBST), blocked with 5% nonfat dry milk in PBS and incubated at room temperature for 60 min. The plates were washed with PBST and then incubated at 37°C for 1 h with 50 μL of specimen per well diluted in 1% milk block. Again the plates were washed with PBST, incubated at room temperature for 1 h with 50 μL of secondary antibody [goat anti-human IgG (heavy and light chains) alkaline phosphatase-conjugate (AP), Bio-Rad 170–6521] diluted 1:1000 in 1% milk block. Plates were washed with PBST. Substrate [p-nitro phenyl phosphate, PNPP, (Bio-Rad, 170–1063)] was added, developed for 30 min and optical densities determined at a 405 nm wavelength using a microplate reader and SoftmaxPro software. Net ODs were determined for each sample by subtracting non-specific ODs (carbonate buffer without antigen wells) from corresponding specific ODs (coated antigen wells).

Four criteria were used for identifying and reporting a positive sample: OD signal > 0.5 OD units in a coated well with a 1:10 diluted sample, a net OD signal of > 0.2 OD units for a 1:10 diluted sample, a positive sample titer compared with the positive control, and a > 4 fold increase in net OD signal between pre and post injection samples.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by the Naval Medical Research and Development Command work units 61102A. S13.F.A0009, 62787A.870.F.A0010, 63002A.810.F.A0011, 603792N.01889.135.A0039 and 60000.000.000.A0062 and by the US. Agency for International Development and the Office of Naval Research Advanced Technology Demonstration. T.L.R., J.E.E., R.C.H., W.O.R., T.C.L., D.A.F., R.A.N., B.W.H. and S.L.H. were active duty military personnel at the time they contributed to this work; Y.C., M. Sedegah and D.E.L. were US Government employees. The work of these individuals was prepared as part of official government duties. Title 17 U.S.C. §105 provides that "Copyright protection under this title is not available for any work of the United States Government." Title 17 U.S.C. §101 defines a US Government work as a work prepared by a military service member or employee of the US Government as part of that person's official duties. The study protocol for the clinical trial presented in this manuscript was approved by the NMRC Institutional Review Board, in compliance with all applicable Federal Regulations governing protection of human subjects. All study subjects gave written informed consent. The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, the Department of the Army, the Department of Defense or the US Government.

The authors thank all the study volunteers who participated in the trial. We are grateful to Richard Stout for providing the Biojector® 2000 and for instructions regarding its optimal use, to Sofyan Masbar and Lori Shatney for reading peripheral blood smears, to June Traylor, Maria Malone, Nicole Freiberg, HM1 Verdell Nelson for supporting the clinical conduct of the study and to Carter Diggs for guidance and steadfast support.

Designed the study: TLR, YC, RW, WOR, DLD, SLH; designed the vaccine: RCH, RM, PMH, JAN, LAS, WOR, DLD, SLH; performed the clinical trial: TLR, JEE, TCL, DAF, RAN, PDLV, MPB, LB, SLH; performed the laboratory-based assessments: YC, RW, JCA, JBS, M Sedegah, VFM, ENA, HG, NOR, JGB, MFBB, TGG, RM, JN; provided key reagents: SK, DEL; analyzed the data: TLR, YC, RW, BWH, M Shi, SLH; wrote the manuscript: TLR, SK, KL, JBS, JCA, MRH, DLD, SLH.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/vaccines/article/22129

References

1. WHO. World Malaria Report. 2011.

2. Murray CJ, Rosenfeld LC, Lim SS, Andrews KG, Foreman KJ, Haring D, et al. Global malaria mortality between 1980 and 2010: a systematic analysis. Lancet 2012; 379:413-31; PMID:22305225; http://dx.doi.org/10.1016/S0140-6736(12)60034-8.
3. Agnandji ST, Lell B, Soulanoudjingar SS, Fernandes JF, Abossolo BP, Conzelmann C, et al. RTS,S Clinical Trials Partnership. First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. N Engl J Med 2011; 365:1863-75; PMID:22007715; http://dx.doi.org/10.1056/NEJMoa1102287.
4. Moorthy VS, Ballou WR. Immunological mechanisms underlying protection mediated by RTS,S: a review of the available data. Malar J 2009; 8:312; PMID:20042088; http://dx.doi.org/10.1186/1475-2875-8-312.
