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Protective Immunity Induced with Malaria Vaccine, RTS,S, Is Linked to *Plasmodium falciparum* Circumsporozoite Protein-Specific CD4$^+$ and CD8$^+$ T Cells Producing IFN-γ[1]

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The *Plasmodium falciparum* circumsporozoite (CS) protein-based pre-erythrocytic stage vaccine, RTS,S, induces a high level of protection against experimental sporozoite challenge. The immune mechanisms that constitute protection are only partially understood, but are presumed to rely on Abs and T cell responses. In the present study we compared CS protein peptide-recalled IFN-γ reactivity of pre- and RTS,S-immune lymphocytes from 20 subjects vaccinated with RTS,S. We observed elevated IFN-γ in subjects protected by RTS,S; moreover, both CD4$^+$ and CD8$^+$ T cells produced IFN-γ in response to CS protein peptides. Significantly, protracted protection, albeit observed only in two of seven subjects, was associated with sustained IFN-γ response. This is the first study demonstrating correlation in a controlled *Plasmodia* sporozoite challenge study between protection induced by a recombinant malaria vaccine and Ag-specific T cell responses. Field-based malaria vaccine studies are in progress to validate the establishment of this cellular response as a possible in vitro correlate of protective immunity to exo-erythrocytic stage malaria vaccines. ***The Journal of Immunology*, 2003, 171: 6961–6967.**

Malaria is an infectious disease caused by the protozoan parasite, *Plasmodium*. It annually affects millions of people throughout the world, and children before the age of 5 years are particularly vulnerable to life-threatening anemia and cerebral malaria (1). The beneficial effects of chemotherapy are diminishing with the development of drug-resistant *Plasmodia* strains. Therefore, an effective antimalaria vaccine that elicits lasting immune responses capable of controlling the infection is urgently needed to combat this serious disease.

We previously reported the results of clinical trials in which substantial protection against experimental sporozoite (spz)[3] challenge was achieved with a recombinant *P. falciparum* circumsporozoite (CS) protein-based vaccine known as RTS,S (2–4). The vaccine consists of a hybrid molecule in which the CS protein central tandem repeat and carboxyl-terminal regions are fused N terminal to hepatitis B surface Ag (HBsAg) and are coexpressed with unfused HBsAg. In one of the studies (2), RTS,S was formulated in three different adjuvants: AS02A (formerly SBAS2), AS03 (formerly SBAS3), and AS04 (formerly SBAS4). Of the three, RTS,S/AS02A (vaccine 3), induced greater protection against spz challenge than RTS,S/AS04 (vaccine 1), or RTS,S/AS03 (vaccine 2) (2). Analyses of immune responses after vaccination with the three formulations of RTS,S confirmed an induction of high levels of CS protein-specific Abs (2) and proliferative T cells (3) in nearly all RTS,S-immunized subjects. The magnitude of proliferation, however, did not correlate with protection against experimental challenge (2, 3). Results from a subsequent RTS,S/AS02A vaccine trial corroborated these findings (4). Extensive testing of PBMC from vaccinees receiving all three RTS,S formulations failed to reveal CD8$^+$ CTLs to CS protein peptides.

There is ample evidence from studies of immune responses elicited by natural exposure to *Plasmodia* parasites and experimental immunization with attenuated *Plasmodia* spz to support a protective role of B cells (5, 6) and CD4$^+$ (7–9) and CD8$^+$ (10–13) T cells specific for exo-erythrocytic Ags. Although both T cell subsets exhibit many functional properties, CD4$^+$ T cells principally help, whereas CD8$^+$ T cells directly exert protective effector function by several distinct mechanisms. Apart from HLA class I-restricted cytolytic activity (11–13), CD8$^+$ T cells engage in pathways of protective immunity that rely on the release of soluble and/or membrane-bound inflammatory mediators that may have direct antiparasitic effects. IFN-γ, in particular, has been considered a key cytokine in protective immunity, particularly against exo-erythrocytic stage malaria infection (14, 15); in its presence the number of liver-stage parasites is drastically reduced, presumably through the NO pathway (16, 17). Hence, in the present study we asked whether IFN-γ-producing, CS protein-specific T cells induced by immunization with RTS,S would segregate protected from nonprotected subjects.

We analyzed PBMC for CS protein-specific T cells producing IFN-γ. The analyses were conducted on PBMC sampled at multiple time points, including pre- and post-immunization, from subjects immunized with the three RTS,S formulations. In addition, we analyzed PBMC for T cell responses from subjects who underwent one or two experimental spz challenges. On the basis of our observations obtained from 20 participants enrolled in this study, the presence of IFN-γ-producing, CS protein-specific T

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[3] Abbreviations used in this paper: spz, sporozoite; CS, circumsporozoite; HBsAg, hepatitis B surface Ag.

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cells distinguished protected from nonprotected subjects. Moreover, both $CD4^+$ and $CD8^+$ T cells secreted IFN-γ specifically in response to CS protein peptides, and T cell reactivity was sustained in subjects with protracted protection. This is the first study demonstrating a correlation in a controlled challenge between malaria vaccine-induced Ag specific-T cell response and protection. The establishment of this cellular response as a possible in vitro correlate of protective immunity awaits confirmatory results from a larger number of subjects to be included in subsequent experimental as well as field-based malaria vaccine studies.

