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Production by activated human T cells of interleukin 4 but not interferon-γ is associated with elevated levels of serum antibodies to activating malaria antigens

(Pf155/ring-infected erythrocyte surface antigen/anti-peptide antibodies/T-cell epitopes)

M. TROYE-BLOMBERG*, E. M. RILEY†, L. KABILAN*, M. HOLMBERG‡, H. PERLMANN*, U. ANDERSSON*, C. H. HEUSSER§, AND P. PERLMANN*

*Department of Immunology, University of Stockholm, S-106 91 Stockholm, Sweden; †Medical Research Council Laboratories, Fajara, Near Banjul, The Gambia; ‡Department of Medical Genetics, Biomedical Center, Uppsala, Sweden; and §CIBA-Geigy, Basel, Switzerland

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ABSTRACT **T cells play a crucial role in antibody-mediated and antibody-independent immunity against *Plasmodium falciparum* malaria. Therefore, a vaccine immunogen should include parasite-derived B- and T-cell epitopes capable of giving rise to protective responses in both systems. The *P. falciparum* antigen Pf155/ring-infected erythrocyte surface antigen (RESA), a vaccine candidate, contains immunodominant T- and B-cell epitopes located in the central (5′) and C-terminal (3′) invariant repeat regions of the molecule. To relate Pf155/RESA-peptide-specific responses of T cells to function, T cells from *P. falciparum* immune donors were activated with peptides corresponding to these immunodominant regions. Activation was measured as induction of interferon-γ secretion, T-cell proliferation (DNA synthesis), or transcription and translation of interleukin 4 (IL-4) mRNA. Peptides from both regions were shown to induce interferon-γ, IL-4, proliferation, or any combination. In individual donors, there was no correlation between these different activities. Rather, they were negatively correlated, demonstrating the importance of examining multiple parameters of T-cell activation when estimating the proportion of individuals responding to a given epitope. However, IL-4 mRNA and intracellular IL-4 could be induced in T cells of donors who had elevated concentrations of serum antibodies to the same peptide that was used for T-cell activation. These results suggest that a causal relationship exists between the activation of IL-4-producing T-cell subsets and production of the anti-Pf155/RESA-specific antibodies in individuals in which immunity has been induced by natural infection. This finding has implications that should be considered for the selection of immunogens to be included in a future *P. falciparum* subunit vaccine and for vaccine development in general.**

Regulatory T cells of the $CD4^+$ phenotype play a crucial role in the acquisition and maintenance of cellular and humoral immunity to malaria. Thus, although antibodies are important for protection, the production of these antibodies is regulated by the T-cell system (1, 2). Furthermore, immunity to malaria involves important cellular mechanisms that are antibody-independent and the induction of parasite-specific anamnestic responses after reinfection after vaccination also requires that the vaccine immunogen contains both B-cell (antibody)- and T-cell-reactive epitopes. Thus, for the rational design and development of a subunit malaria vaccine, it is essential not only to define the sequences making up the immunodominant T-cell epitopes but also to understand the nature and function of the T-cell responses that they induce.

Analysis of murine regulatory $CD4^+$ T-cell clones has revealed that they can be further divided into two subsets, based on their repertoire of lymphokine production. Upon activation, $CD4^+$ cells designated T helper 1 (TH1) produce interleukin 2 (IL-2) and interferon-γ (IFN-γ), and cells designated T helper 2 (TH2) produce the lymphokines interleukin 4 (IL-4) and interleukin 5 (IL-5) (3–7). Although the relationship between these cell types is not clear, there is good evidence that TH1 cells mediate certain antibody-independent responses (e.g., delayed type hypersensitivity) and TH2 cells provide help for specific antibody production (3–7). Analysis of the murine *Plasmodium chabaudi chabaudi* malaria system supports a role for TH1 cells in early antibody-independent protection, whereas the final clearance of the parasite load coincides with the appearance of malaria-antigen-specific TH2 cells and antibody-mediated effector mechanisms (8, 9). Although there is as yet no evidence for the existence of similar $CD4^+$ subsets in the human system, human $CD4^+$ T cells have been shown to be heterogeneous with regard to surface marker characteristics (10–12) and cytokine production (13).

