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Cellular Basis of Early Cytokine Response to *Plasmodium falciparum*

MEIKE HENSMANN AND DOMINIC KWIATKOWSKI*

Department of Paediatrics, Oxford University, Oxford OX3 9DU, United Kingdom

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Uncertainty remains about the cellular origins of the earliest phase of the proinflammatory cytokine response to malaria. Here we show by fluorescence-activated cell sorter analysis that γδ T cells and CD14$^+$ cells from nonimmune donors produce tumor necrosis factor and that γδ T cells also produce gamma interferon within 18 h of contact with mycoplasma-free *Plasmodium falciparum*-infected erythrocytes in vitro. This early cytokine response is more effectively induced by intact than by lysed parasitized erythrocytes. However, the IFN-γ response to lysed parasites is considerably enhanced several days after peripheral blood mononuclear cells are primed with low numbers of intact parasitized erythrocytes, and in this case it derives from both αβ and γδ T cells. These data show that naïve γδ T cells can respond very rapidly to malaria infection but that malaria fever may involve a multistage process in which the priming of both γδ and αβ T-cell populations boosts the cytokine response to lysed parasite products released at schizont rupture.

Malaria-infected individuals produce large amounts of proinflammatory cytokines, such as tumor necrosis factor (TNF) and gamma interferon (IFN-γ). This innate cytokine response is responsible for the high levels of fever that occur within a few days of the onset of blood stage infection in nonimmune individuals (15, 18, 20, 21, 33). Since TNF and IFN-γ have important antiparasitic actions but are also believed to play a major role in the pathogenesis of severe complications, such as cerebral malaria and severe malarial anemia, understanding the cellular basis of the early innate cytokine response may be of considerable importance in relation to both malaria immunity and pathogenesis.

Until recently, it has been widely assumed that the early host response to malaria is broadly similar to that evoked by bacterial endotoxin, whereby parasite factors (toxins) stimulate monocytes and macrophages to release TNF and related cytokines (5, 19). This model was supported by evidence that some isolates of *Plasmodium falciparum* strongly stimulate TNF production by human peripheral blood mononuclear cells (PBMC) (1) and by a significant literature documenting TNF production by monocytes or macrophages within a few hours of exposure to malaria parasite preparations in vitro (examples are given in references 2, 25, and 34). However, it has recently become apparent that many malaria culture preparations are contaminated with mycoplasma species which have potent macrophage-stimulatory factors (22, 30, 36). Mycoplasma species have been found in parasite lines obtained from different laboratories around the world, and even after infection has been eradicated, continuous cultures are susceptible to reinfection within a few months unless their mycoplasma status is continually monitored. These observations have made it necessary to reevaluate the cellular basis of the early cytokine response to malaria.

* Corresponding author. Mailing address: Department of Paediatrics, John Radcliffe Hospital, Oxford University, Oxford OX3 9DU, United Kingdom. Phone: (01865) 221071. Fax: (01865) 220479. E-mail: dominic.kwiatkowski@paediatrics.ox.ac.uk.

We have recently observed that the pattern of early cytokine production by nonimmune human PBMC following stimulation by mycoplasma-free *P. falciparum*-infected erythrocytes (PFE) differs considerably from that induced by bacterial endotoxin (32). Both CD3$^+$ and CD14$^+$ populations are required for this early parasite-induced TNF response, whereas the endotoxin-induced response is unaffected by depletion of the CD3$^+$ population and malaria parasites appear to stimulate much more IFN-γ production at an early stage (i.e. within 1 day of exposure in vitro) than does endotoxin. We have also observed that parasitized erythrocytes fail to stimulate the monocytelike cell line MonoMac6 to express proinflammatory cytokines under conditions in which endotoxin induces a strong TNF response.

These findings suggest that the early inflammatory response to malaria is critically dependent on lymphocyte subpopulations that play a lesser role in the response to bacterial endotoxin. Although previous studies have shown that αβ and γδ T cells from nonimmune human donors produce cytokines, including TNF and interleukin 12 (IL-12) within 5 or 6 days (4, 6, 7, 9, 12, 25, 29, 35, 38), relatively little attention has been paid to the role of T-cell-derived cytokines at the earliest stage of malarial infection, apart from a recent report that γδ T cells can produce IFN-γ within a day of exposure to parasitized erythrocytes in vitro (26). The primary goal of the present study was to investigate the ability of nonimmune αβ and γδ T cells to generate a rapid TNF and IFN-γ response upon stimulation in vitro with malaria parasite preparations known to be free of mycoplasma contamination.

MATERIALS AND METHODS

PBMC preparation. PBMC from four healthy nonimmune adult Caucasian donors were prepared as described previously (32) using a density separation technique (Lymphoprep; Nycomed). The cells were washed once in saline and once in RPMI 1640 and then set up for assay in PBMC medium (RPMI 1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, 0.2% glucose, and 10% human AB$^+$ serum).

