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


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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


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 ELISOPOT 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– (CD45RA–) 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.

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4 Abbreviations used in this paper: PfCSP, *Plasmodium falciparum* circumsporozoite protein; HBsAg, hepatitis B surface Ag; AS02A, adjuvant system number 02A; Pf, *P. falciparum*; SFC, spot forming cell; h, human; TT, tetanus toxin.
HUMAN IMMUNE RESPONSES TO MALARIA DNA AND RECOMBINANT PROTEIN VACCINE

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 for protection against malaria, we initiated a study of a candidate malaria DNA vaccine (PfCSP/HBsAg fusion protein [RTS,S]) containing aa 207–395 of Pf (NF54/1310), and previous studies have shown that these peptides are recognized by HLA-DR class II molecules. Eight synthetic peptides derived from PfCSP and included in the RTS,S/AS02A vaccine were used for sensitization of CTL targets and ELISPOT assays. These peptides were purchased from Chiron Technologies (Clayton, Victoria, Australia) at 95% purity. The panel included four defined CTL epitopes (9) derived from HIV Gag protein (residue 77–85, SLYNTVATL, HLA-A2*0201-restricted), five new peptides derived from HIV Gag protein (residue 77–85, SLYNTVATL, HLA-A2*0201-restricted), four HLA-DR-binding peptides, DR.316 (residue 316–323, EYLQNSLSTEW (1)), DR.336 (residue 363–383, DYEKICKMECKCSSVFVNNS (9)), and DR.346 ((residue 364–365, IKPGSANKPDIELDYANDIE (23)), and previous studies have shown that these peptides are recognized by PBMCs from volunteers immunized with the VR2510 DNA vaccine (9). Antigenic and/or T-cell epitope-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) (residue 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 immuno-stimulant monophosphoryl lipid A and a saponin derivative known as QS-21, an endogenous thimerosal. The vaccine formulations containing 50 μg of RTS,S in 0.5 ml were prepared 30 min beforejection, according to the standard protocol.

Study design

Twenty-four HLA-A*0201-positive, malaria-naive volunteers were recruited as DNA-primed controls. All were negative for Abs to PfCSP, HIV, HBV core Ag, HCV, vaccinia, and ddsDNA. 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 ≥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 90–95% 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 (A3,113,36), and HLA-A*0201 (B3,35,35) (8), and four HLA-DR-binding peptides, DR.316 (residue 316–335, IKEYLQNSLSTEW (1)), DR.336 (residue 363–382, EYLQNSLSTEW (1)), DR.363 (residue 363–383, DYEKICKMECKCSSVFVNNS (9)), and DR.346 (residue 364–365, IKPGSANKPDIELDYANDIE (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 PFC protein-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) (residue 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 immuno-stimulant monophosphoryl lipid A and a saponin derivative known as QS-21, an endogenous thimerosal. The vaccine formulations containing 50 μg of RTS,S in 0.5 ml were prepared 30 min beforejection, according to the standard protocol.

Materials and Methods

Vaccines

The DNA vaccine, VCL-2510, encoding the full-length PICSP (Vical, San Diego, CA) has been described previously (8, 9, 13). The recombinant PICSP/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 immuno-stimulant monophosphoryl lipid A and a saponin derivative known as QS-21, an endogenous thimerosal. The vaccine formulations containing 50 μg of RTS,S in 0.5 ml were prepared 30 min beforejection, according to the standard protocol.

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 × 10⁵ cells/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 CD4⁺ or CD8⁺ 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 RNAeasy 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′-TGTGGGATGTAGGTACACATGGA-3′, hIFN-γ-R, 5′-CCTTTCGACACATGAAAAGAAT-3′, hGDPDH-F, 5′-GAAGTTGGAAGGTCGTCGAC-3′, hGDPDH-R, 5′-GAAGAGTGATGCTATTCATGCTCC-3′, hIFN-γ probe, 5′-TTGTCATCTGAAACACACGTTGCTGAA-3′, hGDPDH probe, 5′-CAAGCTTCCCCGTTCACGC-3′) for amplifying the IFN-γ and GDPDH mRNA were designed and standardized in-house following the manufacturer’s protocol. Amplification of GDPDH 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 fluorescent emission increased above a threshold level. Target gene expression was normalized between different samples based on the values of the expression of the GDPDH gene.
Statistical analysis

The frequency of peptide-specific IFN-γ responses was assessed using the χ² 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. PCP-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 PCP DNA vaccine previously (9).

CTL responses were detected in all four of the peptides containing PCP-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 (EYLKIQNSL-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 PICP 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.

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 PICP-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 PICP peptides which are included in the RTS,S sequence.