The *Plasmodium falciparum* antigen Pf155/ring-infected erythrocyte surface antigen (RESA) (14, 15), a candidate for a blood-stage malaria vaccine (16, 17), induces *in vitro* proliferation, release of IL-2 and IFN-γ by T cells from individuals primed to this antigen by natural infection (18–21). Moreover, by using a T-cell/B-cell cooperation system, Pf155/RESA was found to contain T helper (TH) epitopes capable of inducing the secretion of T-cell-dependent anti-Pf155/RESA antibodies *in vitro* (22). Some of these T-cell epitopes are located within or adjacent to peptide sequences of Pf155/RESA that form the immunodominant B-cell epitopes. These are located in the conserved amino acid repeat regions of the molecule (18–21, 23, 24) and some of them have been shown to be associated with clinical protection (25–27).

To investigate the possible role of TH1- and TH2-like cells in the human response to Pf155/RESA, we have analyzed production of the cytokines IFN-γ and IL-4 by T cells from *P. falciparum*-primed individuals after *in vitro* activation with Pf155/RESA peptides. Peptides corresponding to immunodominant T-cell epitopes induced T-cell proliferation, IFN-γ secretion, and expression of IL-4. In individual donors, there was no association between proliferation and cytokine production. Moreover, in individual donors, there was usually no association between IFN-γ and IL-4 expression. However, a significant association was found between the induc-

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Abbreviations: IFN-γ, interferon-γ; RESA, ring-infected erythrocyte surface antigen; SI, stimulation index; IL-2, -4, and -5, interleukin 2, 4, and 5, respectively; TH cell, T helper cell; TH1 and TH2 cells, T helper 1 and 2 cells, respectively.

tion, by particular peptides, of IL-4 in T cells and the presence of antibodies to the same peptides in the plasma of the T-cell donors. These results suggest that a relationship exists between the activation of IL-4-producing T-cell subsets and antibody production in human systems in which the immune response is induced by natural infection.

MATERIALS AND METHODS

Study Subjects. Samples of venous blood were obtained with informed consent from malaria-immune adult men living in rural villages in The Gambia. In this region malaria is holoendemic but seasonal. The epidemiological pattern of malaria transmission in this area has been reported (24). Samples from Swedish donors not previously exposed to the malaria parasite served as controls.

Preparation and Fractionation of Peripheral Blood Mononuclear Cells. Between 10 and 15 ml of venous blood was diluted 1:2 with RPMI 1640 medium (Flow Laboratories) containing sodium heparin (10 units/ml). Mononuclear cells were isolated by sodium metrizoate density gradient centrifugation (Lymphoprep–Nyecomed, Oslo). T cells were separated by rosette formation with neuraminidase-treated sheep erythrocytes. Erythrocyte-rosette-forming cells were separated from non-erythrocyte-rosette-forming cells by sodium metrizoate density gradient centrifugation (28).

Adherent Cells. These cells were obtained from peripheral blood mononuclear cells by incubation for 1–2 hr at 37°C in Petri dishes in 50% (vol/vol) heat-inactivated human AB^+ serum. Nonadherent cells were washed off and adherent cells were recovered after overnight incubation at 4°C.

Cell Cultures for [^{3}H]Thymidine Incorporation. A total of 5000 adherent cells were seeded into round-bottomed microtiter plates (Linbro) and incubated in triplicate for 4 hr with 200 μl of peptide (1 μM) in complete tissue culture medium [RPMI 1640 medium containing 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (25 μg/ml), 30 mM Hepes, and 0.22% sodium carbonate] supplemented with 10% AB^+ serum. After incubation with the antigen, supernatants were removed and replaced with fresh tissue culture medium and 1×10^5 autologous T cells were added to each well. Plates were incubated at 37°C in humidified 95% air/5% CO_2 for 5 days, and then 100 μl of cell-free supernatant was collected from each well for IFN-γ determination. The remaining cells were radiolabeled with 1 μCi of [^{3}H]thymidine (specific activity, 2 Ci/mmol; 1 Ci = 37 GBq; Amersham) in 100 μl of complete tissue culture medium for 18 hr. Cellular incorporation of [^{3}H]thymidine was measured by liquid scintillation counting. Results were expressed as a stimulation index (SI), defined as mean cpm of test cultures divided by mean cpm of control cultures. The baseline for antigen-specific proliferation (SI ≥ 2.5) was calculated from the response in antigen-free cultures as described (19).