For priming, the PBMC were set up in six-well plates at 5×10^6 to 10×10^6/well and incubated at 37°C with the appropriate stimulants for 8 days. The cells

were then recovered with a cell scraper, counted, and plated out in fresh PBMC medium in the desired format.

Parasite preparation. *P. falciparum* parasites (isolate IT4/25/5; strain A4) were maintained in fresh human erythrocytes at 0.2% hematocrit in PFE medium (RPMI 1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, 0.2% glucose, 25 μg of gentamycin/ml, and 10% AB^+ serum). The cultures and media were regularly tested for mycoplasma contamination by PCR (American Type Culture Collection PCR kit). Washed schizont preparations at >70% parasitemia were obtained by Plasmagel flotation (23). Intact PFE (diluted in PBMC medium) or lysed PFE (added to 5 volumes of sterile endotoxin-free water before being diluted in PBMC medium) were immediately dispensed into microtiter wells containing PBMC. Control preparations of mock-cultured uninfected erythrocytes (URBC) from the same donor were tested in all experiments, and in most experiments, the PBMC also came from the same donor.

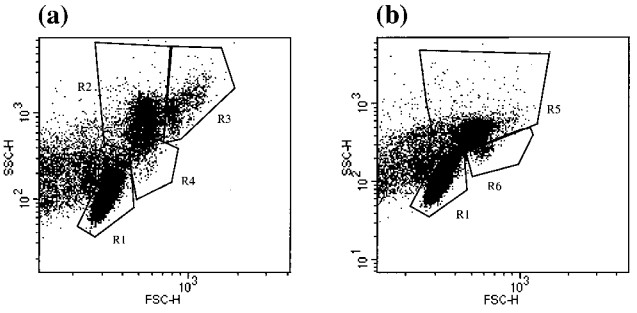
Cytokine protein ELISA. PBMC were plated out in 96-well round-bottom plates at 2×10^5/100-μl/well and rested for 4 h. Stimulants were added in 100 μl of PBMC medium to the desired final concentrations. The supernatants were collected after 16 h, and the cytokine concentrations were measured by enzyme-linked immunosorbent assay (ELISA), TNF was measured as previously described (32), and IFN-γ and IL-12 p40 were measured by using R&D Systems matched antibody pairs or DuoSet ELISA kits. Unless otherwise stated, phytohemagglutinin (PHA) was used at 10 μg/ml and lipopolysaccharide (LPS) was used at 50 ng/ml. Anti-human IL-12 p70 and immunoglobulin G1 (IgG1) isotype control (ITC) were used at 10 μg/ml (both murine monoclonal antibodies were from R&D Systems), a dose sufficient to neutralize 1 ng of IL-12/ml, which was determined to achieve maximum reduction of the IFN-γ response in our experiments (dose-response curve not shown).

Flow cytometric analysis. PBMC were plated out in 24-well plates at 2×10^6/well. Stimulants were added in 100 μl of PBMC medium and incubated for 18 h total. For intracellular staining, brefeldin A (10-μg/ml final concentration) was added to the PBMC cultures after 6 h of stimulation to stop protein secretion. The cells were recovered from the plates by light scraping, washed once in phosphate-buffered saline (PBS) containing 5% AB^+ serum, and transferred to 7-ml polystyrene tubes for further processing. The cells were then surface labeled in PBS–5% AB^+ serum, fixed in 2% paraformaldehyde in PBS, permeabilized and cytokine labeled in PBS–5% AB^+ serum–0.1% azide–0.5% saponin, and fixed again in 2% paraformaldehyde. The following fluorescently labeled antibodies were used according to the manufacturers' recommendations: CD3-fluorescein isothiocyanate (FITC), CD14-FITC, IgG1-phycoerythrin (PE)-FITC, and IgG2b-PE-FITC (Sigma); pan-αβ–T-cell receptor–FITC and Vγ9-FITC (Pharmingen); CD56-FITC (Serotec); and TNF-PE and IFN-γ-PE (R&D Systems). Samples were analyzed on a Becton Dickinson FACScan flow cytometer.

Definition of regions in the fluorescence-activated cell sorter (FACS) spectra. For each stimulus, unlabeled cells were used to identify and gate on discrete cell populations (Fig. 1) as well as to determine the quadrant boundaries for quantitative analysis. Simple surface staining with ITC antibodies was used to ascertain that none of the specific surface antibodies bound spuriously under the conditions used. ITCs for intracellular labels were included in each experiment, but no nonspecific binding was detected. In accordance with the distribution of surface markers in the respective regions (Fig. 1), these were identified as follows: small lymphocyte region, R1 (both PFE-and URBC-stimulated cells); monocyte region, R5 (URBC-stimulated cells) or R2 plus R3 (PFE-stimulated cells); and blasting lymphocyte region, R6 (URBC-stimulated cells) or R4 (PFE-stimulated cells). R2 and R3 were analyzed separately, as they appeared as distinct populations, but no significant difference in surface marker distribution or cytokine secretion was detected. In the results, therefore, monocyte region refers to R5 or to R2 plus R3. For each sample, events were subsequently collected up to a count of 20,000 in the small lymphocyte region (R1).

