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Induction in Humans of CD8$^+$ and CD4$^+$ T Cell and Antibody Responses by Sequential Immunization with Malaria DNA and Recombinant Protein[1]

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Vaccine-induced protection against diseases like malaria, AIDS, and cancer may require induction of Ag-specific CD8$^+$ and CD4$^+$ T cell and Ab responses in the same individual. In humans, a recombinant *Plasmodium falciparum* circumsporozoite protein (PfCSP) candidate vaccine, RTS,S/adjuvant system number 2A (AS02A), induces T cells and Abs, but no measurable CD8$^+$ T cells by CTL or short-term (ex vivo) IFN-γ ELISPOT assays, and partial short-term protection. *P. falciparum* DNA vaccines elicit CD8$^+$ T cells by these assays, but no protection. We report that sequential immunization with a PfCSP DNA vaccine and RTS,S/AS02A induced PfCSP-specific Abs and Th1 CD4$^+$ T cells, and CD8$^+$ cytotoxic and Tc1 T cells. Depending upon the immunization regime, CD4$^+$ T cells were involved in both the induction and production phases of PfCSP-specific IFN-γ responses, whereas, CD8$^+$ T cells were involved only in the production phase. IFN-γ mRNA up-regulation was detected in both CD45RA$^-$ (CD45RO$^+$) and CD45RA$^+$CD4$^+$ and CD8$^+$ T cell populations after stimulation with PfCSP peptides. This finding suggests CD45RA$^+$ cells function as effector T cells. The induction in humans of the three primary Ag-specific adaptive immune responses establishes a strategy for developing immunization regimens against diseases in desperate need of vaccines. ***The Journal of Immunology*****, 2004, 172: 5561–5569.**

RTS,S is a vaccine in which the 19 NANP repeats and the C terminus of the *Plasmodium falciparum* circumsporozite protein (PfCSP; residues 199–387, 189 aa in length) are fused to the hepatitis B surface Ag (HBsAg). It contains several CD4$^+$ and CD8$^+$ T cell epitopes (1). Administration of 2 or 3 doses of RTS,S in the adjuvant system number 2A (AS02A) has protected a mean of 44% of >60 volunteers challenged with *P. falciparum* (Pf) 2–3 wk after last immunization (2–4), and protected 70% of semi-immune Gambians for 2 mo after last immunization (4). However, this protection is of short duration (4, 5). Immunization with RTS,S induces anti-PfCSP Abs and CD4$^+$ T cell dependent IFN-γ responses, but no CD8$^+$ T cell dependent CTL or IFN-γ responses by ex vivo ELISPOT assay have been detected (1). Recently, CD8$^+$ T cells containing IFN-γ have been detected by intracellular staining and flow cytometry (6).

DNA vaccines induce cell-mediated immune responses, including the Ag-specific CD8$^+$ CTLs and Th1-biased CD4$^+$ T cell responses that are the major mechanisms of protection against intracellular pathogens and tumors (7–12). However, thus far, DNA vaccines have proved suboptimal for induction of protective immune responses in humans.

We have conducted two Phase I clinical trials of a single gene DNA vaccine encoding the full-length PfCSP (VCL-2510) (8, 9, 13, 14), and shown that immunization with PfCSP DNA vaccine by either needle or needleless jet injection induces the CD8$^+$ T cell dependent CTLs and CD8$^+$ type 1 IFN-γ responses (9), believed to play a critical role in protection against parasite-infected hepatocytes (7). However, we have been unsuccessful in inducing detectable Ab responses. These volunteers were not challenged, but other volunteers immunized with Pf DNA have been challenged, and none have been shown to be protected (15) (T. R., unpublished observations).

We and others have reported (16–18) that in mice and monkeys, immunization strategies that include priming with DNA and boosting with recombinant virus or recombinant protein in adjuvant (19) are more immunogenic and protective than immunization with DNA alone. Recently, it was reported that priming with Pf DNA and boosting with recombinant modified vaccinia Ankara expressing the same protein as the DNA, elicited excellent CD8$^+$ and CD4$^+$ T cell responses, but no Ab, and minimal protection (15).

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[4] Abbreviations used in this paper: PfCSP, *Plasmodium falciparum* circumsporozoite protein; HBsAg, hepatits B surface Ag; AS02A, adjuvant system number 02A; Pf, *P. falciparum*; SFC, spot forming cell; h, human; TT, tetanus toxin.

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To determine whether a regimen of priming with DNA and boosting with the known protective immunogen RTS,S in AS02A (RTS,S/AS02A) could induce not only the protective $CD4^+$ T cell and Ab responses found in individuals who receive RTS,S/AS02A alone, but also the $CD8^+$ CTL and IFN-γ responses measured by short-term (ex vivo) ELISPOT assay many consider to be critical to sustained protective immunity against the liver stages of Pf (20, 21), 10 volunteers who had received three doses of the PfCSP DNA 12–14 mo previously were boosted with two doses of RTS,S/AS02A. Controls were 14 naive volunteers who received two doses of RTS,S/AS02A. T cell responses were measured by chromium-release, IFN-γ ELISPOT, and quantitative RT-PCR assays. The results of safety and Ab assessment are reported elsewhere (22).

Materials and Methods

Vaccines

The DNA vaccine,VCL-2510, encoding the full-length PfCSP (Vical, San Diego, CA) has been described previously (8, 9, 13). The recombinant PfCSP/HBsAg fusion protein (RTS,S) containing aa 207–395 of Pf (NF54/3D7) CSP protein fused to HBsAg expressed in yeast has been also described previously (2) and was formulated with adjuvant AS02A in an oil-in-water emulsion (GlaxoSmithKline, Rixensart, Belgium). AS02A, also known as SBAS2, is a water-in-oil emulsion containing the immunostimulant monophosphoryl lipid A and a saponin derivative known as QS-21 with the preservative thimerosol. The vaccine formulations containing 50 μg of RTS,S in 0.5 ml were prepared 30 min before injection, according to the standard protocol.

Study design

Twenty-four HLA-A*0201-positive, malaria-naive volunteers were recruited. Of these 24 individuals, 10 had previously received three doses of 2500 μg of the PfCSP DNA vaccine (13). The last dose of the DNA vaccine was given 12–14 mo before the initiation of this study. Fourteen malaria-naive volunteers who had not previously received either the PfCSP DNA or RTS,S/AS02A vaccine were recruited as nonprimed controls. All were negative for Abs to PfCSP, HIV, HBV core Ag, HCV, vaccinia, and dsDNA. Six of the 10 DNA-primed volunteers and 8 of the 14 nonprimed controls were positive for Ab to HBsAg (cutoff for seropositivity defined as anti-HBsAg Ab titers $\geq$1/15). The 14 volunteers received two injections of RTS,S in AS02A at 0 and 8 wk by i.m. injection in the left deltoid. Three HLA-mismatched volunteers did not receive either the PfCSP DNA or RTS,S/AS02A vaccine and provided specimens used as negative controls in the assays.