Analysis of Expression of mRNA Specific for IL-4. T cells supplemented with 5% autologous adherent cells were cultured for 24 hr in 0.5 ml of complete tissue culture medium (1×10^6 cells per ml) in round-bottomed tissue culture tubes (A/S; Nunc) in the presence of peptide (1 μM) or in the absence of any stimulants for 24 hr. Thereafter, the cells were centrifuged, the supernatant was removed, and the cells were lysed with 100 μl of 1% *N*-lauroylsarcosine/50 mM EDTA, pH 8.0. Cesium trifluoroacetate (100 μl; 2 g/ml) (Pharmacia) was added to dissociate protein from nucleic acids and thereafter the vials were kept at room temperature until further processing. The samples were diluted to a final concentration of 10× SSC and applied to nylon filters (Zeta-Probe, Bio-Rad) by using a dot-blot manifold. After baking, the filters were prehybridized at room temperature in 50% (vol/vol) formamide/50 mM Hepes, pH 7.4/6× Denhardt's solution/6× SSC/0.5% SDS/yeast tRNA (100 μg/ml) (1× SSC = 0.15 M NaCl/0.01 M sodium citrate and 1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin). A 36-base antisense oligonucleotide probe [kindly provided by R. M. Locksley (University of California, San Francisco)] was used to detect IL-4 mRNA. The probe was end-labeled with ^{32}P by using T4 polynucleotide kinase, to a specific activity of 1×10^8 cpm/mg. The filters were washed three times in 6× SSC/0.1% SDS at 37°C and exposed to x-ray film overnight at −70°C.

Immunofluorescence Staining for Intracellular IL-4. Cells cultured for 24 hr with peptide (as above) were harvested and washed twice in isotonic phosphate-buffered saline (PBS) and then fixed in suspension in 4% (wt/vol) paraformaldehyde/PBS for 5 min at room temperature as described (29). Briefly, 1×10^6 fixed and washed lymphocytes were incubated for 45 min at room temperature in 100 μl of PBS containing 0.1% saponin (Riedel De Haen, Seelze, F.R.G.) and a mixture of three mouse anti-human IL-4 monoclonal antibodies (C.H.H., and K. Wagner, unpublished data). Thereafter, the cells were washed twice in PBS/saponin and incubated for another 30 min at room temperature with fluorochrome-coupled rabbit anti-mouse immunoglobulin diluted in PBS/saponin. Finally, the cells were washed and resuspended in hypotonic sodium citrate and 15 μl of the cell suspension was air-dried on glass slides. Such mounted preparations could be stored in the dark at 4°C for several weeks without deterioration in the quality of the staining.

Estimation of IFN-γ in Culture Supernatants. IFN-γ was determined by a two-site ELISA with the use of two monoclonal antibodies to human IFN-γ [kindly provided by G. Andersson (Kabi, Stockholm)] as described (30–32).

Peptides. Peptides 15–22 amino acids long were prepared. Briefly, the protected resins were prepared in groups of 100 by the method of simultaneous multiple peptide synthesis and were cleaved in groups of 24 in a multivessel apparatus (33). Typical purities of the crude peptides ranged from 65% to 95%. All peptides were desalted before use and tested for cytotoxicity. None of the peptides were toxic.

Serology. A small aliquot of plasma was obtained from each sample and used for determination of anti-peptide antibodies using an ELISA as described (25). Briefly, 96-well plastic plates were coated with bovine serum albumin-conjugated [2:1 (wt/wt)] peptides with glutaraldehyde. Bound antibodies were detected by alkaline phosphatase-conjugated rabbit antibodies specific for human IgG with *p*-nitrophenyl phosphate as substrate.

RESULTS AND DISCUSSION

Functionally distinct subpopulations of TH ($CD4^+$) lymphocytes with characteristic surface markers have been described in man (13). The helper and suppressor/inducer functions of these subsets have been defined in a T-cell-dependent immunoglobulin production system using polyclonal activation (10, 11). However, no information is available regarding the induction of functionally distinct subpopulations of TH cells in humans primed to specific antigens by natural infection.