RESULTS

TNF and IFN-γ responses in fresh PBMC. Freshly isolated PBMC from different donors were stimulated with intact PFE for 18 h. As reported earlier (32), both TNF and IFN-γ could be detected by protein ELISA at this early time point. Here we used intracellular cytokine staining to identify the cellular source of these cytokines. Erythrocyte preparations enriched to >70% PFE were added to PBMC in 24-well plates (at 2×10^6 PBMC/well) at a ratio of 10:1 PFE-PBMC. In pilot experiments, as in our earlier report (29), this had been determined as the lowest PFE/PBMC ratio (of a range 0.1:1 to 25:1) at which cytokines could readily be detected above background at the 18-h time point (data not shown). At 6 h after stimulation, protein secretion was blocked by the addition of brefeldin A, and cells were harvested for labeling after an additional 12 h. Erythrocytes were lysed during the saponin permeabilization step and thus did not interfere with PBMC detection on the flow cytometer. Figure 1 shows typical FACS spectra of PBMC stimulated with intact PFE (Fig. 1a) or intact URBC (Fig. 1b) at 18 h, as well as proportions of relevant cell types in the indicated regions. αβ T cells are the predominant cell type in the small lymphocyte region, and there are very few blasting lymphocytes in the spectrum. The distribution of surface and intracellular labels was analyzed separately for each of the distinctive regions of cells in the spectrum. In the small lymphocyte region, although the proportion of $V\gamma9^+$ cells varied among donors, the entire TNF production could be attributed to $V\gamma9^+$-cell populations in all donors (Fig. 2A and Table 1). In the monocyte region, all TNF detected could be assigned to $CD14^+$ cells. Typically, 1 to 2% of the cells in the small lymphocyte region and 20 to 25% of the cells in the monocyte region were TNF^+ by intact-PFE stimulation (Table 1). Any TNF^+ cells detected outside these two major regions were $V\gamma9^+$.



	R1 (a) (20,000)	R2 (2,000)	R3 (2,000)	R4 (400)	R1 (b) (20,000)	R5 (5,000)	R6 (600)
CD3	70-86	3-6	3-7	25-86	80-86	n.d.	53-87
TCR αβ	56-73	2-4	n.d.	10-50	50-67	n.d.	39-57
TCR γδ	1-24	0-2	n.d.	0-18	1-18	n.d.	1-50
CD56	3-29	n.d.	0-5	20-73	4-22	n.d.	78-84
CD14	0-1	61-68	80-87	n.d.	0-1	51-65	n.d.

FIG. 1. FACS spectra (forward versus side scatter) of PBMC stimulated with intact PFE (a) or URBC (b) at 10:1 erythrocytes-PBMC for 18 h. Regions R1 to R6, used in subsequent analyses, are indicated in the plots. The percentages of different lymphocyte subpopulations are shown in the table; the numbers represent the range of values observed in eight different experiments with the blood of four different donors. The numbers in parentheses indicate the approximate average number of events detected. n.d., not detectable; TCR, T-cell receptor.

Typically, 1 to 3% of cells in the small lymphocyte region stained positive for IFN-γ (Fig. 2B and Table 1). Virtually all of these were $CD14^-$ and $\alpha\beta^-$. A high proportion of IFN-γ^+ cells (60 to 90%, depending on the donor) bore the $V\gamma9^+$ surface marker. No significant amounts of TNF or IFN-γ were detected in any cell population after control stimulation with mock-cultured URBC (Table 1; also see Fig. 4).

Differences in PBMC responses to intact and water-lysed PFE. We previously showed that intact PFE elicited higher