Peptides

Eight synthetic peptides derived from PfCSP and included in the RTS,S sequence were used for sensitization of CTL targets and ELISPOT assays. These peptides were purchased from Chiron Technologies (Clayton, Victoria, Australia) at 80–95% purity. The panel included four defined CTL epitopes (9–10 aa), restricted by HLA-A*0201 (A2.319), HLA-A*0101 (A1.310), HLA-A*0301 (A3/11.336), and HLA-B*3501 (B35.353) (8), and four HLA-DR-binding peptides, DR.316 (residue 316–335, IKEY LNKIQNSLSTEWSPCS (9)), DR.318 (residue 318–332, EYLNKIQNSL STEW (1)), DR.363 (residue 363–383, DIEKKICKMEKCSSVFNVVNS (9)), and DR.346 ((residue 346–365, IKPGSANKPKDELDYANDIE (23)), and previous studies have shown that these peptides are recognized by PBMCs from volunteers immunized with the VR2510 DNA vaccine (9). A pool of 13 PfCSP-derived peptides and a pool of 19 HBsAg-derived peptides (15 aa), were provided by GlaxoSmithKline (1). Peptides derived from the influenza matrix protein (Flu M A_2) (residue 58–66, GILGFV FTL, HLA-A*0201-restricted (24)), or tetanus toxin (TT) universal Th epitope P30 (TT-DR) (residue 947–969, FNNFTVSFWLRVPKVSASH LET, DR- and DP-restricted (25)), were used as positive controls. Peptides from HIV Gag protein (residue 77–85, SLYNTVATL, HLA-A2*0201-restricted (26)), or Pf protein Exp-1 (residue 82–96, sequence A*GLLGN VSTV*LLGGV, containing known HLA-A*0201 and HLA-DR-binding sequences (27), italicized sequence is HLA-A*0201 epitope), were used as negative controls.

Cytotoxic T cell assays

In vitro restimulation and chromium release assays were performed as previously described (8). In brief, to generate effector cells, we infected 20% of the total PBMCs with recombinant canary-pox expressing the PfCSP (vCP182) at 5 PFU/cell for 90 min at 37°C. After washing twice, these PBMCs were combined with the remaining PBMCs and cultured for 7–10 days. Recombinant human (h)IL-2 (PerkinElmer/Cetus, Emeryville, CA) was added after 48 h (20 U/ml). Target cells were autologous or MHC-mismatched PHA blasts that were sensitized overnight with PfCSP-specific CTL epitopes or control peptide at 10 μg/ml. The CTL activity was assessed by a conventional 6 h chromium release assay. Percent lysis was defined as (experimental release $-$ medium control release)/(maximum release $-$ medium control release) $\times$ 100. Percent of specific lysis was determined by subtracting the percent lysis of targets cultured with the control HIV Gag A2-restricted peptide from the percent lysis of targets incubated with the experimental peptide. CTL responses were considered positive only if percent of specific lysis postimmunization was $>$10% for at least two E:T ratios in the same assay and if percent of specific lysis preimmunization was $<$10%.

IFN-γ ELISPOT assay

The number of PfCSP-specific IFN-γ-producing cells was determined by ELISPOT after 36 h in vitro stimulation in the presence of 10 μg/ml peptides (9). All the ELISPOT assays were conducted in quadruplicate. The number of spots corresponding to IFN-γ-producing cells in wells (spot-forming cells (SFCs)) was enumerated with an automated spot-counting system (Scanalytics, Fairfax, VA). Responses were expressed as the mean number of SFCs per 10^6 PBMCs, and were considered significant if 1) the mean number of cells in wells with experimental peptide was significantly greater ($p < 0.05$) than in wells with control peptide, 2) the net SFCs per well (mean SFCs in experimental peptide wells $-$ mean SFCs in control peptide wells) was $\geq$5 SFCs per 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. Furthermore, if cells obtained before immunization had a positive response to a PfCSP-specific peptide as defined above, the response to the same peptide after the immunization was not considered positive.

Depletion or enrichment of different subsets of T cell populations

ELISPOT assays were conducted with PBMCs depleted of $CD4^+$ or $CD8^+$ T cells before culture using anti-$CD4^+$- or anti-$CD8^+$-coated Dynabeads M-450 (Dynal Biotech, Great Neck, NY). IFN-γ mRNA expression levels were measured by real-time PCR in selectively enriched T cell populations: $CD4^+$/$CD45RA^+$, $CD4^+$/$CD45RA^-$, $CD8^+$/$CD45RA^+$, and $CD8^+$/$CD45RA^-$ T cells. In these assays, frozen PBMCs were recovered by overnight culture in a 24-well plate at 3×10^6 cell/well in 2 ml complete RPMI medium with 10% human AB serum, and then stimulated with short peptide (9–10 aa) for 2 h, or long peptides (15–22 aa) for 4 h at 10 μg/ml. Then, PBMCs were harvested and enriched for $CD8^+$ or $CD4^+$ T cells using MACS MultiSort kit (Miltenyi Biotec, Auburn, CA), and $CD45RA^+$ and $CD45RA^-$ cells were separated by passing the enriched $CD4^+$or $CD8^+$ T cells through CD45RA MicroBeads (Miltenyi Biotec).

Quantification of IFN-γ mRNA by real-time PCR

Total RNA was isolated from enriched T cell subsets using the RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized from the total RNA using random hexamers and the TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA). A relative quantification of IFN-γ mRNA by real-time PCR was done on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) using the TaqMan PCR kit (Applied Biosystems) according to manufacturer's instructions. The primers (hIFN-γ-F, 5′-TTGGTGATGATTTGAACATTGGA-3′, hIFN-γ-R, 5′-CCCAG TTCCTGCAGAGTAGAAAA-3′;hGAPDH-F,5′-GAAGGTGAAGGTCG GAGTC-3′, hGAPDH-R, 5′-GAAGATGGTGATGGGATTTC-3′), probes (hIFN-γ probe, 5′-TGTCACTTGCAAACACACAGCTTGTCGAA-3′; hGAPDH probe, 5′-CAAGCTTCCCGTTCTCAGCC-3′) for amplifying the IFN-γ and GAPDH mRNA were designed and standardized in-house following the manufacturer's protocol. Amplification of GAPDH was done for each experimental sample as an endogenous control to account for differences in the amount and quality of total RNA added to each reaction. Thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of two-step PCR consisting of 15 s at 95°C and 1 min at 60°C. All samples were amplified in triplicate. Threshold cycle, which correlates inversely with the target mRNA levels, was measured as the cycle number at which the reporter florescent emission increased above a threshold level. Target gene expression was normalized between different samples based on the values of the expression of the *GAPDH* gene.

Statistical analysis

The frequency of peptide-specific IFN-γ responses was assessed using the χ^2 test (two-tailed, uncorrected) except when the cell value was <5, in which case Fisher's exact test was used (two-tailed), and the magnitude of responses was assessed using the Student's *t* test (two-tailed). Analysis was conducted using SPSS version 8.0 (SPSS, Chicago, IL) or Epi Info version 6.04b (Centers for Disease Control and Prevention, Atlanta, GA). The level of significance was $p < 0.05$.

Results

CTL responses

Immunization with RTS,S alone induces Ab and $CD4^+$ T cell dependent IFN-γ responses in humans (1), but has not been reported to elicit Ag-specific CTLs in humans. To determine whether DNA-induced memory CTLs could be recalled by RTS,S, Ag-specific CTLs were assessed with PBMCs collected from DNA-primed or nonprimed volunteers 1 or 2 wk before immunization with RTS,S, and 1–2 wk after the first and second doses of RTS,S. No CTLs were detected in DNA-primed or nonprimed volunteers immediately before administration of RTS,S. No CTLs were detected in any of the 14 nonprimed volunteers who received only RTS,S. PfCSP-specific and genetically restricted CTLs were detected in 5 of 10 DNA-primed volunteers (Fig. 1). One of five responders had CTLs 1 wk after the first dose, and the others had CTLs after the second dose of RTS,S. The CTL responses were significantly greater in the DNA-primed ($p = 0.0029$) as compared with nonprimed volunteers. The CTL responses were comparable to those observed after DNA immunization alone among the 15 volunteers who received three doses of PfCSP DNA vaccine previously (9).