Materials and Methods

Subjects and PBMC

The RTS,S vaccine study has been described in detail previously (2, 3). Briefly, 20 malaria-naive subjects received three doses (at wk 0, 4, and 28) of RTS,S as vaccine 1, vaccine 2, or vaccine 3 and subsequently underwent malaria challenge at wk 31 and a rechallenge at wk 55. PBMC were obtained at preimmunity (wk 0) and 10–14 days after each vaccination (wk 2, 6, and 30) as well as after challenge, at wk 40 and 48. PBMC were cryopreserved and stored in liquid nitrogen until analyses. Written informed consent was obtained from all volunteers. Recruitment of volunteers was performed in accordance with existing U.S. Army regulations, and the study was conducted in accordance with guidelines established by and with the approval of the institutional review board of the Walter Reed Army Institute of Research.

Vaccine

RTS,S (2) is a recombinant protein vaccine consisting of a polypeptide chain that contains the central repeat and a C-terminal region of *P. falciparum* (3D7) CS protein encompassing aa 207–395, fused to HBsAg, and an unfused (S) polypeptide of 226 aa of HBsAg. RTS,S was formulated in three adjuvants: alum plus monophosphoryl lipid A (RTS,S/AS04), oil-in-water emulsion (RTS,S/AS03), and oil-in-water emulsion plus monophosphoryl lipid A and QS21, designated RTS,S/AS02A. Details of the vaccine construct, adjuvant formulations, and the safety, immunogenicity, and efficacy have been reported previously (2).

CS peptides

Synthetic, 15-mer, overlapping peptides, p34 (aa 316–330, NEEPSDKHIKEYLNK), p35 (aa 321–335, DKHIKEYLNKIQNSL), p36 (aa 326–340, EYLNKIQNSLSTEWS), p37 (aa 331–345, IQNSLSTEWSPCSVT), and p39 (aa 368–382, KPKDELDYANDIEKK) were used at the indicated concentrations, either singly or as peptide pools in lymphocyte cultures. These peptides are based on the 3D7 strain of *P. falciparum.* The purity of the peptides determined by HPLC (α Diagnostics, San Antonio, TX) ranged from 96–100%.

Lymphocyte culture

Cryopreserved PBMC were thawed, washed, and resuspended at 2×10^6 cells/ml in RPMI 1640 supplemented with 8 mM GlutaMax (Life Technologies, Grand Island, NY), 50 μg/ml penicillin/50 μg/ml streptomycin (Life Technologies), 0.1 mM MEM nonessential amino acids (Life Technologies), 0.042 mM 2-ME (Sigma-Aldrich, St. Louis, MO), 1 mM MEM sodium pyruvate (Life Technologies), and 5% FBS (HyClone, Logan, UT). Cells were seeded into 96-well, round-bottomed tissue culture plates in 100-μl aliquots. One hundred microliters of individual peptides at 40 μg/ml or a peptide pool containing the same concentration of each peptide were added to each culture in triplicate. The peptide pools consisted of the following peptides: p34 plus p36 and p35, p37, plus p39. Control cultures were stimulated with *P. berghei* CS protein peptides at the same concentration. Recombinant IL-2 (Genzyme, Cambridge, MA; 25 μg/ml) and rIL-7 (Genzyme; 2.5 ng/ml) were added, and the cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere for 10–14 days. At the conclusion of the culture period, cells were harvested, washed, and resuspended in complete medium at the indicated concentration for assays.

ELISPOT assay

MultiScreen MAIPS-4510 96-well plates (Millipore, Molsheim, France) were coated with 100 μl/well of 10 μg/ml anti-human IFN-γ (Clone 1-D1K, Mabtech, Sweden) at room temperature for 3 h. The plates were washed with RPMI 1640 and blocked for 1 h. One hundred microliters of the cell suspension (2×10^6 cells/ml) plus 100 μl of each individual peptide at 40 μg/ml or peptide pool were added to triplicate wells, and cultures were incubated for ~18 h at 37°C in a 5% CO_2 humidified atmosphere. The cell cultures were decanted, and the wells were washed six times with PBS/Tween 20 (Mallinckrodt, St. Louis, MO), followed by 2-h incubation at room temperature in the presence of 100 μl/well of 1/1000 biotin-anti-IFN-γ (Mabtech, Nacka, Sweden; clone 7-B-1-biotin). At the conclusion of the reaction time, the wells were washed extensively, and 100 μl/well of a 1/1000 dilution of streptavidin-alkaline phosphatase (Mabtech) was added to each well for an additional 90-min reaction at room temperature. For color development, the plates were thoroughly washed, and 100 μl/well of alkaline phosphatase-conjugated substrate (Bio-Rad, Hercules, CA) was added to each well. When dark spots emerged, the plates were washed under tap water to end the reaction. The spots were counted using a computerized image analyzer (model 3.2, Scananalytics, Fairfax, VA). IFN-γ responses specific to CS peptides were scored as the mean number of spots in triplicate cultures containing CS peptides after subtracting the mean number of spots in triplicate cultures containing medium alone. The data are expressed as the mean of the ELISPOTS per 10^6 cells. The SD of the mean was <20%.

Intracellular staining

PBMC (5×10^6 cells/ml) in CM were stimulated with 20 μg/ml of CS peptides and incubated at 37°C in a 5% CO_2 humidified atmosphere for 24–48 h. Cells were harvested and stained with PE-anti-CD4 (BD Biosciences, Mountain View, CA) and Cy-anti-CD8 (BD PharMingen, San Diego, CA) and then fixed with IC-Fix (BioSource, Camarillo, CA) for 10 min at room temperature. The cells were permeabilized with IC-Perm (1×; BioSource) for 2 min, after which FITC-anti-IFN-γ (BioSource) was added for detection of intracellular cytokine. FITC-mouse IgG1 (BioSource) was used as the isotype control. For analysis, between 30,000 and 50,000 cells/sample were acquired on a FACScan. The results were analyzed using CellQuest (BD Biosciences).