T-cell epitope mapping of the *P. falciparum* malaria antigen Pf155/RESA has revealed that the most frequently recognized T-cell activating peptides correspond to sequences within the 3′ and 5′ repeat regions of the molecule (18–21, 23). The repeat regions are also known to contain some of the immunodominant B-cell epitopes of the molecule (25). When short peptides corresponding to these sequences were used to study *in vitro* proliferative responses and/or IFN-γ production in *P. falciparum*-primed donors from The Gambia, it was found that approximately 66% of the donors responded by either proliferation or IFN-γ secretion and 30–35% responded in both assays (20). Similar results were also ob-

tained when studying donors from Liberia where year-round transmission of malaria occurs (19). Although IFN-γ secretion and proliferation by cells from individual donors tended to correlate when a crude Pf155/RESA preparation was used for stimulation (19), there was no correlation between the two measurements within an individual after peptide activation. These differences reflect the fact that the crude antigens contain several antigenic epitopes capable of activating many different T-cell clones, whereas short peptides are seen by a more restricted populations of T cells.

Table 1 shows the poor and frequently negative correlation between *in vitro* secretion of IFN-γ and the induction of DNA synthesis by two short peptides representing two distinct but often cross-reacting epitopes (20). Proliferation and IFN-γ release were also negatively correlated with the concentration of serum antibodies to the peptides used for T-cell activation. This confirms and extends our earlier results (18–21) and underlines the importance of examining several parameters of T-cell activation for estimating the total proportion of individuals responding to a given epitope. The sole use of the lymphocyte proliferation test as an indicator of T-cell activation not only underestimates the frequency of recognition but is also unsatisfactory as it gives no information regarding the function of the activated cells, a question that is of importance for the selection of vaccine immunogens.

Since the development of Pf155/RESA-specific IgG antibodies is T-cell-dependent (22), the lack of correlation between T-cell activation and antibody concentration may indicate that neither of the activation measurements used reflects activation of those T cells that help B cells to produce antibodies. In analogy to what has been seen in mice (8, 9), this may imply that the T cells important for the regulation of anti-Pf155/RESA antibody production are TH2-like cells, distinct from TH1-like cells producing IFN-γ, a well-established mediator of cell-mediated immunity in malaria (34–41).

Table 1. Immune responses to peptides from the two repeat domains of Pf155/RESA in immune adult Gambians

	$(DDEHVEEPTVA)_2$			$(EENV)_4$		
Donor	SI	IFN-γ, units/ml	Ab, μg/ml	SI	IFN-γ, units/ml	Ab, μg/ml
G128w	[3.2]	0	3	[3.0]	0	3
G102w	[5.5]	0	0	0.9	0	0
G143w	2.4	0	[4]	[16.9]	0	[5]
G152w	[5.1]	0	1	4.2	0	[5]
G153w	3.1	[3]	0	9.2	0	1
G130w	[7.0]	[11]	[6]	5.8	1	3
G101w	1.8	0	[4]	[2.7]	[16]	0
G112w	1.2	[9]	2	0.9	[7]	[4]
G116w	1.4	5	1	1.4	[3]	0
G125w	[3.0]	[14]	[5]	1.4	[8]	2
G129w	2.3	[2]	3	[2.7]	1	2
G107w	[2.6]	0	[13]	2.2	0	[80]
G108w	[2.6]	0	[37]	1.2	0	[23]
G115w	1.0	0	[20]	0.8	0	[97]
G134w	[4.4]	[2]	[16]	2.2	0	[62]
G135w	1.5	0	[16]	1.4	0	[68]
G147w	2.1	0	[13]	2.3	0	[66]

Blood was collected from Gambian donors at the end of the wet (w) season. T-cell stimulating peptides were a dimer of the sequence Asp-Asp-Glu-His-Val-Glu-Glu-Pro-Thr-Val-Ala (in one-letter code) representing the consensus sequence from the 5′ repeat block of Pf155/RESA and a tetramer of the sequence Glu-Glu-Asn-Val, repeated 30 times in the 3′ repeat block. Values ≥2.5 (boxed) were considered antigen-specific according to ref. 19. Values ≥2 (boxed) were considered antigen-specific according to ref. 30. Ab, anti-peptide antibody. Values ≥4 (boxed) were considered specifically elevated above background according to ref. 25.