CTL responses were detected in all four of the peptides containing PfCSP-specific class I-restricted epitopes that were tested in this study. Of five positive responders, four had CTLs to the HLA-A2-restricted epitope A2.319, one responded to the A1-restricted epitope A1.310 (V2), and 2 of 4 A2.319 responders also responded to the A3- and B7-restricted epitopes, A3.336 (V8), and B7.285 (V9), respectively. We were unable to detect CTLs directly against the reported $CD4^+$ CTL epitope DR.318 (E*YLNKIQNSL*-STEWS) (28) that contains the $CD8^+$ CTL epitope A2.319 (*YLNKIQNSL*).

Among the five DNA-primed volunteers who had positive CTL responses after RTS,S boost, three previously had not been shown to have detectable CTLs against the same epitopes when tested after the second and third doses of PfCSP DNA immunization (9), one year before the RTS,S boost. In contrast, two of the four volunteers previously shown to have had detectable CTLs against peptides included within RTS,S after DNA immunization alone did not respond to the RTS,S boost.

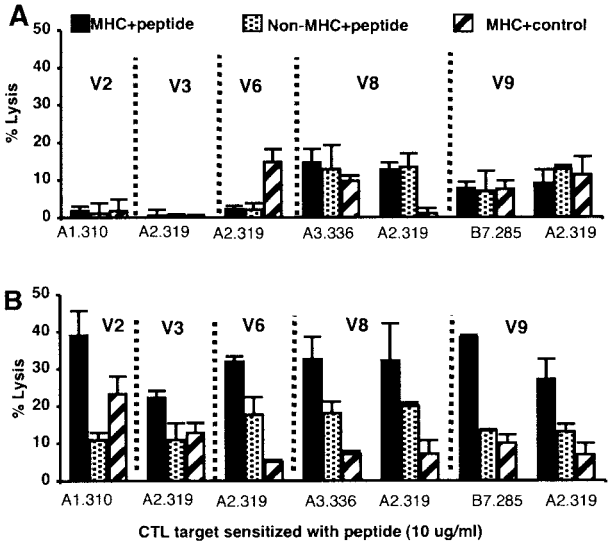

FIGURE 1. PfCSP DNA-primed CTLs recalled by the PfCSP recombinant protein RTS,S. CTLs before the RTS,S boost (*A*), and CTLs for each positive subject and each positive peptide post-RTS,S boost (*B*). Fresh PBMCs, from 2 wk after the first (V6) or second (V2, 3, 8, and 9) doses of RTS,S were stimulated with recombinant canary-pox expressing the PfCSP and assayed against HLA class I-matched (MHC + peptide) or -mismatched targets (Non-MHC + peptide) incubated with the PfCSP-derived peptides or control peptide (HLA-A*0201-restricted HIV Gag) (MHC + control) in a chromium release assay. Shown is the percent lysis for each peptide with its simultaneously assessed controls at a single E:T ratio (20:1 or 40:1).

IFN-γ responses

IFN-γ responses were evaluated by ELISPOT assays with freshly isolated PBMCs, 1–2 wk before, and 1, 2, and 6 wk after the first and second dose of RTS,S. In the assays, PBMCs were incubated with eight PfCSP-specific peptides (four containing HLA class I-restricted epitopes, three containing a class I- and class II-restricted epitope, and one containing only a class II-restricted epitope), or a pool of 13 PfCSP peptides which are included in the RTS,S sequence.

IFN-γ responses were detected to multiple PfCSP-specific peptides containing class I- and/or class II-restricted epitopes in all DNA-primed volunteers after they received three doses of DNA vaccine 12–14 mo before. There were no detectable PfCSP-specific IFN-γ responses in DNA-primed or nonprimed volunteers before RTS,S immunization. At no time after immunization were there IFN-γ responses detected to the short peptides containing only class I-restricted epitopes. After the first dose, IFN-γ responses were detected against all four 15- to 20-mer PfCSP peptides in 6 of 10 DNA-primed volunteers compared with one such peptide in 2 of 14 nonprimed volunteers ($p = 0.019$). The frequency of responses was significantly greater in DNA-primed as compared with nonprimed volunteers (positive assays/total assays, 20 of 116 (18.1%) vs 4 of 164 (2.4%), $p = 0.00001$) regardless of the individuals' anti-HBsAg Ab status (Table I).

After the second dose of RTS,S, IFN-γ responses were detected in 8 of 10 DNA-primed volunteers and in 11 of 14 nonprimed volunteers. Although there was no difference between the two groups in terms of number of responders, there was a significantly greater number of positive assays among the DNA-primed as compared with the nonprimed volunteers (61 of 238 (25.6%) vs 44 of 320 (13.8%), $p = 0.0004$) (Table I). This difference was directly related to the HBsAg Ab status of the volunteers. The frequency of positive assays was significantly greater in DNA-primed than in nonprimed volunteers among HBsAg Ab-positive individuals (31.9 vs 13.1%, $p = 0.0078$), but not in HBsAg Ab-negative individuals (37.5 vs 40.3%).

At the epitope level, we compared IFN-γ responses against peptides DR.316 and DR.363 between the DNA-primed and nonprimed groups. DR.316 contains overlapping $CD4^+$ and $CD8^+$ T cell epitopes while DR.363 contains only a $CD4^+$ T cell epitope. We chose 15-mer peptides instead of 9- to 10-mer peptides including only $CD8^+$ T epitopes based on the assumption that RTS,S alone would not induce $CD8^+$ T cell responses, but was expected to be able to boost the DNA-primed $CD8^+$ T cell responses through $CD4^+$ T cell help.

IFN-γ responses against peptide DR.316 were detected in 4 of 10 DNA-primed vs 0 of 14 nonprimed volunteers after the first dose of RTS,S ($p = 0.0095$), and in 6 of 10 DNA-primed vs 5 of

Table I. *Frequency and magnitide of IFN-γ/responses to PfCSP-specific peptides*

Group	Number of Responders/Number Tested		Number of Positive Assays/Total Assays (%)[a]			Range of Net SFCs/10° PBMCs (geomean)	
	DNA-primed volunteers	Nonprimed volunteers	DNA-primed volunteers	Nonprimed volunteers	*p* value between two groups	DNA-primed volunteers	Nonprimed volunteers
After first immunization							
HBsAg (+)	4/6 (66.7)	2/8 (25.0)	13/69 (18.8)	4/95 (4.2)	0.001	13.1–105.5 (38.5)	20.0–63.1 (39.6)
HBsAg (−)	2/4 (50.0)	0/6 (0)	7/47 (14.9)	0/69 (0)	0.0009	13.8–82.5 (32.7)	Neg
Total	6/10 (60.0)[b]	2/14 (14.3)[b]	20/116 (18.1)	4/164 (2.4)	<0.00001	13.1–105.5 (36.3)	20.0–63.1 (39.6)
After second immunization							
HBsAg (+)	5/6 (83.0)	6/8 (75.0)	23/72 (31.9)	11/84 (13.1)	0.0078	11.9–82.5 (32.1)	14.4–96.9 (37.8)
HBsAg (−)	3/4 (75.0)	6/6 (100.0)	18/48 (37.5)	29/72 (40.3)	0.76	11.7–122.5 (33.4)	17.5–125.6 (41.0)
Total	8/10 (80.0)	11/14 (84.6)	41/120 (34.2)	40/156 (25.6)	0.12	11.7–122.5 (32.6)	14.4–125.6 (39.6)
Overall							
HBsAg (+)	5/6 (83.0)	6/8 (75.0)	36/141 (25.5)	15/179 (8.4)	0.00003	11.9–105.0 (34.3)	14.4–96.9 (38.1)
HBsAg (−)	3/4 (75.0)	6/6 (100.0)	25/95 (26.3)	29/141 (20.6)	0.3	11.7–122.5 (33.2)	17.5–125.6 (41.0)
Total	8/10 (80.0)	11/14 (84.6)	61/238 (25.6)	44/320 (13.8)	0.0004	11.7–122.5 (33.9)	14.4–125.6 (39.6)