5. Clyde DF. Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites. Am J Trop Med Hyg 1975; 24:397-401; PMID:808142.
6. Rieckmann KH, Carson PE, Beaudoin RL, Cassells JS, Sell KW. Letter: Sporozoite induced immunity in man against an Ethiopian strain of Plasmodium falciparum. Trans R Soc Trop Med Hyg 1974; 68:258-9; PMID:4608063; http://dx.doi.org/10.1016/0035-9203(74)90129-1.
7. Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, et al. Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. J Infect Dis 2002; 185:1155-64; PMID:11930326; http://dx.doi.org/10.1086/339409.
8. Roestenberg M, McCall M, Hopman J, Wiersma J, Luty AJ, van Gemert GJ, et al. Protection against a malaria challenge by sporozoite inoculation. N Engl J Med 2009; 361:468-77; PMID:19641203; http://dx.doi.org/10.1056/NEJMoa0805832.
9. Roestenberg M, Teirlinck AC, McCall MB, Teelen K, Makamdop KN, Wiersma J, et al. Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. Lancet 2011; 377:1770-6; PMID:21514658; http://dx.doi.org/10.1016/S0140-6736(11)60360-7.
10. Epstein JE, Tewari K, Lyke KE, Sim BK, Billingsley PF, Laurens MB, et al. Live attenuated malaria vaccine designed to protect through hepatic CD8$^+$ T cell immunity. Science 2011; 334:475-80; PMID:21903775; http://dx.doi.org/10.1126/science.1211548.
11. Hoffman SL, Weiss W, Mellouk S, Sedegah M. Irradiated sporozoite vaccine induces cytotoxic T lymphocytes that recognize malaria antigens on the surface of infected hepatocytes. Immunol Lett 1990; 25:33-8; PMID:2283160; http://dx.doi.org/10.1016/0165-2478(90)90087-7.
12. Weiss WR, Mellouk S, Houghten RA, Sedegah M, Kumar S, Good MF, et al. Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes. J Exp Med 1990; 171:763-73; PMID:1689762; http://dx.doi.org/10.1084/jem.171.3.763.
13. Hoffman SL, Sedegah M, Hedstrom RC. Protection against malaria by immunization with a Plasmodium yoelii circumsporozoite protein nucleic acid vaccine. Vaccine 1994; 12:1529-33; PMID:7879419; http://dx.doi.org/10.1016/0264-410X(94)90078-7.
14. Sedegah M, Hedstrom R, Hobart P, Hoffman SL. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. Proc Natl Acad Sci USA 1994; 91:9866-70; PMID:7937907; http://dx.doi.org/10.1073/pnas.91.21.9866.
15. Sedegah M, Jones TR, Kaur M, Hedstrom R, Hobart P, Tine JA, et al. Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine. Proc Natl Acad Sci USA 1998; 95:7648-53; PMID:9636204; http://dx.doi.org/10.1073/pnas.95.13.7648.
16. Rogers WO, Weiss WR, Kumar A, Aguiar JC, Tine JA, Gwadz R, et al. Protection of rhesus macaques against lethal Plasmodium knowlesi malaria by a heterologous DNA priming and poxvirus boosting immunization regimen. Infect Immun 2002; 70:4329-35; PMID:12117942; http://dx.doi.org/10.1128/IAI.70.8.4329-4335.2002.
17. Dunachie SJ, Walther M, Epstein JE, Keating S, Berthoud T, Andrews L, et al. A DNA prime-modified vaccinia virus ankara boost vaccine encoding thrombospondin-related adhesion protein but not circumsporozoite protein partially protects healthy malaria-naive adults against Plasmodium falciparum sporozoite challenge. Infect Immun 2006; 74:5933-42; PMID:16988273; http://dx.doi.org/10.1128/IAI.00590-06.
18. Webster DP, Dunachie S, Vuola JM, Berthoud T, Keating S, Laidlaw SM, et al. Enhanced T cell-mediated protection against malaria in human challenges by using the recombinant poxviruses FP9 and modified vaccinia virus Ankara. Proc Natl Acad Sci USA 2005; 102:4836-41; PMID:15781866; http://dx.doi.org/10.1073/pnas.0406381102.
19. Sedegah M, Tamminga C, McGrath S, House B, Ganeshan H, Lejano J, et al. Adenovirus 5-vectored P. falciparum vaccine expressing CSP and AMA1. Part A: safety and immunogenicity in seronegative adults. PLoS ONE 2011; 6:e24586; PMID:22003383; http://dx.doi.org/10.1371/journal.pone.0024586.