Statistical analyses

The p values were calculated for statistical comparisons using one-way ANOVA of the mean IFN-γ responses between protected and nonprotected responses. A value of $p < 0.05$ was considered significant. Spearman's rank test was used to calculate the correlation between RTS,S-induced IFN-γ responses and CS protein repeat region-specific Ab responses reported as total IgG in micrograms per milliliter (2).

Results and Discussion

RTS,S immunization induces CS protein-specific lymphocytes to produce IFN-γ

Eighty-six percent (six of seven) efficacy was achieved against experimental spz challenge of seven malaria-naive subjects immunized with RTS,S/AS02A (vaccine 3) (2). We therefore used this study as a model system to investigate whether protective immunity induced by exo-erythrocytic stage vaccine might be linked to CS protein-specific T cells producing IFN-γ. The IFN-γ response was examined in lymphocyte cultures established from 20 subjects who received one of the three RTS,S vaccines: six subjects in vaccine 1 group and seven subjects each in vaccine 2 and vaccine 3 groups. Specifically, IFN-γ responses were evaluated by the ELISPOT assay under conditions of in vitro recall with a pool of *P. falciparum* CS protein peptides (p34, p35, p36, p37, and p39; Fig. 1). These particular CS protein peptides were chosen on the basis of their ability to bind to a wide spectrum of HLA class I (18) and class II (19, 20) alleles and activate $CD4^+$ and $CD8^+$ T cells (18, 21).

RTS,S immunization induced a vigorous CS protein peptide-specific IFN-γ response readily distinguished from the low reactivity of preimmune lymphocytes (mean ± SD, 5 ± 15 ELISPOTs/10^6 cells (termed culture); $n = 20$; Fig. 2). Based on the formula, mean + 3 SD, we determined that <50 ELISPOTs/culture constituted background reactivity. Accordingly, 10 of 20 subjects consistently responded in a positive range of 100–500 ELISPOTs/culture, and two of 20 subjects responded in a background range of 40–50 ELISPOTs/culture; responses were undetectable in the remaining eight subjects.

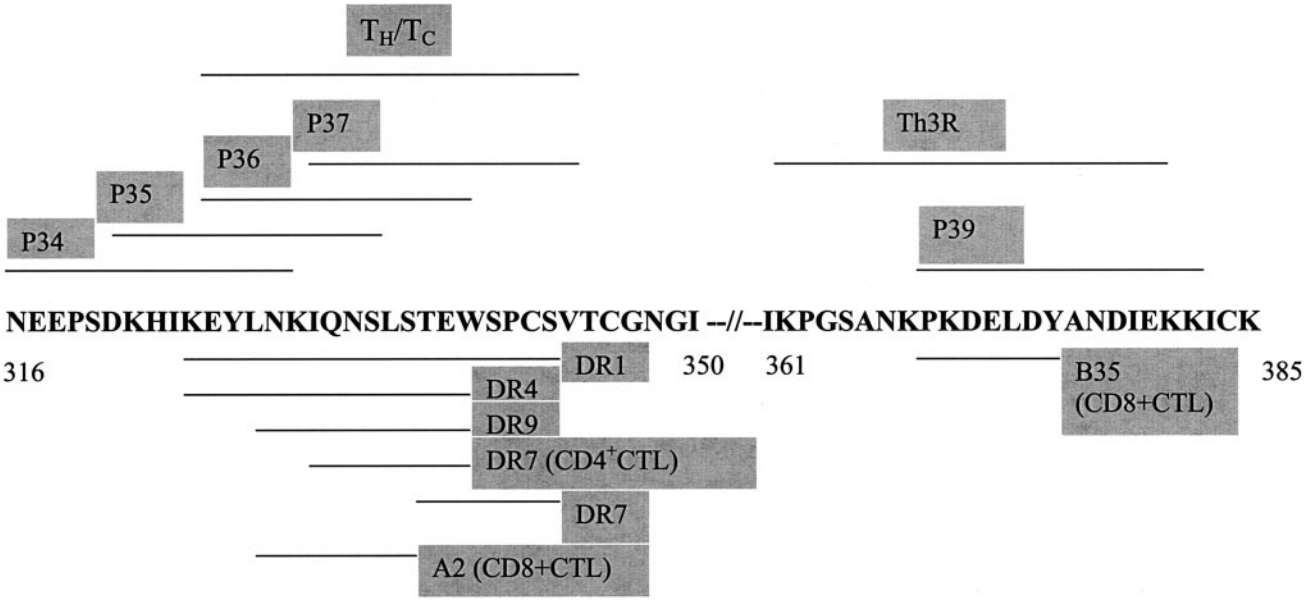

FIGURE 1. *P. falciparum* (strain 3D7) carboxyl-terminus CS protein peptides. The CS protein fragment including aa residues 316 −350 and 361–385 are shown. The individual peptides designated p34 (aa 316–330, NEEPSDKHIKEYLNK), p35 (aa 321–335, DKHIKEYLNKIQNSL), p36 (aa 326–340, EYLNKIQNSLSTEWS), p37 (aa 331–345, IQNSLSTEWSPCSVT), and p39 (aa 368–382, KPKDELDYANDIEKK) are marked by horizontal lines. The regions of the *P. falciparum* CS protein designated Th/Tc and Th3R are also indicated. HLA class I and class II alleles that bind particular CS protein peptides are shown.