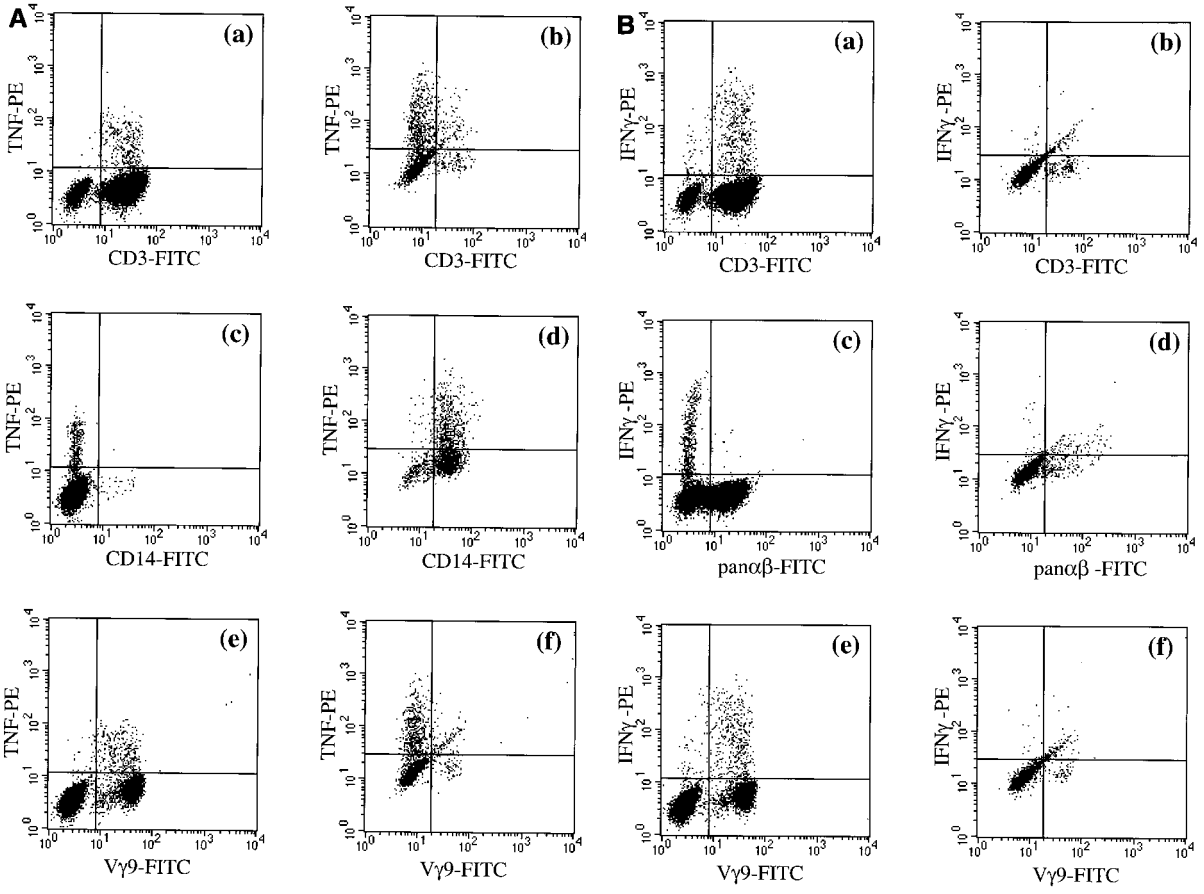


FIG. 2. Intracellular cytokine staining for TNF (A) and IFN-γ (B) after stimulation of PBMC with intact PFE (10:1 PFE-PBMC) for 18 h. The cells were surface stained for CD3 (a and b), CD14 (c and d), or Vγ9 (e and f) and intracellularly stained for TNF or IFN-γ. The gate in each case was set either on the small lymphocyte region (a, c, and e) or on the monocyte region (b, d, and f) of the FACS spectrum. Quadrant markers were set using unlabeled cells to facilitate recognition of positive and negative cell populations (the intrinsic fluorescence of cells in the monocyte region is higher than that of cells in the small lymphocyte region)—the cells in the upper right quadrant are positive for both surface and intracellular markers. These spectra were obtained from the same batch of cells in one experiment and are qualitatively representative of eight experiments performed with the cells of four different donors. The quantitative analysis of these results is shown in Table 1.

concentrations of TNF from fresh PBMC than equivalent amounts of water-lysed PFE (32). Here we extended this observation to IFN-γ production. PBMC were stimulated with equivalent amounts of intact (prepared as described above) and water-lysed PFE (the same preparation in 5:1 sterile H_2O-PFE) in 96-well plates at 10:1 PFE-PBMC. LPS and PHA were included as positive control stimuli. While PHA induced similar amounts of IFN-γ from all donors, there was some degree of variation in the responses to the remaining stimuli (Figure 3), both among donors and within the same donor in different experiments. The experiments were repeated at least three times for each donor. The same qualitative pattern emerged throughout: intact PFE elicited larger amounts of IFN-γ than water-lysed PFE. As previously observed at the mRNA level (32), LPS induced substantially less IFN-γ than PFE under these conditions. Intact or lysed URBC were tested in parallel but also did not induce significant amounts of the cytokine.

The different responses to intact and lysed PFE were inves-

TABLE 1. Quantitative analysis of intracellular-staining results for eight experiments with PBMC of four different donors[a]

Region and cell type	% Double-positive cells stimulated with:			
	TNF^+		$IFN\text{-}\gamma^+$	
	Intact PFE	Intact URBC	Intact PFE	Intact URBC
SLR $\alpha\beta^+$	ND	ND	0.04 ± 0.01 (0.00–0.06)	ND
SLR $\gamma\delta^+$	1.51 ± 0.35 (0.85–2.15)	0.01 ± 0.002 (0.00–0.002)	2.34 ± 0.35 (1.07–3.74)	0.01 ± 0.002 (0.00–0.02)
MR $CD14^+$	21.83 ± 1.83 (16.25–28.5)	ND	ND	ND

[a] Cells were stimulated with intact PFE or URBC: (Fig. 2). The numbers represent the number of cytokine and surface marker double-positive cells as percentages of the total cells in the respective gated region (SLR, small lymphocyte region; MR, monocyte region) with the standard error and (in parentheses) the total range of measured responses. ND not detectable.