[a] Total assays "included ELISPOT assays conducted to detect IFN-γ responses to nine individual peptides and a pool of PfCSP peptides with fresh PBMCs from before or after the first or second immunizations with RTS,S/AS02A. Three repeated assays were conducted at 1, 2, and 6 wk after each immunization with RTS,S/AS02A.

[b] After the first immunization, number of positive responders in DNA-primed volunteers was significantly greater than that in nonprimed volunteers (6/10 vs 2/14, $p = 0.019$).

14 nonprimed volunteers after the second dose of RTS,S ($p = 0.35$). When all assays were considered (after the first and second doses of RTS,S), the DNA primed group had a greater frequency of positive assays (17 of 80 vs 8 of 81, $p = 0.0046$), but there was no difference in the magnitude of responses ($p = 0.21$; data not shown).

IFN-γ responses against peptide DR.363 were detected in 3 of 10 DNA-primed vs 2 of 14 nonprimed volunteers after the first dose of RTS,S ($p = 0.35$), and in 4 of 10 DNA-primed vs 9 of 14 nonprimed volunteers after the second dose of RTS,S ($p = 0.24$). When all assays were considered, there was no significant difference between the DNA-primed and RTS-alone groups in frequency of positive assays (8 of 60 vs 18 of 81, $p = 0.178$). However, there was a significantly greater magnitude of responses in nonprimed as compared with DNA-primed volunteers after the second dose of RTS,S (range of SFCs: 13.1–58.8 (26.4) vs 14.0–140.6 (47.9), $p = 0.004$).

In general, the individuals in the DNA-primed group responded to significantly more of the peptides tested than did the volunteers who only received RTS,S. Of the 8 responders in the 10 DNA-primed volunteers, one had responses against all four of the long peptides tested, one responded to three peptides, five responded to two peptides, and only one responded to one peptide. Of the 11 responders in the 13 nonprimed volunteers, eight responded to only one peptide (2 of 8 responded to DR.316, and 6 of 8 responded to DR.363). Overall, 7 of 8 DNA-primed vs 3 of 11 nonprimed volunteers responded to at least two peptides tested ($p = 0.009$).

HBsAg component of immunogen, pre-existing Abs to HbsAg, and T cell responses

Because RTS,S is a fusion protein of PfCSP and HBsAg, and the findings regarding the influence of HBsAg Ab status on responses to the PfCSP peptides, we expanded the studies. IFN-γ responses to PfCSP and HBsAg were compared by ELISPOT assays with a pool of 13 PfCSP peptides and a pool of 19 HBsAg peptides simultaneously in PBMCs at all study time points after the RTS,S immunization.

In nonprimed volunteers, IFN-γ responses to HBsAg were high in all individuals regardless of whether or not they had Abs to HBsAg (Table II). After the first dose of RTS,S, the magnitude of responses to the HBsAg was significantly greater in individuals with pre-existing Abs to HBsAg than in those without such Abs (range of SFCs/10^6 PBMCs (geomean): 13.1–222.9 (60.1) vs 13.1–132.5 (33.9), $p = 0.013$). This difference was not present after the second immunization. The responses to HBsAg in HBsAg Ab-negative individuals were significantly increased after the second dose of RTS,S compared with after the first dose in terms of frequency ($p = 0.035$) and magnitude ($p = 0.0003$) (data not shown).

Compared with the responses to HBsAg in 13 of 14 nonprimed volunteers after one dose of RTS,S/AS02A, the IFN-γ responses to

Table II. *Comparison of IFN-γ responses in DNA-primed and nonprimed groups after RTS,S/AS02A immunization*[a]

Volunteers with (HBsAg(+)) or without (HBsAg (−)) abs to HBsAg	IFN-γ to PfCSP		IFN-γ to HBsAg	
	HBsAg (+)	HBsAg (−)	HBsAg (+)	HBsAg (−)
Nonprimed group				
After first dose	Neg	Neg	A, B, C	A, B, C
After second dose	A, B	A, B	A, B, C	A, B, C, D
DNA primed group				
After first dose	A, B	C	A, B	B
After second dose	A, B	A, B, D	A, B	A, B

[a] Criteria for the scores of responsiveness was based on statistical significance analysis ($p < 0.05$): A) frequency-1 (no. positive responders/total volunteers), B) frequency-2 (positive assays/total assays), C) magnitude (net SFCs/10^6 PBMC) of positive assays as compared to the baseline, D) a statistically significant increase in the frequency or magnitude of positive assays (no. positive assays/total assays) after the second immunization compared to after the first immunization. Neg, no responses.

PfCSP were only detected in 1 of 14 individuals ($p = 0.0049$). Overall, RTS,S-induced IFN-γ responses were significantly lower to PfCSP than to HBsAg in all volunteers who were not primed with DNA, and even lower in individuals with pre-existing anti-HBsAg Abs in terms of the frequency of positive responders and positive assays after the first and second doses of RTS,S/AS02A (Table II). Likewise, the magnitude of IFN-γ responses was lower after each immunization in both HBsAg Ab-positive ($p < 0.05$–0.0032) and Ab-negative individuals ($p = 0.0001$) (data not shown). These data demonstrated that in nonprimed individuals, RTS,S-elicited T cell responses to HBsAg was significantly more robust than it was to PfCSP.

In contrast, PfCSP DNA priming appeared to balance this immunodominance directing T cell responses toward PfCSP. In DNA-primed, HBsAg Ab-positive volunteers, IFN-γ responses (positive assays/total assays, 12 of 15 vs 4 of 15, $p = 0.0034$) after the first dose of RTS,S was greater to HBsAg than it was to PfCSP. However, after the second dose of RTS,S/AS02A in HBsAg Ab-positive volunteers, and at no time in HBsAg Ab-negative volunteers was the frequency of positive assays to PfCSP different than it was to HBsAg. The magnitude of responses to PfCSP and HBsAg were similar after the first dose regardless of HBsAg Ab status (Table II). After the second dose, in HBsAg Ab-positive individuals, the magnitude of responses to HBsAg was increased significantly as compared with the magnitude of responses to PfCSP (range of SFCs per 10^6 PBMCs (geomean): 18.1–68.8 (33.8) vs 18.8–131.3 (52.8), $p = 0.024$), indicating that responses to HBsAg may eventually predominate over the responses to PfCSP if multiple doses of RTS,S vaccine are given.