In addition, PBMC from a few subjects in each vaccine group, selected on the basis of available cells, were tested for IFN-γ reactivity recalled by individual peptides, p34, p35, p36, p37, and p39. The pattern of responses among the 11 subjects reflected their reactivity to peptide pools, without dominant CS protein peptide reactivity (data not shown).

The RTS,S-induced responses were specific for the *P. falciparum* CS protein as cultures stimulated with *P. berghei* CS protein peptides or medium alone produced responses in a range of 0–15 ELISPOTs/culture (data not shown). Preimmune and immune lymphocytes stimulated with PHA (2 μg/ml) uniformly produced a strong reactivity, defined as >500 ELISPOTs/culture (data not shown).

IFN-γ responses correlate with protection

Most importantly, however, the availability of protection data allowed us to ask whether a link could be established between protective immunity and T cells producing IFN-γ. Comparison of responses among the 20 subjects shows that the majority of protected subjects were indeed high IFN-γ responders (Fig. 2). Among the six protected subjects in the vaccine 3 group, only subject 34 was a nonresponder to a recall with CS protein peptides as well as RTS,S (data not shown). A single nonprotected person, subject 35, in this group showed negligible IFN-γ. The two protected subjects (no. 22 and 25) in the vaccine 2 group also showed vigorous responses (500 and 150 ELISPOTs/culture, respectively). One of the five nonprotected subjects (no. 30) consistently responded in the range of 90–100 ELISPOTs/culture. Among the vaccine 1 recipients, a single protected subject (no. 2) was a high responder (≥500 ELISPOTs/culture); however, an elevated response (150 ELISPOTs/culture) was also noted in a nonprotected subject (no. 8).

Protective immunity in each vaccine group was accompanied by a strong IFN-γ response detected after the third immunization ($p = 0.001$; Fig. 2). Clearly, the presence of the AS02A adjuvant enhanced acquired immunity to RTS,S because vaccines 1 and 2, consisting of the same protein, albeit formulated in different adjuvants, proved less effective in generating CS protein-specific T cells to produce IFN-γ. Adjuvants typically promote Ag-specific immune responses by a prerequisite strong proinflammatory reaction (22). On the basis of the IFN-γ response in the vaccine 3 group, this appears to be true for AS02A. The diverse profiles of both HLA class I and II alleles among the 20 recipients of the three RTS,S formulations argue against any biased distribution that might have favored the induction of a particular specificity of CS protein recognizing T cells (data not shown).

The antimalarial action of IFN-γ has been amply demonstrated by its capacity to prevent development of exo-erythrocytic parasites (15). In addition to its general immune-enhancing effect, such as the potent activation of macrophages, IFN-γ reduces the number of liver stage parasites, presumably through the NO pathway (17). Furthermore, by enhancing the Ab response (14), an important protective mechanism against the spz stage infection, IFN-γ might be a key cytokine that integrates the multifactorial protective immune responses to exo-erythrocytic parasites. RTS,S vaccination induces a substantial CS protein repeat region-specific Ab response (2–4), which generally exceeds the anti-repeat Ab levels described in subjects protected by attenuated *P. falciparum* spz (6). A statistical analysis by Spearman's rank test demonstrated a positive correlation between the RTS,S-induced IFN-γ responses shown here and CS protein repeat-specific Ab titers (micrograms per milliliter) reported previously for the same group of subjects (2), with a coefficient of 0.573 and $p < 0.008$. It appears, therefore, that a substantial involvement of both these responses may indeed form the basis for protective immunity induced by RTS,S. It should be noted, however, that some subjects were susceptible to infection despite showing relatively high levels of both IFN-γ and Ab (150 ELISPOTS and 43.58 μg/ml, respectively, for subject 30), whereas others (subject 44) with a similar IFN-γ response (180 ELISPOTS) but a somewhat lower Ab level (18.5 μg/ml) were nonetheless protected. It is possible that not only titers, but