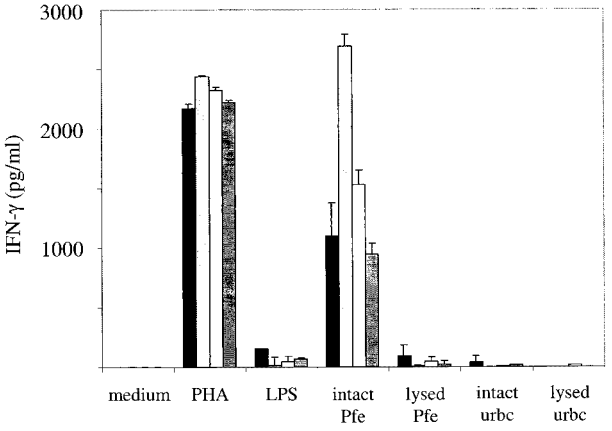
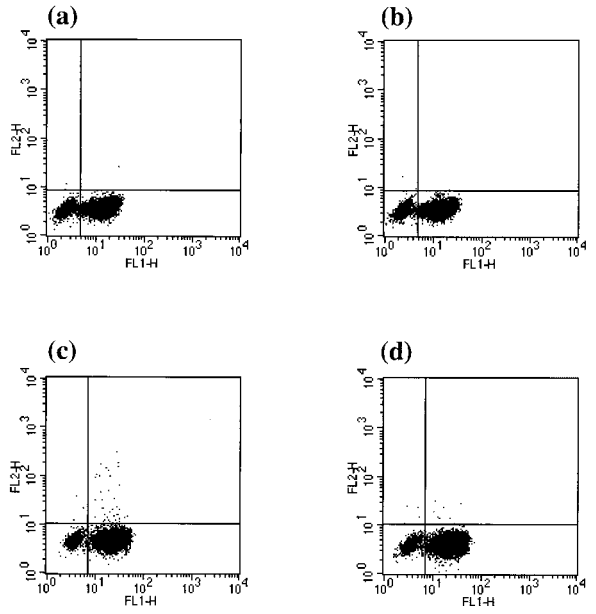


FIG. 3. Concentrations of IFN-γ measured in the supernatants of PBMC incubated for 18 h in the presence of the indicated stimuli (PHA at 10 μg/ml, LPS at 50 ng/ml, and parasite and URBC preparations at 10:1 erythrocytes-PBMC). The data are shown as averages of quadruplicate stimulations of the cells from four different donors (solid bars, donor 1; lightly shaded bars, donor 2; open bars, donor 3; darkly shaded bars, donor 4). The error bars indicate the standard errors.



	% IFNγ$^+$ cells in response to intact uninfected rbc	% IFNγ$^+$ cells in response to lysed uinfected rbc
Fresh PBMC (a and b)	0.01 ±0.002 (0.00-0.02)	0.02 ±0.002 (0.00-0.02)
Pfe-primed PBMC (c and d)	0.20 ±0.06 (0.08-0.54)	0.15 ±0.03 (0.05-0.32)

FIG. 4. Representative control FACS spectra of PBMC stimulated for 18 h with intact (a and c) or water-lysed (b and d) URBC (10:1 URBC-PBMC). The cells were either fresh (a and c) or incubated in the presence of intact PFE (0.1:1 PFE-PBMC) for 8 days prior to stimulation (c and d). Each spectrum shows surface CD3-FITC fluorescence on the *x* axis and intracellular IFN-γ–PE fluorescence on the *y* axis. The gate in each case was set on the small lymphocyte region of the FACS spectrum. Quadrant markers were set using unlabeled cells to facilitate recognition of positive and negative cell populations. These spectra were obtained from the same batch of cells in one experiment and are qualitatively representative of eight experiments performed with the cells of four different donors. The quantitative analysis of the quadrant statistics of all experiments is given in the table below the plots. The numbers represent CD3$^+$ IFN-γ$^+$ double-positive cells as a percentage of the total gated cells, with standard error and (in parentheses) ranges.

tigated by intracellular cytokine staining. Although little IFN-γ could be detected by this method in lysate-stimulated PBMC, it appeared to derive from the same cell types as with intact-PFE stimulation. In all donors, Vγ9$^+$ but not αβ$^+$ cells in the small lymphocyte region stained positive for the cytokine (Table 2). Any IFN-γ$^+$ cells outside this region were also αβ$^-$, and most were Vγ9$^+$. In agreement with the protein data, the number of lysed-PFE-induced IFN-γ$^+$ cells varied among donors and experiments but never exceeded 10% of that induced by intact PFE from the same preparation. Intact or water-lysed URBC did not elicit significant numbers of IFN-γ$^+$ cells (Table 2 and Fig. 4).