To investigate this question, we looked for the induction of mRNA specific for IL-4 in *in vitro*-activated T cells using a ^{32}P-labeled oligonucleotide probe specific for human IL-4 mRNA. We chose to use a dot-blot hybridization technique because no specific bioassays or reliable ELISAs are yet available for measuring human IL-4 secretion. As seen in Table 2, in individual donors, IL-4 mRNA expression was correlated with neither proliferation nor IFN-γ secretion. However, with one exception out of eight individuals studied, IL-4 mRNA was induced only in T cells from donors who had high concentrations of serum antibodies to the same peptide that had been used for T-cell activation (a relationship which was statistically significant; $P < 0.01$, χ^2 analysis). In these donors, the three distinct but related peptides had the capacity to induce IFN-γ, IL-4, proliferation, or any combination thereof. None of these responses were detected when T cells from *P. falciparum*-unprimed donors were activated in the same manner (data not shown).

The presence of mRNA does not necessarily indicate that it is translated and that the corresponding protein is synthe-

Table 2. DNA synthesis, IFN-γ secretion, and expression of mRNA specific for IL-4 in *P. falciparum*-immune T cells after *in vitro* activation with Pf155/RESA-derived peptides and comparison with the serum levels of IgG antibodies to the corresponding peptides

Donor	Antigen	SI	IFN-γ, units/ml	IL-4	Ab, μg/ml
G102d	None	–	–	–	–
	$(EENV)_4$*	0.9	–	+	3
	EENVEHDA $(EENV)_2$†	3.0	–	+	6
	TVAEEHVEEPTVAEE‡	4.4	–	+	5
G107d	None	–	–	–	–
	$K(EENVEHDA)_2$§	0.4	–	–	9
	EENVEHDA $(EENV)_2$	0.5	–	++	110
	TVAEEHVEEPTVAEE	0.9	ND	++	77
G109d	None	–	–	–	–
	$K(EENVEHDA)_2$	5.8	–	–	4
	EENVEHDA $(EENV)_2$	6.1	2	++	65
	TVAEEHVEEPTVAEE	4.5	–	–	11
G112d	None	–	–	–	–
	$K(EENVEHDA)_2$	3.4	12	–	5
	EENVEHDA $(EENV)_2$	1.0	11	++	38
	TVAEEHVEEPTVAEE	1.3	7	–	5
G113d	None	–	–	–	–
	$(EENV)_4$	0.3	–	±	13
	EENVEHDA $(EENV)_2$	4.3	–	–	20
	TVAEEHVEEPTVAEE	0.7	–	±	10
G114d	None	–	–	–	–
	$(EENV)_4$	6.6	–	++	27
	EENVEHDA $(EENV)_2$	3.8	–	++	59
	TVAEEHVEEPTVAEE	2.5	–	–	5
G117d	None	–	–	–	–
	$(EENV)_4$	0.7	–	+	27
	EENVEHDA $(EENV)_2$	1.0	–	+	24
	TVAEEHVEEPTVAEE	1.3	2	–	3
G120d	None	–	–	–	–
	$(EENV)_4$	0.7	–	±	6
	EENVEHDA $(EENV)_2$	0.5	–	++	30
	TVAEEHVEEPTVAEE	ND	–	+	15

Blood was collected from donors at the end of the dry (d) season. Ab, anti-peptide antibody; ND, not determined. Expression of IL-4-specific mRNA, as determined by dot blot after 24 hr of culture (–, ±, +, or ++, no, weak, medium, or strong expression, respectively).

*$(Glu\text{-}Glu\text{-}Asn\text{-}Val)_4$.

†Glu-Glu-Asn-Val-Glu-His-Asp-Ala-$(Glu\text{-}Glu\text{-}Asn\text{-}Val)_2$.

‡Thr-Val-Ala-Glu-Glu-His-Val-Glu-Glu-Pro-Thr-Val-Asn-Glu-Glu.

§Lys-$(Glu\text{-}Glu\text{-}Asn\text{-}Val\text{-}Glu\text{-}His\text{-}Asp\text{-}Ala)_2$.

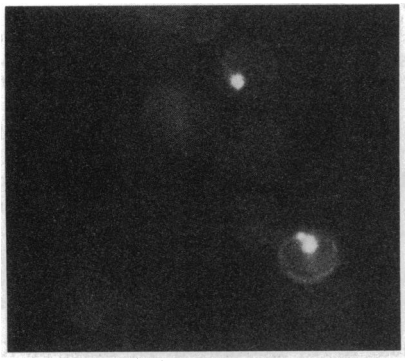

FIG. 1. Cytoplasmic immunofluorescence staining of IL-4 in activated T cells showing the typical local perinuclear cytoplasmic pattern, reflecting accumulation in the Golgi stacks.