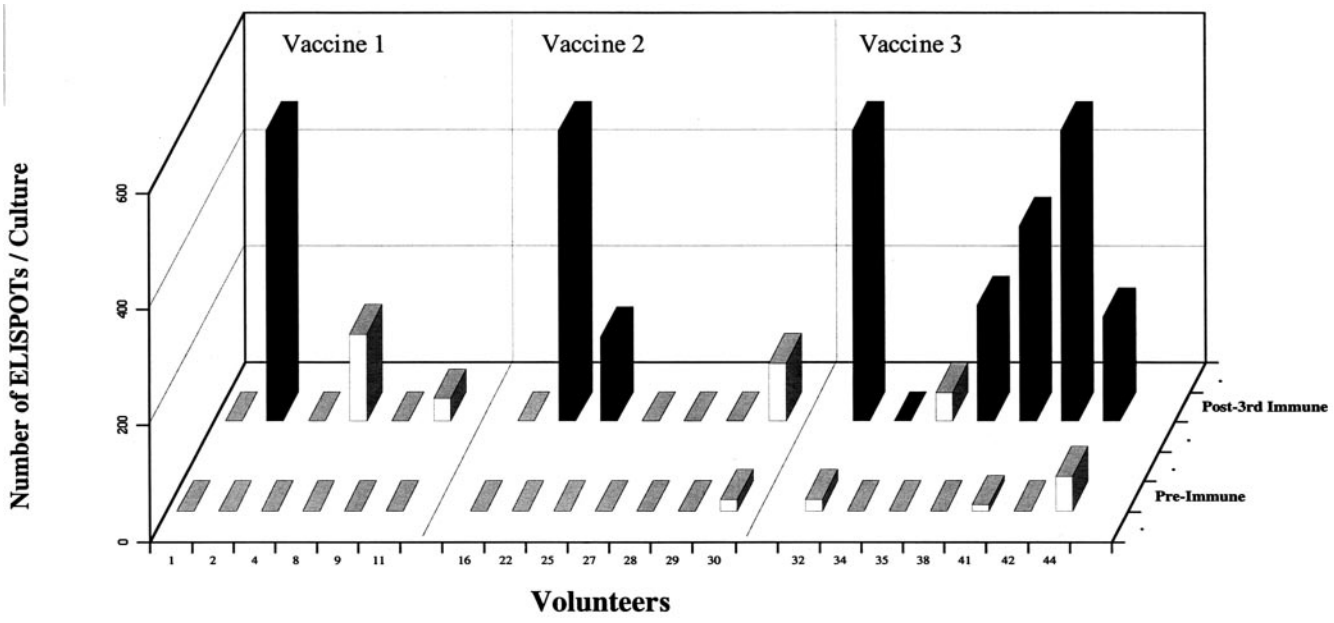

FIGURE 2. IFN-γ responses elicited by the RTS,S vaccine. These composite data show IFN-γ responses recalled by CS protein peptides in cultures from subjects vaccinated with RTS,S. The analyses were performed on PBMC obtained from subjects ($n = 20$) on days 0 (preimmune) and 10–14 after the third vaccination with RTS,S (before spz challenge). The subjects are identified by an arbitrary number from 1–20 and are grouped according to the vaccine formulation they received: vaccine 1, vaccine 2, or vaccine 3. Preimmune and RTS,S-immune PBMC from each subject were assayed concurrently. The IFN-γ responses represent the mean of triplicate cultures (SD, <20%) and are expressed as the number of ELISPOTs per 10^6 cells, termed culture. ■, Responses of protected volunteers; □, responses of subjects who were not protected against spz challenge (2) ($p = 0.001$). PBMC from each subject were assayed at least twice, yielding similar responses.

also affinities and particularly the function of the CS protein-specific Abs, had an effect on the overall protective response. This is consistent with our recent observation that only sera from subjects protected by RTS,S immunization are able to opsonize *P. falciparum* spz (23). Thus, the contribution and relative importance of Abs and T cell-mediated cytokine responses to the integrated protective immunity still remain only partially understood.

Persistence of IFN-γ responses

One of the central issues in vaccine development is the duration of protective immunity, a property inherent in the immunologic memory of T and B cells. In the present study, vaccination with RTS,S occurred at wk 0, 4, and 28; the first spz challenge occurred at wk 31; spz rechallenge of the seven previously protected subjects occurred at wk 55 (3). To determine the persistence of Ag-specific T cells producing IFN-γ, we evaluated IFN-γ production at wk 0, 2, 6, 30, 40, and 48 among five subjects (no. 2, 38, 25, 42, and 44) who were challenged twice (Fig. 3, *a–e*) and two subjects (no. 22 and 41) who were challenged only once (Fig. 3, *f* and *g*). These subjects were chosen on the basis of the availability of cryopreserved lymphocytes.

Robust IFN-γ responses ranging from 150–500 ELISPOTs/culture were observed in all protected subjects after the third immunization (Fig. 3, *a–g*), but variations in the magnitude of the responses were quite apparent among the subjects. As expected, at wk 40 and 48, IFN-γ responses generally declined. Because 9 and 17 wk have elapsed between the first spz challenge (wk 31) and PBMC sampling for the postchallenge analyses, a spz boost effect was difficult to assess. Significantly, substantial IFN-γ responses (200–250 ELISPOTS/culture) were sustained for subjects 2 and 38 (Fig. 3, *a* and *b*), who remained protected at rechallenge (wk 55). Conversely, substantially reduced responses were observed in the three subjects who became susceptible to infection upon rechallenge (Fig. 3, *c–e*). Subjects whose responses are shown in Fig. 3, *f* and *g*, received a single challenge at wk 31. Although these data do not directly address the induction of memory T cell responses, they suggest that a certain level of a recall response, in this case IFN-γ activity, is required for the maintenance of protracted protection.

$CD4^+$ and $CD8^+$ T cells produce IFN-γ

Having shown a correlation between IFN-γ reactivity and protection induced by RTS,S/AS02A, we proceeded to identify and quantify CS protein-specific T cells producing IFN-γ by an intracellular detection assay (24). We hoped that this approach would not only provide a direct identification of T cells that produce the cytokine, but would also independently confirm results obtained by the ELISPOT method. The identification of T cell subsets as a source of IFN-γ became quite important because interactions among regulatory T cell subsets reactive to a nominal protein Ag critically influence the outcome of an immune response (25). Hence, understanding how the *P. falciparum* CS protein-specific $CD4^+$ and $CD8^+$ T cell responses are integrated might reveal the type of cellular interactions that are required for the achievement of protective immunity. Moreover, observations from a separate RTS,S/AS02A vaccine trial that did not include experimental challenge (26) showed that although depletion of $CD8^+$ T cells preserved IFN-γ responses elicited by CS protein peptides, depletion of $CD4^+$ T cells did not.