Different IL-12 dependences of the IFN-γ response to intact and lysed PFE. Intact and lysed PFE were compared for the ability to stimulate PBMC to produce IL-12 p40 following incubation for 18 h (Fig. 5a). Despite variation among experiments and donors in the absolute level of IL-12 p40, in all cases the amount induced by intact PFE was significantly larger than that induced by lysed PFE ($P < 0.05$). Intact or lysed URBC elicited low levels of IL-12 p40 that were similar to those elicited by lysed PFE.

To address the question of whether the IFN-γ induced by PFE is secondary to IL-12 production, a neutralizing monoclonal antibody to IL-12 p70 or an ITC antibody was added to the cells 20 min prior to addition of the respective stimuli. This eliminated some, but not all, of the IFN-γ elicited by intact PFE, PHA, and LPS (Fig. 5b). The amount of cytokine detected in the presence of anti-IL-12 was 42% ± 20% (95% confidence interval) of that detected in the presence of ITC. In contrast, anti-IL-12 had no detectable effect on the modest observed IFN-γ response to lysed PFE (94% ± 14% of ITC levels).

Altered IFN-γ response in PFE-primed PBMC. In order to determine whether prior exposure to PFE may alter the 18-h cytokine response to either intact or lysed PFE, PBMC were incubated in the presence of a low concentration of intact PFE (0.5:1 PFE-PBMC) for 8 days. They were then washed and restimulated in 96-well plates as described above with parasite

TABLE 2. Quantitative analysis of intracellular-staining results for six experiments with PBMC of four different donors[a]

Stimulant	% Double-positive cells	
	αβ$^+$ IFNγ$^+$	γδ$^+$ IFNγ$^+$
Intact PFE	0.04 ± 0.01 (0.00–0.06)	2.34 ± 0.35 (1.07–3.74)
Lysed PFE	ND	0.22 ± 0.03 (0.12–0.31)
Intact URBC	ND	0.01 ± 0.002 (0.00–0.02)
Lysed URBC	ND	0.02 ± 0.002 (0.00–0.03)

[a] Cells were stimulated for 18 h with intact or lysed PFE or URBC (10:1 erythrocytes-PBMC). The numbers represent the number of cytokine and surface marker double-positive cells as percentages of the total cells in the small lymphocyte region R1, with the standard error and (in parentheses) the total range of measured responses (Fig. 2). ND, not detectable.

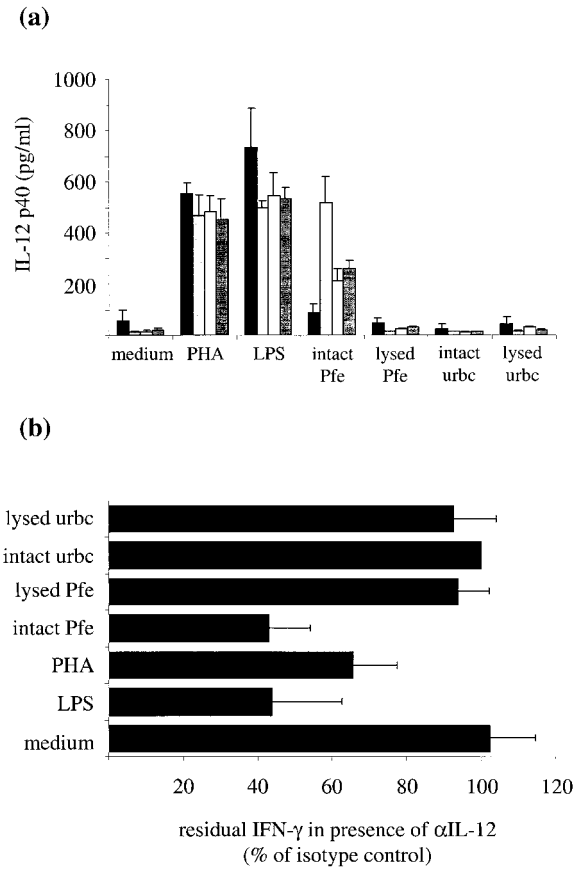


FIG. 5. (a) Concentrations of IL-12 p40 measured in the supernatants of PBMC incubated for 18 h in the presence of the indicated stimuli (PHA at 10 μg/ml, LPS at 50 ng/ml, and parasite and URBC preparations at 10:1 erythrocytes-PBMC). The data are shown as averages of quadruplicate stimulations of the cells from four different donors (solid bars, donor 1; lightly shaded bars, donor 2; open bars, donor 3; darkly shaded bars, donor 4). The error bars indicate the standard errors. (b) Effect of IL-12 p70 neutralization on the amount of IFN-γ measured in the supernatant of PBMC stimulated for 18 h with the indicated stimuli. The data are presented as the residual concentrations of IFN-γ detected in the presence of the neutralizing antibodies as percentages of the concentrations detected in the presence of an ITC antibody. Each bar represents the average of duplicate measurements in six experiments with the cells of three different donors. The error bars indicate the standard errors.

preparations at 10:1 PFE-PBMC. LPS and PHA were included as control stimuli. As shown in Fig. 6, the IFN-γ response to lysed PFE was significantly greater than that to intact PFE ($P < 0.02$) in cells that had been primed 8 days previously with intact PFE. This contrasts with fresh unprimed cells, where the opposite was true (Fig. 3), although the responses to PHA were similar in the two sets of experiments. Intact or lysed URBC were tested in each experiment but induced only small amounts of cytokine.