IFN-γ responses were detected to multiple PICP-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 PICP-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 PICP 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 1).

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 1). 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...
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: 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

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Responders/Number Tested</th>
<th>Number of Positive Assays/Total Assays (%)</th>
<th>Range of Net SFCs/10^6 PBMCs (geomean)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA-primed volunteers</td>
<td>Nonprimed volunteers</td>
<td>DNA-primed volunteers</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------</td>
<td>---------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>After first immunization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg (+)</td>
<td>4/6 (66.7)</td>
<td>2/8 (25.0)</td>
<td>13/69 (18.8)</td>
</tr>
<tr>
<td>HBsAg (−)</td>
<td>2/4 (50.0)</td>
<td>0/6 (0)</td>
<td>7/47 (14.9)</td>
</tr>
<tr>
<td>Total</td>
<td>6/10 (60.0)</td>
<td>b</td>
<td>2/14 (14.3)</td>
</tr>
<tr>
<td>After second immunization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg (+)</td>
<td>5/6 (83.0)</td>
<td>6/8 (75.0)</td>
<td>23/72 (31.9)</td>
</tr>
<tr>
<td>HBsAg (−)</td>
<td>3/4 (75.0)</td>
<td>6/6 (100.0)</td>
<td>18/48 (37.5)</td>
</tr>
<tr>
<td>Total</td>
<td>8/10 (80.0)</td>
<td>11/14 (84.6)</td>
<td>41/120 (34.2)</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg (+)</td>
<td>5/6 (83.0)</td>
<td>6/8 (75.0)</td>
<td>36/141 (25.5)</td>
</tr>
<tr>
<td>HBsAg (−)</td>
<td>3/4 (75.0)</td>
<td>6/6 (100.0)</td>
<td>25/95 (26.3)</td>
</tr>
<tr>
<td>Total</td>
<td>8/10 (80.0)</td>
<td>11/14 (84.6)</td>
<td>61/238 (25.6)</td>
</tr>
</tbody>
</table>

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*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.

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, $p = 0.019$).

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

<table>
<thead>
<tr>
<th>Volunteers with (HBsAg (+)) or without (HBsAg (−)) Abs to HBsAg</th>
<th>IFN-γ to PfCSP</th>
<th>IFN-γ to HBsAg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonprimed group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After first dose</td>
<td>Neg</td>
<td>A, B, C</td>
</tr>
<tr>
<td>After second dose</td>
<td>A, B</td>
<td>A, B</td>
</tr>
<tr>
<td>DNA primed group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After first dose</td>
<td>A, B</td>
<td>C</td>
</tr>
<tr>
<td>After second dose</td>
<td>A, B</td>
<td>A, B, D</td>
</tr>
</tbody>
</table>

*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.*
**DNA-primed, HBsAg Ab-positive volunteers, 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 were significantly more robust than it was to PfCSP.

In contrast, PICP DNA priming appeared to balance this immunodominance directing T cell responses toward PICP. 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 PICP. 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 PICP different than it was to HBsAg. The magnitude of responses to PICP 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 PICP (range of SFCs per 10⁶ PBMCs (geometric mean): 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 PICP if multiple doses of RTS,S vaccine are given.

DNA induces Th1 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 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 PICP 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 PICP 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. 2A). In contrast, IFN-γ responses to peptide TT-DR were 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. 2A).
completely CD4+, but not CD8+ T cell dependent, in all three positive responders tested (Fig. 2B).

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. 2E). 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. 2E). 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 PICSP 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. 2C). 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. 2E). 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 (Fig. 2E). 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 PICSP-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. 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 DNA-alone and RTS,S-boosted individuals.

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 DNA-alone and RTS,S-boosted individuals.
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. 4A), 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. 4B), 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. 2E; 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 CD8+ T-cells ranged from 3.0 to 28.3-fold (geometric mean, 6.6-fold) compared with that in CD8+ T-cells, which ranged from 4.0 to 281.03 (geometric, 19.7-fold) after the RTS,S boost. 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 PICSP 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. 2F) 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.

**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 PICSP 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 PICSP 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 PICSP recombinant protein and synthetic peptide vaccines (31, 32). The studies reported herein demonstrate that priming with the PICSP 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 Th1 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 PICSP-HBsAg fusion particle vaccine. Among individuals with Abs to HBsAg, those individuals primed with the PICSP DNA vaccine produced significantly better T cell responses after administration of RTS,S/AS02A than did volunteers who had never received PICSP 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 PICSP-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.

**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.
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+ T cell 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+ T cell 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+ T cell 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 simultane-}

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**References**