The detection of intracellular IFN-γ was performed on preimmune and, in some instances, both pre- and postchallenge lymphocytes from subjects in each of the three groups. Representative data (Fig. 4) from a single subject show dot plot details of IFN-γ responses observed in lymphocytes cultured with a peptide pool and medium control. Clearly, RTS,S immunization induced CS protein-specific $CD4^+$ as well as $CD8^+$ T cells to produce IFN-γ. In contrast to an extremely low reactivity of preimmune $CD4^+$ T

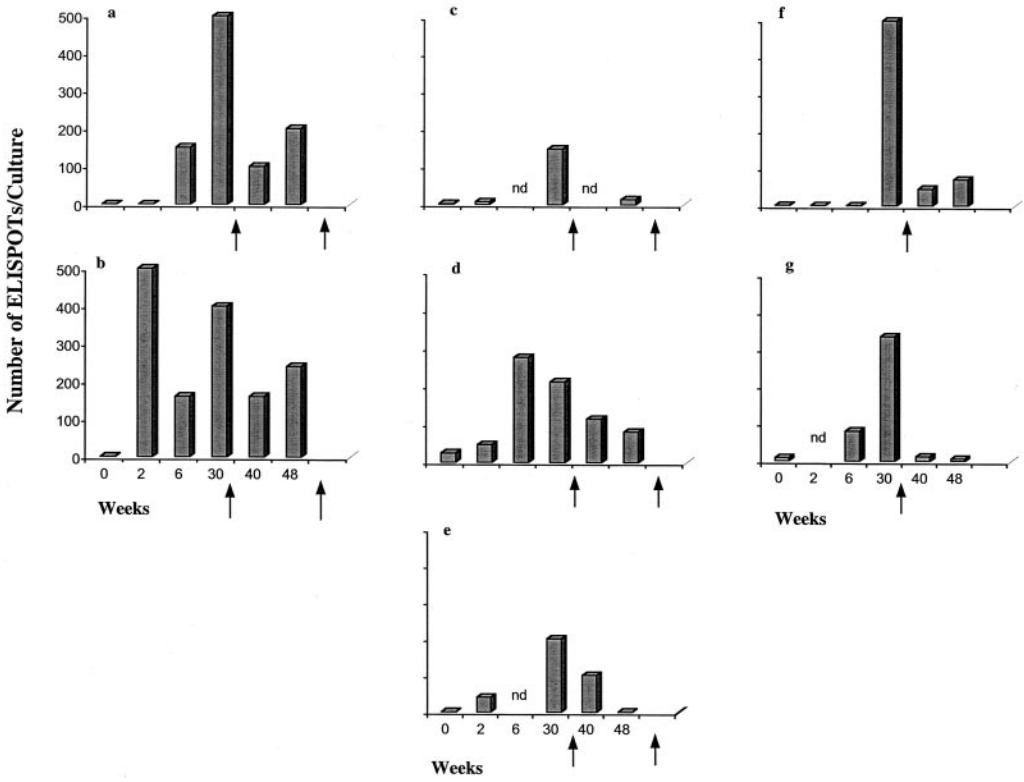

FIGURE 3. Persistence of IFN-γ responses in protected subjects. These composite data show profiles of IFN-γ responses at the indicated time points (weeks) during the study. The arrows indicate challenge (wk 31) and rechallenge (wk 55). *a* and *b*, Responses of subjects 2 and 38, respectively, who were protected at challenge and rechallenge; *c–e*, responses for subjects 25, 42, and 44, respectively, who were protected at challenge, but not at rechallenge; *f* and *g*, responses for subjects 22 and 41, respectively, who were protected at challenge, but were not rechallenged. The IFN-γ responses representing the mean of triplicate cultures (SD, <20%) are expressed as the number of ELISPOTs per 10^6 cells. For details, see Fig. 2.

cells cultured in the presence of CS protein peptide pool or medium alone (0.03 and 0.06% of $CD4^+$ T cells were positive for IFN-γ, respectively), RTS,S-immune $CD4^+$ T cells positive for intracellular IFN-γ rose to 0.30%, hence increasing 10-fold over the preimmune response. Similarly, preimmune $CD8^+$ T cells positive for IFN-γ were negligible in response to CS protein peptide pool or medium (0.04 and 0.02%), whereas RTS,S-immune $CD8^+$ T cells positive for IFN-γ rose to 0.34%. Isotype-matched controls yielded only background responses with 0.02% for $CD4^+$ T cells and 0.05% for $CD8^+$ T cells. To confirm the specificity of the intracellular IFN-γ, we performed inhibition studies by adding soluble IFN-γ (0.01 μg/ml) to cultures during the assay. Under these conditions, neither $CD4^+$ nor $CD8^+$ T cells positive for intracellular IFN-γ exceeded the background responses of 0.02 and 0.04%, respectively.

Composite data (Fig. 5, *a* and *b*) showing IFN-γ responses of T cells detected by an intracellular approach confirm that protective immunity is indeed associated with CS protein peptide-recalled IFN-γ and unambiguously demonstrate that both $CD4^+$ and $CD8^+$ T cells are the producers of this cytokine. The level of IFN-γ-producing T cells varied among the eight subjects tested, but among protected subjects the response ranged between 0.18 and 0.57% for $CD8^+$ T cells (0.33 ± 0.13; $p = 0.009$) and between 0.1 and 0.3% for $CD4^+$ T cells (0.2 ± 0.1; $p = 0.042$; Fig. 5*a*). In contrast, the responses of nonprotected persons were considerably lower for both T cell subsets (0.11 ± 0.035 for $CD8^+$ T cells and 0.066 ± 0.042 for $CD4^+$ T cells; Fig. 5*b*).