DNA induces Tc1 and Th1 responses, whereas RTS,S induces only Th1 responses in humans

Both DNA and RTS,S alone are capable of inducing IFN-γ responses so that after the second dose of RTS,S/AS02A, the IFN-γ responses in both groups were equivalent in terms of the positive responders (8 of 10 vs 11 of 14). Nevertheless, as reported previously, the pathway for IFN-γ production induced by DNA or RTS,S alone were different. Immunization with DNA induces both $CD4^+$ and $CD8^+$ T cell dependent IFN-γ responses measured by ex vivo ELISPOT assay (9), and RTS,S induces only $CD4^+$ T cell dependent responses by this assay (1).

To distinguish the T cell profiles of Ag-specific IFN-γ responses in the volunteers immunized with DNA alone, RTS,S alone, or from the DNA-primed/RTS,S-boosted volunteers, depleted T cell populations were incubated with defined PfCSP peptides before the ELISPOT assays to identify which subsets of T cell were involved in the induction of IFN-γ responses in vitro, which we defined as the induction phase. In contrast, IFN-γ mRNA expression levels were assessed by real-time PCR in enriched subsets of T cell populations after incubation of PBMCs with the same peptides used for the ELISPOT assays. This was done to delineate the IFN-γ-producing T cells, which we defined as the effector phase. Responses to peptide DR.363 (containing only a $CD4^+$ T epitope) and DR.316 (containing overlapping $CD4^+$ and $CD8^+$ T epitopes) were assessed to compare the mechanisms underlying the IFN-γ responses against PfCSP by different vaccine delivery systems. Responses to the conserved and HLA-A2-restricted $CD8^+$ T cell epitope from influenza matrix protein (Flu M A2) and the HLA-DR-restricted $CD4^+$ T cell epitope TT-DR from TT were also evaluated in parallel to provide internal standardization between different epitopes, assays, and volunteers.

IFN-γ by ELISPOT to the Flu M A2 peptide were $CD8^+$ but not $CD4^+$ T cell dependent because depletion of $CD8^+$, but not $CD4^+$, T cells immediately before culture of PBMCs completely abrogated or significantly reduced responses in all 17 individuals tested, regardless of what type of antimalaria vaccine they received (Fig. 2*A*). In contrast, IFN-γ responses to peptide TT-DR were

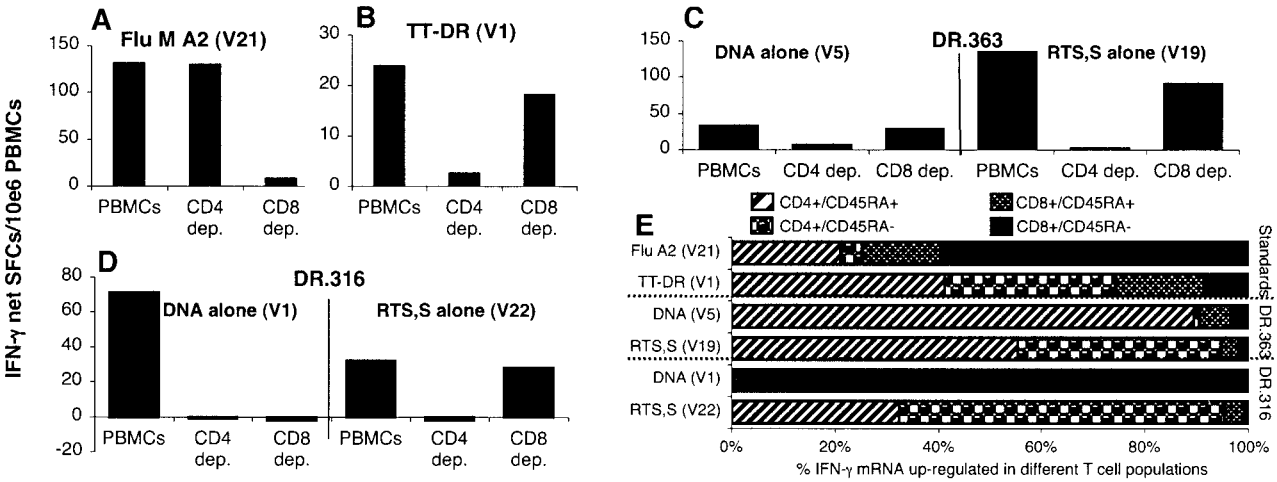

FIGURE 2. Different T cell populations were involved in the induction and production of IFN-γ. ELISPOT assays were conducted with frozen PBMCs from volunteers immunized with PfCSP DNA alone (V1 and V5) or RTS,S alone (V19, V21, and V22), either treated with control Dynabeads (Dynal Biotech) or anti-$CD4^+$ or anti-$CD8^+$ beads immediately before culture with peptides. *A*, Flu M A2; *B*, TT-DR; *C*, PfCSP DR.363; or *D*, PfCSP DR.316. *E*, IFN-γ mRNA expression levels were measured by real-time PCR in selectively enriched T cell populations ($CD4^+$/CD45RA$^+$, $CD4^+$/CD45RA$^-$, $CD8^+$/CD45RA$^+$, and $CD8^+$/CD45RA$^-$) from the same volunteer at the same study time points immediately after culture with the same sets of peptides tested in ELISPOT assays. ELISPOT assays in which depletion was done before culture demonstrated IFN-γ responses to Flu M A2 (*A*) were dependent on $CD8^+$, but not $CD4^+$ T cells, TT-DR (*B*) were dependent on $CD4^+$, but not $CD8^+$ T cells, DR.363 (PfCSP $CD4^+$ T epitope) were dependent on $CD4^+$, but not $CD8^+$ T cells in volunteers immunized with DNA alone or RTS,S alone (*C*), and DR.316 (PfCSP overlapping $CD4^+$ and $CD8^+$ T epitope) (*D*) were dependent on both $CD4^+$ and $CD8^+$ T cells in volunteers immunized with DNA alone, and were dependent on $CD4^+$, but not $CD8^+$, T cells in volunteers immunized with RTS,S alone. IFN-γ mRNA expression measured by real-time PCR demonstrated at the end of the 36-h incubation (*E*) that the IFN-γ-secreting cells were $CD8^+$ cells when incubated with the Flu M A2 peptide, $CD4^+$ cells when incubated with the TT-DR peptide, $CD4^+$ T cells when incubated with the PfCSP DR.363 peptide in both DNA and RTS,S immunized volunteers, and $CD8^+$ T cells when incubated with the DR.316 peptide in DNA-immunized volunteers, and $CD4^+$ cells when incubated with the DR.316 in RTS,S-immunized individuals.

completely $CD4^+$, but not $CD8^+$ T cell dependent, in all three positive responders tested (Fig. 2*B*).

IFN-γ mRNA expression levels measured by real-time PCR in four enriched T cell populations ($CD4^+/CD45RA^+$, $CD4^+/CD45RA^-$, $CD8^+/CD45RA^+$, and $CD8^+/CD45RA^-$) were consistent with the findings obtained from the ELISPOT assays. IFN-γ mRNA was up-regulated predominantly in $CD8^+$ T cells after stimulation with the Flu M A2 peptide (Fig. 2*E*). The IFN-γ mRNA expression levels increased 6.8-fold (range, 3.4- to 12.9-fold) in $CD8^+$ T cells compared with 2.2-fold (range, 0.98- to 7.58-fold) in $CD4^+$ T cells ($p = 0.03$). IFN-γ mRNA up-regulation in $CD8^+$ accounted for a mean of 78% of IFN-γ production (range, 62–99%). In contrast, IFN-γ mRNA was up-regulated predominantly in $CD4^+$ T cells after stimulation with TT-DR (Fig. 2*E*). The IFN-γ mRNA levels increased 7.6-fold (range, 2.4- to 18.3-fold) in $CD4^+$ compared with 2.2-fold (range, 1.1–4.6) in $CD8^+$ T cells ($p = 0.02$). IFN-γ mRNA up-regulation in $CD4^+$ T cells accounted for a mean of 79% of IFN-γ production (range, 74–100%). These results indicated that $CD8^+$ T cells are functional effectors of IFN-γ responses against the Flu M A2 peptide whereas $CD4^+$ T cells are the effectors against the TT-DR peptide.