20. Tamminga C, Sedegah M, Regis D, Chuang I, Epstein JE, Spring M, et al. Adenovirus-5-vectored P. falciparum vaccine expressing CSP and AMA1. Part B: safety, immunogenicity and protective efficacy of the CSP component. PLoS ONE 2011; 6:e25868; PMID:22003411; http://dx.doi.org/10.1371/journal.pone.0025868.
21. Reyes-Sandoval A, Sridhar S, Berthoud T, Moore AC, Harty JT, Gilbert SC, et al. Single-dose immunogenicity and protective efficacy of simian adenoviral vectors against Plasmodium berghei. Eur J Immunol 2008; 38:732-41; PMID:18266272; http://dx.doi.org/10.1002/eji.200737672.
22. Reyes-Sandoval A, Berthoud T, Alder N, Siani L, Gilbert SC, Nicosia A, et al. Prime-boost immunization with adenoviral and modified vaccinia virus Ankara vectors enhances the durability and polyfunctionality of protective malaria CD8+ T-cell responses. Infect Immun 2010; 78:145-53; PMID:19858306; http://dx.doi.org/10.1128/IAI.00740-09.
23. Narum DL, Thomas AW. Differential localization of full-length and processed forms of PF83/AMA-1 an apical membrane antigen of Plasmodium falciparum merozoites. Mol Biochem Parasitol 1994; 67:59-68; PMID:7838184; http://dx.doi.org/10.1016/0166-6851(94)90096-5.
24. Robson KJ, Hall JR, Jennings MW, Harris TJ, Marsh K, Newbold CI, et al. A highly conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite. Nature 1988; 335:79-82; PMID:3045563; http://dx.doi.org/10.1038/335079a0.
25. Rogers WO, Malik A, Mellouk S, Nakamura K, Rogers MD, Szarfman A, et al. Characterization of Plasmodium falciparum sporozoite surface protein 2. Proc Natl Acad Sci USA 1992; 89:9176-80; PMID:1409621; http://dx.doi.org/10.1073/pnas.89.19.9176.
26. Doolan DL, Hoffman SL. DNA-based vaccines against malaria: status and promise of the Multi-Stage Malaria DNA Vaccine Operation. Int J Parasitol 2001; 31:753-62; PMID:11403765; http://dx.doi.org/10.1016/S0020-7519(01)00184-9.
27. Kumar S, Epstein JE, Richie TL, Nkrumah FK, Soisson L, Carucci DJ, et al. A multilateral effort to develop DNA vaccines against falciparum malaria. Trends Parasitol 2002; 18:129-35; PMID:11854091; http://dx.doi.org/10.1016/S1471-4922(01)02207-3.
28. Le TP, Coonan KM, Hedstrom RC, Charoenvit Y, Sedegah M, Epstein JE, et al. Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers. Vaccine 2000; 18:1893-901; PMID:10699338; http://dx.doi.org/10.1016/S0264-410X(99)00407-7.
29. Epstein JE, Gorak EJ, Charoenvit Y, Wang R, Freydberg N, Osinowo O, et al. Safety, tolerability, and lack of antibody responses after administration of a PfCSP DNA malaria vaccine via needle or needle-free jet injection, and comparison of intramuscular and combination intramuscular/intradermal routes. Hum Gene Ther 2002; 13:1551-60; PMID:12228010; http://dx.doi.org/10.1089/10430340260201644.
30. Wang R, Doolan DL, Le TP, Hedstrom RC, Coonan KM, Charoenvit Y, et al. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. Science 1998; 282:476-80; PMID:9774275; http://dx.doi.org/10.1126/science.282.5388.476.
31. Wang R, Richie TL, Baraceros MF, Rahardjo N, Gay T, Banania JG, et al. Boosting of DNA vaccine-elicited gamma interferon responses in humans by exposure to malaria parasites. Infect Immun 2005; 73:2863-72; PMID:15845492; http://dx.doi.org/10.1128/IAI.73.5.2863-2872.2005.
32. Nussenzweig V, Nussenzweig RS. Circumsporozoite proteins of malaria parasites. Cell 1985; 42:401-3; PMID:2411417; http://dx.doi.org/10.1016/0092-8674(85)90093-5.