Although exogenously presented Ags induce mainly $CD4^+$ T cells, instances when they also induce $CD8^+$ T cells are rare. $CD8^+$ T cells are presumed to arise specifically in response to endogenously presented Ags. There is growing evidence, however, that Ags present in extracellular fluids can induce $CD8^+$ T cells after gaining entry into the cytosol. In fact, processing/presentation studies have shown that particulate exogenous forms of Ags can enter both HLA class II and class I pathways for activation of $CD4^+$ and $CD8^+$ T cells (27, 28). RTS,S represents a particulate form of an Ag, and the portion of the CS protein included in the RTS,S vaccine contains both CD4 and CD8 T cell epitopes (7). In an earlier study we observed that RTS,S/MPL/Alum or RTS,S/AS04 could induce a CS protein peptide-specific $CD8^+$ CTL response (29). Moreover, we previously demonstrated that inclusion of CS protein in liposomes allows MHC class I presentation of CS to $CD8^+$ T cells in mice (30) and to a less detectable degree in humans (31). We speculate, therefore, that AS02A enhanced RTS,S entry into the cytosol of an APC for the generation of CS protein peptide-specific $CD8^+$ T cells.

The limited availability of lymphocytes prevented us from addressing several important questions concerning mechanisms of RTS,S induction of CS protein peptide-specific $CD4^+$ and $CD8^+$ T cells. On the basis of information concerning the overlap between CD4 and CD8 epitopes (7), it is possible that both T cell sets responded to the same or overlapping CS protein peptides. It can be envisaged that epitope overlaps facilitate interdependent responses between $CD4^+$ and $CD8^+$ T cells across a single APC. At present, the importance of these observations is not completely understood, but other examples of an overlap between CD4 and CD8 epitopes that induce HLA class II- and class I-restricted T cell responses have been reported (32, 33). Preliminary evidence (P. Sun, unpublished observations) show a dependence of $CD8^+$ T cells on $CD4^+$ T cells, as the addition of anti-HLA class II mAbs