Conducting the assays in parallel with two standards as described above, we were able to clarify the T cell profiles of IFN-γ responses induced by DNA or RTS,S to two different PfCSP peptides (DR.363 and DR.316). After stimulation with peptide DR.363, which contains a $CD4^+$ T cell epitope, the ELISPOT results with depleted T cell populations showed that IFN-γ to peptide DR.363 were completely $CD4^+$ T cell dependent in volunteers who received DNA alone (2 of 2 tested, V1 and V5) or RTS,S alone (6 of 6 tested) (Fig. 2*C*). IFN-γ mRNA expression levels in enriched T cell populations were correlated with the T cell dependence by ELISPOT. IFN-γ mRNA was up-regulated predominantly in $CD4^+$ T cells in both DNA- and RTS,S-immunized volunteers (Fig. 2*E*). In five DNA-immunized volunteers tested, IFN-γ mRNA levels increased 5.3-fold (range, 2.6–11.5) in $CD4^+$ T cells compared with a 1.7-fold (range, 0.99–3.2) in $CD8^+$ T cells ($p = 0.014$). IFN-γ mRNA up-regulation in $CD4^+$ T cells accounted for a mean of 74% of IFN-γ production (range, 64–91%). The same pattern was seen in four RTS,S-immunized volunteers tested (Fig. 2*E*), IFN-γ mRNA levels increased 9.2-fold (range, 2.9–53.5) in $CD4^+$ compared with a 0.9-fold (range, 0.6–1.1) in $CD8^+$ T cells, and that IFN-γ mRNA up-regulation in $CD4^+$ T cells accounted for a mean of 86% of IFN-γ production (range, 73–98%). These results provided us with the first evidence that PfCSP-specific and $CD4^+$ T cell dependent, in addition to $CD8^+$ T cell dependent, IFN-γ responses were also induced in humans by the DNA vaccine.

Interestingly, IFN-γ responses to DR.316 (overlapping $CD4^+$ and $CD8^+$ T epitope) were dependent upon different subsets of T cells in volunteers receiving DNA or RTS,S alone. The responses were both $CD4^+$ and $CD8^+$ T cell dependent in volunteers who received DNA alone (V1) (Fig. 2*D*) as previously reported (9), compared with only $CD4^+$, but not $CD8^+$ T cell dependent in volunteers who received RTS,S alone (3 of 3 volunteers tested) (Fig. 2*D*). Furthermore, IFN-γ mRNA was up-regulated predominantly in $CD8^+$ T cells in DNA-immunized volunteers (Fig. 2*E*), although the response was both $CD4^+$ and $CD8^+$ T cell dependent by ELISPOT. IFN-γ mRNA expression levels increased 64.7-fold in $CD8^+$ T cells compared with 0.36-fold in $CD4^+$ T cells, and IFN-γ mRNA up-regulation in $CD8^+$ T cells accounted for a mean of 99.6% of IFN-γ production. In contrast, IFN-γ mRNA was up-regulated predominantly in $CD4^+$ T cells in RTS,S-immunized volunteers (Fig. 2*E*). IFN-γ mRNA expression levels increased 24.7-fold (range, 5.3–176.7) in $CD4^+$ compared with 2.5-fold (range, 1.1–5.6) in $CD8^+$ T cells. IFN-γ mRNA up-regulation in $CD4^+$ T cells accounted for a mean of 86% of IFN-γ production (range, 69–98%). These findings indicate that in DNA-immunized individuals after stimulation of their PBMCs with peptide DR.316, $CD4^+$ T cells are involved only in the induction phase of IFN-γ responses, whereas $CD8^+$ T cells actually produce IFN-γ. In contrast, in RTS,S-immunized individuals, our results indicate that $CD4^+$ T cells are involved in production of IFN-γ after stimulation with peptide DR.316.

DNA-prime/RTS,S-boost broadens the repertoire of IFN-γ-producing T cells

The final step in our analysis was to delineate the repertoire of IFN-γ-producing T cells recalled by RTS,S in DNA-primed volunteers. IFN-γ responses to peptide DR.363 (does not contain a $CD8^+$ T cell epitope) were only $CD4^+$ T cell dependent in volunteers immunized with DNA or RTS,S alone. The same type of response to DR.363 was detected in 2 of 3 responders (V1 and V5) in the DNA-primed group after the RTS,S boost. Strikingly, IFN-γ mRNA expression levels in $CD4^+$ T cells were increased 94.9-fold in volunteer V1 and 46.7-fold in V5 after the RTS,S boost as compared with a 7.6-fold increase in V1 and 12.5-fold in V5 after the immunization with three doses of DNA alone. The magnitude of responses after the RTS,S boost were 12.5 times higher in V1 and 3.7 times higher in V5 than that after the DNA immunization (Fig. 3).

IFN-γ responses to peptide DR.316 (an overlapping $CD4^+$ and $CD8^+$ T epitope) were dependent upon different subsets of T cells in volunteers receiving DNA or RTS,S alone. DNA-induced responses were both $CD4^+$ and $CD8^+$ T cell dependent in the induction phase, but only $CD8^+$ T cell dependent in the effector phase. RTS,S-induced responses were only $CD4^+$ T cell dependent in both induction and effector phases. Thus, it was not surprising that the responses to DR.316 in DNA-primed volunteers after the RTS,S boost was a mixture of the two patterns seen in

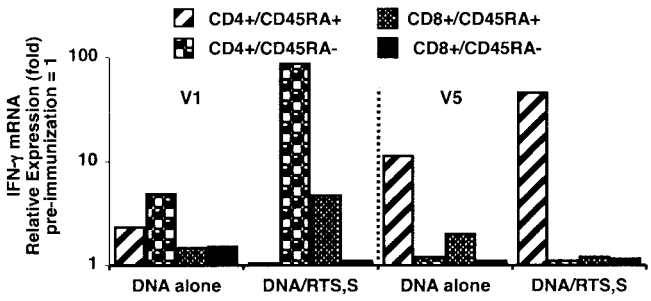

FIGURE 3. IFN-γ mRNA expression levels in different T cell populations. Frozen cells from two volunteers (V1 and V5) acquired after three doses of PfCSP DNA (DNA alone), and after the individuals had been boosted with two doses of RTS,S/AS02A (DNA/RTS,S), were studied. Cells were incubated with peptide DR.363, selectively enriched, and assessed for IFN-γ mRNA expression. The IFN-γ mRNA expression was normalized against GAPDH mRNA and the results were presented as the fold of up-regulation relative to preimmune cells. The IFN-γ mRNA copy numbers per 10^4 copies GAPDH mRNA in preimmune cells ranged from 105 to 338 in enriched $CD4^+$ cells, and 156–365 in enriched $CD8^+$ cells in volunteer V1, and in volunteer V5, the copy numbers ranged from 441 to 1320 in enriched $CD4^+$ cells, and from 425 to 1355 in enriched $CD8^+$ cells. After DNA immunization IFN-γ mRNA expression was modestly (5- to 10-fold) up-regulated in $CD4^+$ T cells in the $CD45RA^-$ subset in volunteer V1 and in the $CD45RA^+$ subset in volunteer V5, but not in $CD8^+$ T cells. RTS,S boosting was associated with a significant (80- to 100-fold) increase in IFN-γ mRNA expression in the $CD4^+$ but not $CD8^+$ T subset in both volunteers.