33. Doolan DL, Hedstrom RC, Rogers WO, Charoenvit Y, Rogers M, de la Vega P, et al. Identification and characterization of the protective hepatocyte erythrocyte protein 17 kDa gene of Plasmodium yoelii, homolog of Plasmodium falciparum exported protein 1. J Biol Chem 1996; 271:17861-8; PMID:8663412; http://dx.doi.org/10.1074/jbc.271.30.17861.
34. Zhu J, Hollingdale MR. Structure of Plasmodium falciparum liver stage antigen-1. Mol Biochem Parasitol 1991; 48:223-6; PMID:1840628; http://dx.doi.org/10.1016/0166-6851(91)90117-O.
35. Prieur E, Druilhe P. The malaria candidate vaccine liver stage antigen-3 is highly conserved in Plasmodium falciparum isolates from diverse geographical areas. Malar J 2009; 8:247; PMID:19874576; http://dx.doi.org/10.1186/1475-2875-8-247.
36. Weiss WR, Ishii KJ, Hedstrom RC, Sedegah M, Ichino M, Barnhart K, et al. A plasmid encoding murine granulocyte-macrophage colony-stimulating factor increases protection conferred by a malaria DNA vaccine. J Immunol 1998; 161:2325-32; PMID:9725227.
37. Hamilton JA. Colony-stimulating factors in inflammation and autoimmunity. Nat Rev Immunol 2008; 8:533-44; PMID:18551128; http://dx.doi.org/10.1038/nri2356.
38. Mirza S, Walker A, Chen J, Murphy JM, Young IG. The Ig-like domain of human GM-CSF receptor alpha plays a critical role in cytokine binding and receptor activation. Biochem J 2010; 426:307-17; PMID:20078425; http://dx.doi.org/10.1042/BJ20091745.
39. Parker SE, Monteith D, Horton H, Hof R, Hernandez P, Vilalta A, et al. Safety of a GM-CSF adjuvant-plasmid DNA malaria vaccine. Gene Ther 2001; 8:1011-23; PMID:11438836; http://dx.doi.org/10.1038/sj.gt.3301491.
40. Chen Y, Webster RG, Woodland DL. Induction of CD8+ T cell responses to dominant and subdominant epitopes and protective immunity to Sendai virus infection by DNA vaccination. J Immunol 1998; 160:2425-32; PMID:9498786.
41. Chulay JD, Schneider I, Cosgriff TM, Hoffman SL, Ballou WR, Quakyi IA, et al. Malaria transmitted to humans by mosquitoes infected from cultured Plasmodium falciparum. Am J Trop Med Hyg 1986; 35:66-8; PMID:3511753.

42. Sinden RE. A biologist's perspective on malaria vaccine development. Hum Vaccin 2010; 6:3-11; PMID:19946205; http://dx.doi.org/10.4161/hv.6.1.9604.
43. Khusmith S, Charoenvit Y, Kumar S, Sedegah M, Beaudoin RL, Hoffman SL. Protection against malaria by vaccination with sporozoite surface protein 2 plus CS protein. Science 1991; 252:715-8; PMID:1827210; http://dx.doi.org/10.1126/science.1827210.
44. Baer K, Klotz C, Kappe SH, Schnieder T, Frevert U. Release of hepatic Plasmodium yoelii merozoites into the pulmonary microvasculature. PLoS Pathog 2007; 3:e171; PMID:17997605; http://dx.doi.org/10.1371/journal.ppat.0030171.
45. Jiang G, Charoenvit Y, Moreno A, Baraceros MF, Banania G, Richie N, et al. Induction of multi-antigen multi-stage immune responses against Plasmodium falciparum in rhesus monkeys, in the absence of antigen interference, with heterologous DNA prime/poxvirus boost immunization. Malar J 2007; 6:135; PMID:17925026; http://dx.doi.org/10.1186/1475-2875-6-135.
46. Jiang G, Shi M, Conteh S, Richie N, Banania G, Geneshan H, et al. Sterile protection against Plasmodium knowlesi in rhesus monkeys from a malaria vaccine: comparison of heterologous prime boost strategies. PLoS ONE 2009; 4:e6559; PMID:19668343; http://dx.doi.org/10.1371/journal.pone.0006559.
47. Collins WE, Contacos PG, Jumper JR, Smith CS, Skinner JC. Studies on the exoerythrocytic stages of simian malaria. 8. Plasmodium knowlesi. J Parasitol 1973; 59:344-52; PMID:4633177; http://dx.doi.org/10.2307/3278832.