sized and secreted as an active lymphokine (42). To establish production of IL-4, T cells from seven immune individuals were stimulated with a Pf155/RESA peptide representing a dominant epitope from its 3′ repeat region. The presence of intracytoplasmic IL-4 was detected with a pool of three mouse monoclonal anti-human IL-4 antibodies. IL-4 was visualized by the characteristic morphology of a granular or ring-shaped accumulation of staining close to the nucleus (Fig. 1) (43). Intracytoplasmic IL-4 was seen in four of the seven donors after peptide activation. As shown in Fig. 2, in individual donors the presence of IL-4-producing cells correlated well with the presence of elevated concentrations of serum antibodies to the peptide used for T-cell activation. Antigen specific B and T cells frequently have different epitope specificities and thus, it may be expected that the epitopes activating IL-4 producing T cells would be distinct from the epitopes recognized by antibodies. However, in the present situation, the immunogens are made up of short tandemly repeated amino acid sequences that are known to contain several B- and T-cell epitopes. It is therefore not surprising that T cells and antibodies react with the same peptide. IL-4-producing cells are usually present only at low frequency after primary *in vitro* antigen activation, but are more easily detected after secondary activation *in vitro* (44). The high percentage of IL-4$^+$ cells found here (1–3%) probably reflects the continuous exposure to *P. falciparum* in

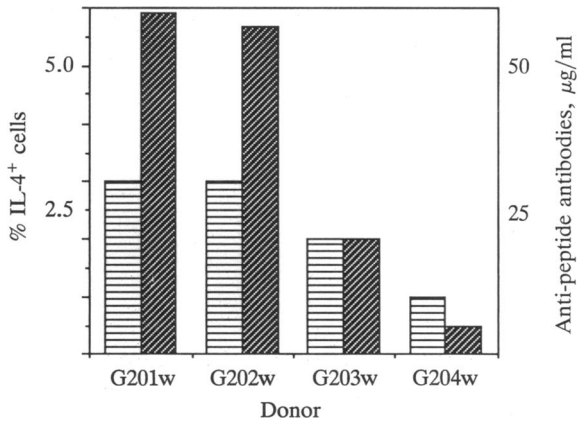

FIG. 2. Correlation of cytoplasmatic IL-4 and the presence of serum antibodies to the same peptide. Percentage of IL-4$^+$ cells in peptide [Glu-Glu-Asn-Val-Glu-His-Asp-Ala-(Glu-Glu-Asn-Val)$_2$]-activated T-cell cultures (horizontal hatched bars). Cells were cultured with the peptide for 24 hr. The presence of IL-4 was recorded as intracellular staining in an immunofluorescence assay. Anti-peptide antibody concentration (μg/ml) in serum measured by an ELISA is shown (diagonal hatched bars). Blood was collected from donors at the end of the wet (w) season.

these individuals. Two of the donors who had IL-4$^+$ cells after peptide activation also had cells that produced IFN-γ (data not shown). The finding that there is no absolute dissociation between induction of IL-4 mRNA and IFN-γ secretion or proliferation was to be expected and suggests either that T cells of both subsets are present or that a population of multipotential T cells capable of producing several cytokines (i.e., cells belonging to a THO-like subset) (45, 46) is activated. Also, since the studies described here were performed with unseparated T-cell preparations (i.e., mixed CD3$^+$, CD4$^+$, and CD8$^+$ cells), we cannot exclude the possibility that CD8$^+$ cells were also stimulated by the same peptide epitopes to produce IFN-γ. However, this appears unlikely since CD8$^+$ cells have been shown to suppress rather than induce *P. falciparum*-dependent T-cell proliferation and IFN-γ production in some malaria-immune individuals (47). These questions will be resolved by studying lymphokine production at the single-cell level and by the use of double labeling techniques (43).

In conclusion, the data presented here suggest that IL-4-producing TH cells are involved in the induction of Pf155/RESA-specific IgG antibody production in people who have acquired functional immunity to malaria as a result of long-term natural exposure to the parasite. Although we cannot be sure that this lymphokine directly or indirectly triggers antibody production, measurement of IL-4 induction represents a functional assay for TH-cell activity and could be used to identify and characterize TH-cell epitopes on this and other candidate vaccine antigens. This represents a step forward in the rational selection of immunogens for subunit vaccines.

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