volunteers immunized with either DNA or RTS,S alone. In the induction phase, both $CD4^+$ and $CD8^+$ T cell dependent IFN-γ responses to DR.316 were detected in 3 of 5 responders after the first dose of RTS,S/AS02A. Completely $CD4^+$ T cell dependent, but only partially $CD8^+$ T cell dependent IFN-γ responses, were detected in 4 of 6 responders after the second dose of RTS,S/AS02A. Depletion of $CD8^+$ T cells was unable to abrogate IFN-γ production (Fig. 4*A*), indicating that, in addition to $CD8^+$ T cells, $CD4^+$ T cells produce IFN-γ as well after the RTS,S boost. Concurrently, in the effector phase, IFN-γ mRNA expression levels were up-regulated not only in $CD8^+$ T cells (8 of 8 responders), but also in $CD4^+$ T cells (4 of 8 responders after the first dose, 6 of 8 responders after the second dose of RTS,S/AS02A) (Fig. 4*B*), as compared with being up-regulated in only $CD8^+$ T cells in volunteers immunized with DNA alone, or in only $CD4^+$ T cells in volunteers immunized with RTS,S alone (Fig. 2*E*; DR.316). Overall, up-regulation of IFN-γ mRNA in both $CD8^+$ and $CD4^+$ T cells were detected in 6 of 8 responders, and the up-regulation of IFN-γ mRNA in $CD4^+$ T cells ranged from 3.0 to 28.3-fold (geomean, 6.6-fold) compared with that in $CD8^+$ T cells, which ranged from 4.0 to 281.03 (geomean, 19.7-fold) after the RTS,S boosts. IFN-γ mRNA up-regulation in $CD4^+$ T cells accounted for a mean of 23.5% of IFN-γ production (range, 6.5–45.1%). The results here demonstrated that DR.316-specific $CD4^+$ T cells in DNA-primed volunteers after the RTS,S boost functioned as both T helper that are required for $CD8^+$ T cell production of IFN-γ (a feature of the DNA-induced IFN-γ response) and effectors that produced IFN-γ (a feature of the RTS,S-induced IFN-γ response).

Finally, we have demonstrated IFN-γ mRNA up-regulation in $CD45RA^+$ and $CD45RA^-$ ($CD45RO^+$) $CD4^+$ and/or $CD8^+$ T cell populations in response to PfCSP peptides (DR.363 and DR.316) and positive control peptides (Flu M A2 and TT-DR) after immunization with DNA or RTS,S alone (Fig. 2*E*) or both (Fig. 4). The ratio of $CD45RA^+$ to $CD45RA^-$ ($CD45RO^+$) in $CD4^+$ or $CD8^+$ T cells with IFN-γ mRNA up-regulation varied depending on the donors and which peptides were used for the in vitro stimulation.

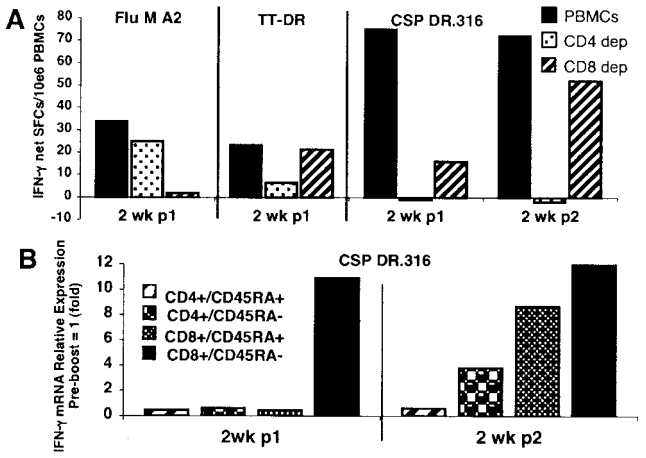

FIGURE 4. Shifting patterns of IFN-γ responses after the RTS,S boost. DNA-induced pattern of IFN-γ response to peptide DR.316 ($CD8^+$ Tc1 only) was shifted to a mixture of two patterns ($CD8^+$ Tc1 and $CD4^+$ Th1) after two doses of RTS,S. *A*, In the ex vivo ELISPOT conducted with cells from volunteer V2, IFN-γ responses were significantly reduced by $CD4^+$ and $CD8^+$ T cell depletion before culture after the first dose of RTS,S/AS02A. After the second dose, only $CD4^+$ T cell depletion significantly reduced activity. *B*, IFN-γ mRNA expression was up-regulated in $CD8^+$, but not in $CD4^+$ T cells after the first dose of RTS,S/AS02A, and was up-regulated in both $CD8^+$ and $CD4^+$ T cells after the second dose of RTS,S/AS02A.

Discussion

The process of developing an effective, sustainable vaccine against difficult to prevent infections like Pf, *Mycobacterium tuberculosis*, and HIV has proven to be slower, more difficult, and complex than expected. Although vaccines that primarily elicit a specific type of immune response may be effective, it has been the contention of many in the field that a vaccine that elicits protective $CD8^+$ and $CD4^+$ T cell responses and Abs against multiple proteins has the best chance for success (29, 30). The most progress with a malaria subunit vaccine has been made with RTS,S in AS02A adjuvant (3, 4). Immunization with RTS,S/AS02A provides modest, short-term protection against Pf-malaria in humans, which is not optimal. In humans, this vaccine elicits excellent Ab and Th1 $CD4^+$ T cell responses (1, 3), but not $CD8^+$ CTLs or IFN-γ responses as measured by short-term (ex vivo) ELISPOT assay (1), a finding confirmed in this study. However, recently there has been a report of the demonstration by intracellular staining and flow cytometry of up-regulation of PfCSP peptide-specific $CD4^+$ and $CD8^+$ T cells containing IFN-γ, and a significant correlation between the presence of either cell population and protection (6). This suggests that increasing the magnitude and quality of $CD8^+$ T cell responses to the point that they are detectable by CTL and ex vivo ELISPOT assays may increase the protective efficacy of the vaccine. In humans, the PfCSP DNA vaccine has been shown to elicit $CD8^+$ CTLs (8) and IFN-γ responses (9), but has not been shown to elicit Ab or Th1 $CD4^+$ T cell responses. Priming with Pf DNA and boosting with recombinant modified vaccinia Ankara (virus) expressing the same Pf proteins elicits much higher $CD8^+$ and $CD4^+$ T cell responses than does DNA alone (15). However, it does not elicit Ab or significant protection against malaria, but is associated with a delay in the onset of parasitemia (15), not dissimilar from that seen in the first studies of PfCSP recombinant protein and synthetic peptide vaccines (31, 32). The studies reported herein demonstrate that priming with the PfCSP DNA and boosting with RTS,S leads to the same levels of Ab (22) and $CD4^+$ T cell responses as does immunization with RTS,S/AS02A alone; immune responses associated with protective immunity. However, in addition, this regimen elicits cytotoxic and Tc1 $CD8^+$ T cell responses measurable by short-term (ex vivo) ELISPOT assay, and thus may provide a first step toward improving the protection found after immunization with RTS,S/AS02A alone and other recombinant protein vaccines.