48. Doolan DL, Sedegah M, Hedstrom RC, Hobart P, Charoenvit Y, Hoffman SL. Circumventing genetic restriction of protection against malaria with multigene DNA immunization: CD8+ cell-, interferon gamma-, and nitric oxide-dependent immunity. J Exp Med 1996; 183:1739-46; PMID:8666931; http://dx.doi.org/10.1084/jem.183.4.1739.
49. Hedstrom RC, Doolan DL, Wang R, Kumar A, Sacci JB Jr., Gardner MJ, et al. In vitro expression and in vivo immunogenicity of Plasmodium falciparum pre-erythrocytic stage DNA vaccines. Int J Mol Med 1998; 2:29-38; PMID:9854139.
50. Sedegah M, Charoenvit Y, Minh L, Belmonte M, Majam VF, Abot S, et al. Reduced immunogenicity of DNA vaccine plasmids in mixtures. Gene Ther 2004; 11:448-56; PMID:14973538; http://dx.doi.org/10.1038/sj.gt.3302139.
51. Bacon DJ, Sedegah M. Reduced production of RNA transcripts from individual DNA plasmids given in a multivalent DNA vaccine formula. Hum Vaccin 2007; 3:48-53; PMID:17312401; http://dx.doi.org/10.4161/hv.3.2.3833.
52. Robertson JS. Safety considerations for nucleic acid vaccines. Vaccine 1994; 12:1526-8; PMID:7879418; http://dx.doi.org/10.1016/0264-410X(94)90077-9.
53. Chen TT, Levy R. Induction of autoantibody responses to GM-CSF by hyperimmunization with an Id-GM-CSF fusion protein. J Immunol 1995; 154:3105-17; PMID:7897201.
54. Clarkson PM, Kearns AK, Rouzier P, Rubin R, Thompson PD. Serum creatine kinase levels and renal function measures in exertional muscle damage. Med Sci Sports Exerc 2006; 38:623-7; PMID:16679975; http://dx.doi.org/10.1249/01.mss.0000210192.49210.fc.
55. Kenney K, Landau ME, Gonzalez RS, Hundertmark J, O'Brien K, Campbell WW. Serum creatine kinase after exercise: drawing the line between physiological response and exertional rhabdomyolysis. Muscle Nerve 2012; 45:356-62; PMID:22334169; http://dx.doi.org/10.1002/mus.22317.
56. Gilbert SC, Moorthy VS, Andrews L, Pathan AA, McConkey SJ, Vuola JM, et al. Synergistic DNA-MVA prime-boost vaccination regimes for malaria and tuberculosis. Vaccine 2006; 24:4554-61; PMID:16150517; http://dx.doi.org/10.1016/j.vaccine.2005.08.048.
57. Gorse GJ, Newman MJ, deCamp A, Hay CM, De Rosa SC, Noonan E, et al.; NIAID HIV Vaccine Trials Network. DNA and modified vaccinia virus Ankara vaccines encoding multiple cytotoxic and helper T-lymphocyte epitopes of human immunodeficiency virus type 1 (HIV-1) are safe but weakly immunogenic in HIV-1-uninfected, vaccinia virus-naive adults. Clin Vaccine Immunol 2012; 19:649-58; PMID:22398243; http://dx.doi.org/10.1128/CVI.00038-12.
58. Narum DL, Kumar S, Rogers WO, Fuhrmann SR, Liang H, Oakley M, et al. Codon optimization of gene fragments encoding Plasmodium falciparum merozoite proteins enhances DNA vaccine protein expression and immunogenicity in mice. Infect Immun 2001; 69:7250-3; PMID:11705894; http://dx.doi.org/10.1128/IAI.69.12.7250-7253.2001.
59. Vasan S, Hurley A, Schlesinger SJ, Hannaman D, Gardiner DF, Dugin DP, et al. In vivo electroporation enhances the immunogenicity of an HIV-1 DNA vaccine candidate in healthy volunteers. PLoS ONE 2011; 6:e19252; PMID:21603651; http://dx.doi.org/10.1371/journal.pone.0019252.
60. Sedegah M, Weiss W, Sacci JB Jr., Charoenvit Y, Hedstrom R, Gowda K, et al. Improving protective immunity induced by DNA-based immunization: priming with antigen and GM-CSF-encoding plasmid DNA and boosting with antigen-expressing recombinant poxvirus. J Immunol 2000; 164:5905-12; PMID:10820272.