To determine whether the effect of priming is to alter the responsive cell type, we carried out intracellular cytokine staining of cells that had been primed and restimulated as described above (Fig. 7 and Table 3). The spectra represent the subpopulations of cells in the small lymphocyte region of the FACS spectrum. In this region, $V\gamma9^+$ cells were again the predominant IFN-γ^+ cell type observed upon stimulation by both intact and lysed PFE. The depicted spectra are qualitatively representative of the responses in all five experiments with the cells of three donors, and the results of the respective quantitative quadrant analyses are shown in Table 3. Neither whole nor lysed URBC induced large numbers of IFN-γ^+ cells under these experimental conditions (Fig. 4c and d and Table 3).

PFE-responsive blasting $\alpha\beta^+$ cells in PFE-primed PBMC. In spectra c and d of Fig. 7, a very small number of $\alpha\beta^+$ IFN-γ^+ cells can be observed for stimulation by both intact and lysed PFE. Upon examination of other regions of cells in the entire FACS spectrum, a population of blasting lymphocytes was detected outside of the small lymphocyte region (in the location of R4 in Fig. 1a). In this region, a substantial number of $\alpha\beta^+$ IFN-γ^+ cells were induced by intact-PFE stimulation when the cells had been primed with PFE but not URBC (Table 3). Restimulation with lysed PFE produces similar spectra, while both intact and lysed URBC had very little effect.

DISCUSSION

Despite a considerable body of data on proinflammatory cytokine production by lymphocytes after several days of stimulation with malaria antigens, relatively little is known about how lymphocytes respond within the first day of encountering a parasitized erythrocyte. This is an issue of some biological relevance, since the proinflammatory response is thought to represent one of the first lines of antiparasitic host defence in nonimmune individuals. Having excluded mycoplasma contamination, we found that naïve $\gamma\delta^+$ T cells produce both TNF and IFN-γ within 18 h of exposure to intact parasitized erythrocytes in vitro. Naïve $\alpha\beta^+$ T cells do not manifest this early response, but after PBMC are primed with parasitized RBC several days beforehand, subsequent exposure to parasite ly-

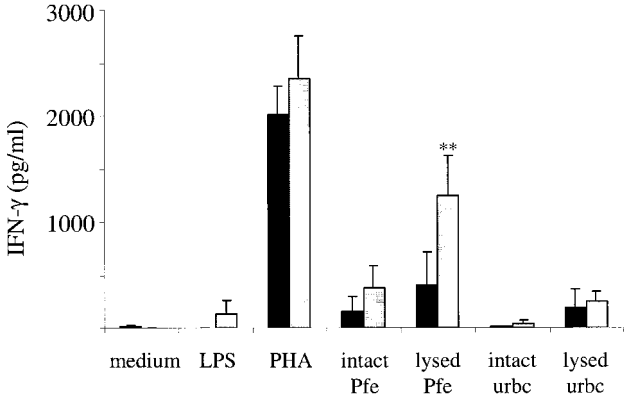


FIG. 6. Concentrations of IFN-γ measured in the supernatants of PBMC incubated for 18 h in the presence of the indicated stimuli (PHA at 10 μg/ml, LPS at 50 ng/ml, and parasite and URBC preparations at 10:1 erythrocytes-PBMC). Prior to stimulation, the PBMC were primed with URBC (solid bars) or PFE (shaded bars) at 0.5:1 erythrocytes-PBMC for 8 days. The data are shown as averages of triplicate stimulations in five independent experiments (three donors). The error bars indicate the standard deviations, and the asterisks signify a P value of less than 0.02 for the difference between stimulations by intact and lysed PFE.