RTS,S is a PfCSP-HBsAg fusion particle vaccine. Among individuals with Abs to HBsAg, those individuals primed with the PfCSP DNA vaccine produced significantly better T cell responses after administration of RTS,S/AS02A than did volunteers who had never received PfCSP DNA. There is now considerable effort being directed to producing recombinant fusion proteins and recombinant viruses and bacteria that express the target protein(s). In many cases, for HBsAg, vaccinia, poliovirus, and *Salmonella typhi*, immunized individuals will have pre-existing Ab against these backbone components of the vaccine. The fact that in individuals with Abs to the backbone component of the vaccine (e.g., HBsAg), priming with DNA encoding target proteins significantly enhanced the T cell responses to these proteins as compared with priming with recombinant protein alone may be an extremely important advantage of this prime boost strategy of immunization.

DNA-primed PfCSP-specific CTL responses were recalled in 50% of the volunteers by boosting with RTS,S 12–14 mo after the last vaccination with DNA, indicating that the DNA vaccine was effective at the induction of long-lived memory T cell responses.

Two of the five volunteers with recalled CTL responses after the RTS,S injection had no detectable CTLs after immunization with DNA alone, suggesting that immunization with the DNA vaccine was superior for the induction of memory CTLs in these individuals, but may not have been optimal for induction of effector T cell responses (33, 34). Because there were no CTLs detected in nonprimed volunteers who received RTS,S alone, RTS,S was not capable of priming PfCSP-specific CTLs but had the capacity to boost the CTL responses initiated by the DNA vaccine. DNA-primed PfCSP-specific IFN-γ responses were also boosted strongly by RTS,S, particularly after the first dose. Six of the 10 DNA-primed volunteers had IFN-γ responses against all four peptides tested as compared with 2 of 14 nonprimed volunteers, who had responses against only one of the four peptides. The breadth of IFN-γ responses at the epitope level was also significantly greater in DNA-primed than in nonprimed volunteers.

The results also suggest that DNA-prime/RTS,S boost broadens the repertoire of IFN-γ-producing T cells. DNA priming initiated two profiles of IFN-γ-producing T cells: (1) $CD4^+$ T cell dependent $CD8^+$ type 1 responses against overlapping $CD4^+/CD8^+$ T epitopes (DR.316), and (2) $CD4^+$ Th1 IFN-γ responses against the DR-restricted $CD4^+$ T epitope (DR.363). RTS,S alone, in contrast, induced only $CD4^+$ Th1 IFN-γ responses. With regard to DR.316, an overlapping $CD4^+/CD8^+$ T epitope, DNA alone induced $CD4^+$-dependent $CD8^+$ type 1 responses and RTS,S alone induced $CD4^+$ Th1 responses against this peptide. However, priming with DNA and boosting with RTS,S induced both patterns of responses to DR.316 simultaneously.

We reported previously that PfCSP DNA induced $CD4^+$ T cell dependent $CD8^+$ type 1 IFN-γ responses in humans as measured by T cell depletion and enrichment ELISPOT assays, and we speculated that $CD4^+$ T cells may function in a bystander helper capacity for $CD8^+$ T cell production of IFN-γ (8). In this study, we confirmed this hypothesis by conducting ELISPOT assays and real-time RT-PCR in parallel, in depleted or enriched T cell populations before and after the stimulation of PBMCs with peptide in vitro, respectively. Comparison of the numbers of IFN-γ-producing cells and IFN-γ mRNA expression levels before or after the peptide stimulation delineated the functional profiles of T cells involved in IFN-γ responses induced by DNA and RTS,S alone.

IFN-γ mRNA up-regulation was detected to PfCSP-specific peptides as expected in $CD45RA^-$ ($CD45RO^+$) $CD4^+$ and $CD8^+$ T cell populations. Cells with this profile are considered to be memory T cells, and should respond. Of interest was the fact that we also detected IFN-γ responses in $CD45RA^+CD4^+$ and $CD8^+$ T cell populations. This was unexpected as these populations are generally considered to be naive. These findings challenge the current definitions of naive, memory, and effector subsets of T cells with different phenotypes in humans. Similar results have been reported recently in human responses to EBV and other viral infections (35–37). Clearly, more work will be required to determine the relative roles of $CD45RA^+$ and $CD45RA^-$ T cells in protective immunity against Pf.

IFN-γ responses to PfCSP showed significant differences between the DNA-primed and nonprimed volunteers among those individuals who had existing anti-HBsAg Abs. Parallel comparison of the responses to HBsAg and PfCSP individually revealed that the RTS,S-induced IFN-γ responses were significantly lower to PfCSP than to HBsAg in all volunteers who were not primed with DNA, and were even lower in individuals with pre-existing anti-HBsAg. Although 13 of 14 control volunteers responded to HBsAg after one dose of RTS,S/AS02A, IFN-γ responses to PfCSP were only detected in 1 of 14 individuals. In contrast, the responses to the backbone Ag in DNA-primed volunteers had little or no impact on induction of IFN-γ responses to PfCSP, because both the frequency and the magnitude of IFN-γ responses to PfCSP were equivalent between HBsAg sero-positive and -negative individuals after the RTS,S boost. The results suggest that DNA priming directed postboost responses to the primed Ag. In regard to immunization with RTS,S, this appears to be of particular importance. RTS,S was designed with HBsAg as a carrier which would enhance T cell responses to the malaria Ag (PfCSP). Individuals in this study with baseline Abs against HBsAg had been previously immunized with the hepatitis B vaccine. We would expect that a malaria vaccine, delivered in sub-Saharan Africa, would have a target population with significant natural exposure or previous vaccination with HBsAg. Thus, our results suggest that the use of HBsAg in the vaccine may not permit the maximal induction of T cell responses to the malarial Ag. These findings should also have great implications for vaccination against other infectious diseases, particularly when vaccinated individuals have had previous exposure to proteins in delivery systems like recombinant viruses. Given the fact that there has been widespread smallpox vaccination, the prevalence of immunity to vaccinia virus could potentially limit the efficacy of vaccinia virus-based vaccines against other infectious diseases and cancer. Our results clearly demonstrated that a critical role for DNA in the face of exposure is to initiate and direct the T cell responses toward the specific Ag, and to balance the desired immunity along with the background responses. The prime-boost approach with priming with the DNA vaccine may prove useful in the development of vaccines in humans.

There is now an emerging body of data suggesting that combined vaccination regimens involving different vaccine vehicles or means of Ag presentation to the immune system induce immune responses more efficiently than vaccination with a single vehicle (11, 38, 39). Numerous data generated with prime-boost strategies in animal models have shown that priming with DNA and boosting with other delivery systems (e.g., recombinant poxviruses, recombinant protein, and recombinant adenovirus) can result in an increase in Ag-specific Ab and T cell responses and protective efficacy (40–44). In this study, Ag-specific $CD4^+$ helper, $CD8^+$ T cell dependent CTL and IFN-γ responses, Th1-type $CD4^+$ T cell dependent IFN-γ responses, and Ab responses were all simultaneously elicited in human volunteers by a DNA priming/recombinant protein-boosting immunization strategy. This strategy, capable of inducing all arms of the adaptive immune response, may offer unique advantages for preventive and therapeutic vaccines. Furthermore, we have provided experimental evidence for novel mechanisms by which this approach may have induced broad immune responses that are not obtained by immunization with a single vaccine vehicle.

Acknowledgments

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