61. Kumaratilake LM, Ferrante A, Jaeger T, Rzepczyk C. GM-CSF-induced priming of human neutrophils for enhanced phagocytosis and killing of asexual blood stages of Plasmodium falciparum: synergistic effects of GM-CSF and TNF. Parasite Immunol 1996; 18:115-23; PMID:9223165; http://dx.doi.org/10.1046/j.1365-3024.1996.d01-64.x.
62. Singh PP, Singh S. Protection of mice from malaria after co-administration of recombinant mouse granulocyte-macrophage colony- stimulating factor and methionine-enkephalin. Eur Cytokine Netw 2001; 12:528-36; PMID:11566634.
63. Schneider J, Langermans JA, Gilbert SC, Blanchard TJ, Twigg S, Naitza S, et al. A prime-boost immunisation regimen using DNA followed by recombinant modified vaccinia virus Ankara induces strong cellular immune responses against the Plasmodium falciparum TRAP antigen in chimpanzees. Vaccine 2001; 19:4595-602; PMID:11535306; http://dx.doi.org/10.1016/S0264-410X(01)00260-2.
64. Haddad D, Ramprakash J, Sedegah M, Charoenvit Y, Baumgartner R, Kumar S, et al. Plasmid vaccine expressing granulocyte-macrophage colony-stimulating factor attracts infiltrates including immature dendritic cells into injected muscles. J Immunol 2000; 165:3772-81; PMID:11034382.
65. Sedegah M, Brice GT, Rogers WO, Doolan DL, Charoenvit Y, Jones TR, et al. Persistence of protective immunity to malaria induced by DNA priming and poxvirus boosting: characterization of effector and memory CD8(+)-T-cell populations. Infect Immun 2002; 70:3493-9; PMID:12065488; http://dx.doi.org/10.1128/IAI.70.7.3493-3499.2002.
66. Sedegah M, Belmonte M, Epstein JE, Siegrist CA, Weiss WR, Jones TR, et al. Successful induction of CD8 T cell-dependent protection against malaria by sequential immunization with DNA and recombinant poxvirus of neonatal mice born to immune mothers. J Immunol 2003; 171:3148-53; PMID:12960342.
67. Kumar S, Miller LH, Quakyi IA, Keister DB, Houghten RA, Maloy WL, et al. Cytotoxic T cells specific for the circumsporozoite protein of Plasmodium falciparum. Nature 1988; 334:258-60; PMID:2456467; http://dx.doi.org/10.1038/334258a0.
68. Sakai T, Horii T, Hisaeda H, Zhang M, Ishii K, Nakano Y, et al. DNA immunization with Plasmodium falciparum serine repeat antigen: regulation of humoral immune response by coinoculation of cytokine expression plasmid. Parasitol Int 1999; 48:27-33; PMID:11269323; http://dx.doi.org/10.1016/S1383-5769(98)00036-1.
69. Sanchez GI, Sedegah M, Rogers WO, Jones TR, Sacci J, Witney A, et al. Immunogenicity and protective efficacy of a Plasmodium yoelii Hsp60 DNA vaccine in BALB/c mice. Infect Immun 2001; 69:3897-905; PMID:11349057; http://dx.doi.org/10.1128/IAI.69.6.3897-3905.2001.
70. Li N, Yu YZ, Yu WY, Sun ZW. Enhancement of the immunogenicity of DNA replicon vaccine of Clostridium botulinum neurotoxin serotype A by GM-CSF gene adjuvant. Immunopharmacol Immunotoxicol 2011; 33:211-9; PMID:21284488; http://dx.doi.org/10.3109/08923971003782327.
71. Bråve A, Ljungberg K, Boberg A, Rollman E, Isaguliants M, Lundgren B, et al. Multigene/multisubtype HIV-1 vaccine induces potent cellular and humoral immune responses by needle-free intradermal delivery. Mol Ther 2005; 12:1197-205; PMID:16112909; http://dx.doi.org/10.1016/j.ymthe.2005.06.473.
72. Lai L, Kwa S, Kozlowski PA, Montefiori DC, Ferrari G, Johnson WE, et al. Prevention of infection by a granulocyte-macrophage colony-stimulating factor co-expressing DNA/modified vaccinia Ankara simian immunodeficiency virus vaccine. J Infect Dis 2011; 204:164-73; PMID:21628671; http://dx.doi.org/10.1093/infdis/jir199.