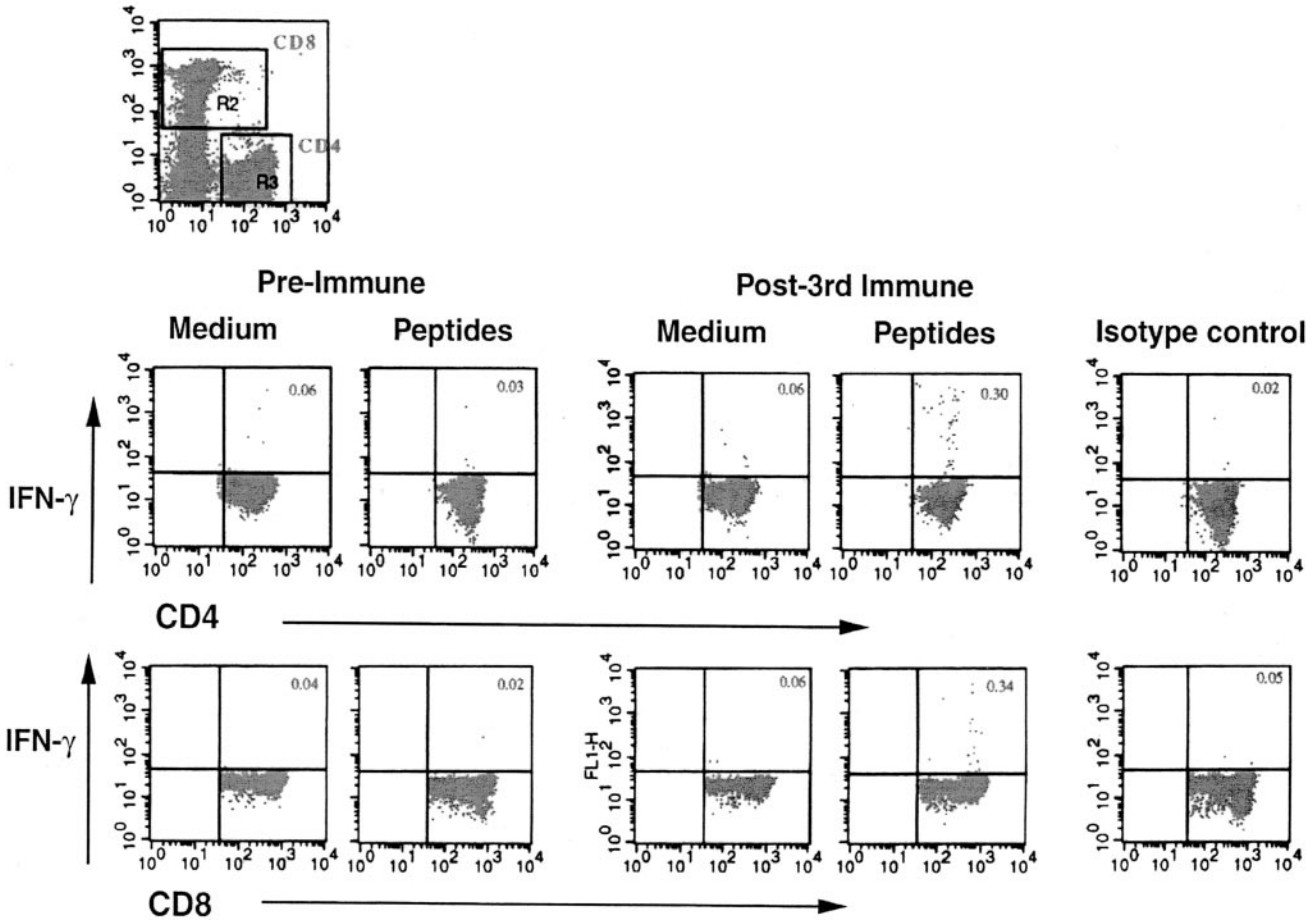

FIGURE 4. Identification of IFN-γ-producing T cells. PBMC from a single protected subject were stimulated for 24 h with a pool of *P. falciparum* CS protein peptides (p35, p36, plus p37) or medium control. Lymphocytes were gated by forward and side scatter and T cells labeled with PE-anti-CD4 and CyChrome-anti-CD8 were identified by their respective surface markers. Preimmune and post-third RTS,S-immune $CD4^+$ and $CD8^+$ T cells were analyzed by intracellular staining for the presence of IFN-γ using FITC-anti-IFN-γ. The numbers in each *upper right panel* show the percentage of IFN-γ-positive T lymphocytes from gated $CD4^+$ or $CD8^+$ T cells. Isotype controls included FITC-mouse-IgG1 and show baseline response.

abrogated IFN-γ production by both T cell sets, whereas inclusion of anti-HLA class I mAb interfered only with the reactivity of $CD8^+$ T cells. Most importantly, the helper/inducer activity of RTS,S-immune $CD4^+$ T cells was evident only in cultures containing RTS,S-immune, but not naive $CD8^+$ T cells. Despite the preliminary nature of these observations, there is growing evidence that expansion as well as responses of *Plasmodia* Ag-generated $CD8^+$ T cells depend upon support from $CD4^+$ T cells (34, 35).

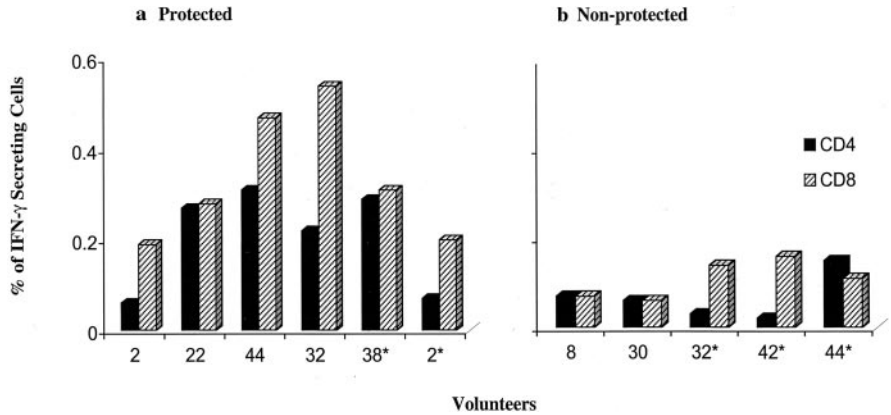

FIGURE 5. Correlation between IFN-γ-producing CS protein-specific $CD4^+$ and $CD8^+$ T cells and protection. PBMC, obtained before challenge and rechallenge, were cultured under conditions as described in Fig. 4. The data are expressed as a percentage of gated $CD4^+$ and $CD8^+$ T cells positive for intracellular IFN-γ determined from protected (*a*; $n = 6$) and nonprotected (*b*; $n = 5$) subjects. $p < 0.042$ for IFN-γ $CD4^+$ T cells from protected vs nonprotected and $p < 0.009$ for IFN-γ $CD8^+$ T cells from protected vs nonprotected. Subject number followed by an asterisk indicates a rechallenge. The percentage of IFN-γ-positive $CD4^+$ and $CD8^+$ T cells in cultures stimulated with medium alone were negligible and were not subtracted from the responses in peptide-stimulated cultures. PBMC from each subject tested were analyzed at least twice, yielding similar results.

Therefore, it is possible that depletion of $CD4^+$ T cells from cultures of RTS,S-immune lymphocytes (26) might have prevented the reactivity of $CD8^+$ T cells. Generally, it is thought that $CD4^+$ T cells either directly help $CD8^+$ T cells or, by licensing APC, provide indirect support (36).

In conclusion, these observations provide the first evidence that a relationship exists between recombinant protein malaria vaccine-induced protection and peptide-specific $CD4^+$ and $CD8^+$ T cells producing IFN-γ. The significance of this observation cannot be overstated, as other studies of experimental malaria vaccines have observed a robust induction of Ag-specific T cell response marked by either IFN-γ (34) or CTL (37) in the absence of protection. Furthermore, the persistence of elevated IFN-γ responses observed in subjects with protracted protection suggests that memory T cells may have been induced by RTS,S. Experiments addressing these and other issues concerning the interaction of RTS,S-induced $CD4^+$ and $CD8^+$ T cells are currently in progress. Malaria vaccine studies are also being conducted in malaria endemic areas, and it is hoped that results from these studies will validate present conclusions regarding Ag-specific $CD4^+$ and $CD8^+$ T cells producing IFN-γ as an in vitro correlate of protective immunity induced by a pre-erythrocytic malaria vaccine.

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