73. Sedegah M, Charoenvit Y, Aguiar J, Sacci J, Hedstrom R, Kumar S, et al. Effect on antibody and T-cell responses of mixing five GMP-produced DNA plasmids and administration with plasmid expressing GM-CSF. Genes Immun 2004; 5:553-61; PMID:15318164; http://dx.doi.org/10.1038/sj.gene.6364125.
74. Rodríguez AM, Pascutti MF, Maeto C, Falivene J, Holgado MP, Turk G, et al. IL-12 and GM-CSF in DNA/MVA immunizations against HIV-1 CRF12_BF Nef induced T-cell responses with an enhanced magnitude, breadth and quality. PLoS ONE 2012; 7:e37801; PMID:22655069; http://dx.doi.org/10.1371/journal.pone.0037801.
75. Dobaño C, Widera G, Rabussay D, Doolan DL. Enhancement of antibody and cellular immune responses to malaria DNA vaccines by in vivo electroporation. Vaccine 2007; 25:6635-45; PMID:17669562; http://dx.doi.org/10.1016/j.vaccine.2007.06.036.
76. Wunderlich G, Moura IC, del Portillo HA. Genetic immunization of BALB/c mice with a plasmid bearing the gene coding for a hybrid merozoite surface protein 1-hepatitis B virus surface protein fusion protects mice against lethal Plasmodium chabaudi chabaudi PC1 infection. Infect Immun 2000; 68:5839-45; PMID:10992493; http://dx.doi.org/10.1128/IAI.68.10.5839-5845.2000.
77. Cassaday RD, Sondel PM, King DM, Macklin MD, Gan J, Warner TF, et al. A phase I study of immunization using particle-mediated epidermal delivery of genes for gp100 and GM-CSF into uninvolved skin of melanoma patients. Clin Cancer Res 2007; 13:540-9; PMID:17255276; http://dx.doi.org/10.1158/1078-0432.CCR-06-2039.
78. Perales MA, Yuan J, Powel S, Gallardo HF, Rasalan TS, Gonzalez C, et al. Phase I/II study of GM-CSF DNA as an adjuvant for a multipeptide cancer vaccine in patients with advanced melanoma. Mol Ther 2008; 16:2022-9; PMID:18797450; http://dx.doi.org/10.1038/mt.2008.196.
79. Senzer N, Barve M, Kuhn J, Melnyk A, Beitsch P, Lazar M, et al. Phase I trial of "bi-shRNAi(furin)/GMCSF DNA/autologous tumor cell" vaccine (FANG) in advanced cancer. Mol Ther 2012; 20:679-86; PMID:22186789; http://dx.doi.org/10.1038/mt.2011.269.

80. Kumar S, Villinger F, Oakley M, Aguiar JC, Jones TR, Hedstrom RC, et al. A DNA vaccine encoding the 42 kDa C-terminus of merozoite surface protein 1 of Plasmodium falciparum induces antibody, interferon-gamma and cytotoxic T cell responses in rhesus monkeys: immuno-stimulatory effects of granulocyte macrophage-colony stimulating factor. Immunol Lett 2002; 81:13-24; PMID:11841841; http://dx.doi.org/10.1016/S0165-2478(01)00316-9.
81. Kusakabe K, Xin KQ, Katoh H, Sumino K, Hagiwara E, Kawamoto S, et al. The timing of GM-CSF expression plasmid administration influences the Th1/Th2 response induced by an HIV-1-specific DNA vaccine. J Immunol 2000; 164:3102-11; PMID:10706700.
82. Kraynyak KA, Kutzler MA, Cisper NJ, Laddy DJ, Morrow MP, Waldmann TA, et al. Plasmid-encoded interleukin-15 receptor alpha enhances specific immune responses induced by a DNA vaccine in vivo. Hum Gene Ther 2009; 20:1143-56; PMID:19530914; http://dx.doi.org/10.1089/hum.2009.025.
83. Ramanathan MP, Kutzler MA, Kuo YC, Yan J, Liu H, Shah V, et al. Coimmunization with an optimized IL15 plasmid adjuvant enhances humoral immunity via stimulating B cells induced by genetically engineered DNA vaccines expressing consensus JEV and WNV E DIII. Vaccine 2009; 27:4370-80; PMID:19497647; http://dx.doi.org/10.1016/j.vaccine.2009.01.137.
84. Morrow MP, Pankhong P, Laddy DJ, Schoenly KA, Yan J, Cisper N, et al. Comparative ability of IL-12 and IL-28B to regulate Treg populations and enhance adaptive cellular immunity. Blood 2009; 113:5868-77; PMID:19304955; http://dx.doi.org/10.1182/blood-2008-11-190520.
85. Muthumani G, Laddy DJ, Sundaram SG, Fagone P, Shedlock DJ, Kannan S, et al. Co-immunization with an optimized plasmid-encoded immune stimulatory interleukin, high-mobility group box 1 protein, results in enhanced interferon-gamma secretion by antigen-specific CD8 T cells. Immunology 2009; 128(Suppl):e612-20; PMID:19740322; http://dx.doi.org/10.1111/j.1365-2567.2009.03044.x.
86. Belisle SE, Yin J, Shedlock DJ, Dai A, Yan J, Hirao L, et al. Long-term programming of antigen-specific immunity from gene expression signatures in the PBMC of rhesus macaques immunized with an SIV DNA vaccine. PLoS ONE 2011; 6:e19681; PMID:21701683; http://dx.doi.org/10.1371/journal.pone.0019681.
87. Yin J, Dai A, Lecureux J, Arango T, Kutzler MA, Yan J, et al. High antibody and cellular responses induced to HIV-1 clade C envelope following DNA vaccines delivered by electroporation. Vaccine 2011; 29:6763-70; PMID:21195801; http://dx.doi.org/10.1016/j.vaccine.2010.12.055.
88. Institute NC. Common Toxicity Criteria Version 2.0. In: http://ctepcancergov/protocolDevelopment/electronic_applications/ctchtm; 1999.
89. Rickman LS, Jones TR, Long GW, Paparello S, Schneider I, Paul CF, et al. Plasmodium falciparum-infected Anopheles stephensi inconsistently transmit malaria to humans. Am J Trop Med Hyg 1990; 43:441-5; PMID:2240371.
90. Wang R, Epstein J, Baraceros FM, Gorak EJ, Charoenvit Y, Carucci DJ, et al. Induction of CD4(+) T cell-dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine. Proc Natl Acad Sci USA 2001; 98:10817-22; PMID:11526203; http://dx.doi.org/10.1073/pnas.181123498.
91. Aguiar JC, Hedstrom RC, Rogers WO, Charoenvit Y, Sacci JB Jr., Lanar DE, et al. Enhancement of the immune response in rabbits to a malaria DNA vaccine by immunization with a needle-free jet device. Vaccine 2001; 20:275-80; PMID:11567774; http://dx.doi.org/10.1016/S0264-410X(01)00273-0.
92. Szarfman A, Lyon JA, Walliker D, Quakyi I, Howard RJ, Sun S, et al. Mature liver stages of cloned Plasmodium falciparum share epitopes with proteins from sporozoites and asexual blood stages. Parasite Immunol 1988; 10:339-51; PMID:2457864; http://dx.doi.org/10.1111/j.1365-3024.1988.tb00225.x.
93. Charoenvit Y, Majam VF, Corradin G, Sacci JB Jr., Wang R, Doolan DL, et al. CD4(+) T-cell- and gamma interferon-dependent protection against murine malaria by immunization with linear synthetic peptides from a Plasmodium yoelii 17-kilodalton hepatocyte erythrocyte protein. Infect Immun 1999; 67:5604-14; PMID:10531206.
94. Dodoo D, Hollingdale MR, Anum D, Koram KA, Gyan B, Akanmori BD, et al. Measuring naturally acquired immune responses to candidate malaria vaccine antigens in Ghanaian adults. Malar J 2011; 10:168; PMID:21689436; http://dx.doi.org/10.1186/1475-2875-10-168.
95. Doolan DL, Wizel B, Hoffman SL. Class I HLA-restricted cytotoxic T lymphocyte responses against malaria--elucidation on the basis of HLA peptide binding motifs. Immunol Res 1996; 15:280-305; PMID:8988396; http://dx.doi.org/10.1007/BF02935313.
96. Weiss WR, Jiang CG. Protective CD8+ T lymphocytes in primates immunized with malaria sporozoites. PLoS ONE 2012; 7:e31247; PMID: 22355349.