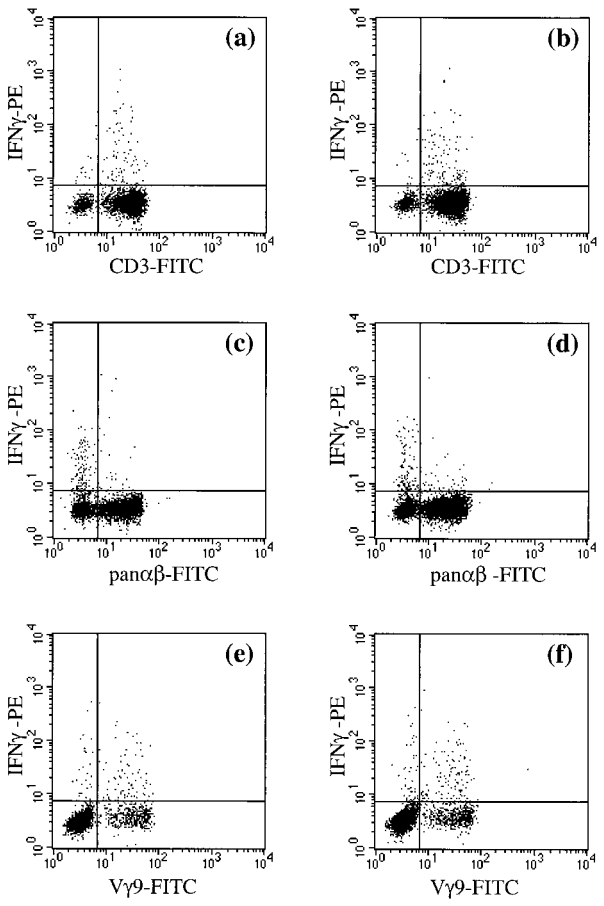


FIG. 7. FACS spectra of PBMC stimulated for 18 h with intact (a, c, and e) or water-lysed (b, d, and f) PFE (10:1 PFE-PBMC) after 8 days of preincubation with low numbers of intact PFE (0.5:1 PFE-PBMC). The cells were surface stained for CD3 (a and b), pan-αβ T-cell receptors (c and d), or Vγ9 (e and f) and intracellularly stained for IFN-γ. The gate in each case was set on the small lymphocyte region of the FACS spectrum. Quadrant markers were set using unlabeled PBMC to facilitate recognition of positive and negative cell populations. These spectra were obtained from the same batch of the cells in one experiment and are qualitatively representative of five experiments performed with the cells of three different donors. The quantitative analysis of these results is presented in Table 3.

sates causes both $\alpha\beta^+$ and $\gamma\delta^+$ T cells to release IFN-γ within a matter of hours.

These observations go against the widespread assumption, based mainly on analogy with bacterial infection, that the first phase of the cytokine response to malaria is derived mainly from monocytes and macrophages while lymphocytes contribute to proinflammatory cytokine production only at a later stage in the infection process. We have previously reported that the ability of PBMC to produce TNF within the first day of exposure to *P. falciparum* in vitro is greatly reduced by depletion of either the monocyte or the lymphocyte subpopulation. The present data show rapid TNF production by both $CD14^+$ and $CD3^+$ cells and demonstrate that $\gamma\delta^+$ T cells are the major lymphocyte subpopulation involved in both TNF and IFN-γ production at this early time point.

A striking aspect of these data is that intact parasitized erythocytes are much more efficient than lysed parasitized erythrocytes at inducing rapid IFN-γ production by $\gamma\delta^+$ T cells. A previous investigation of peripheral blood γδ T-cell responses after 5 days of stimulation in vitro gave a similar result (38). These observations suggest two possibilities: either some specific cell-cell interaction is involved or natural schizont rupture rather than lysis is required to release the stimulatory factor(s). Further experiments are needed to resolve this issue, though it is worth noting that close physical contact has been observed between intact parasitized erythrocytes and human dendritic cells (37). Other protozoan parasites are known to stimulate dendritic cells to produce IL-12, a potent inducer of IFN-γ (8, 14, 27). We find that the amount of IFN-γ produced by PBMC within 18 h of exposure to intact parasitized erythrocytes is significantly reduced in the presence of antibodies that inhibit IL-12. In contrast, lysed parasitized erythrocytes induce a lower IFN-γ response that is unaffected by inhibition of IL-12. Several previous studies have identified proinflammatory components of lysed parasites that appear to act directly on specific cell types, notably plasmodial glycosylphosphatidylinositols, which stimulate macrophages to release various inflammatory mediators (34), and phosphorylated compounds, which stimulate $\gamma\delta^+$ T cells to proliferate and release cytokines (4, 26). Taken together, these observations suggest that there exist both IL-12-independent and IL-12-dependent pathways for rapid IFN-γ production and that the latter involves intact parasitized erythrocytes.

During blood stage malarial infection, the immune system is repeatedly exposed to large quantities of parasite antigens and other debris released at schizont rupture, so it is important to consider how the cytokine response to this antigenic challenge may evolve over time. To examine the initial phase of this process, we primed PBMC with intact parasitized erythrocytes and rechallenged them after 8 days with either intact or lysed parasitized erythrocytes, measuring IFN-γ production 18 h later. The result was markedly different from that with naïve PBMC, with lysed parasitized erythrocytes now inducing a higher level of IFN-γ production than intact parasitized erythrocytes. FACS analysis indicates that this derives from both $\gamma\delta^+$ and $\alpha\beta^+$ T cells, the latter being primarily located within a blasting lymphocyte population. It is known that during the first few days of primary *P. falciparum* and *Plasmodium vivax* infections, $\gamma\delta^+$ T-cell populations, particularly the Vγ9 subclasses, expand in peripheral blood (16, 17, 24, 29; W. L. Chang, H. van der Heyde, D. G. Maki, M. Malkovsky, and W. P. Weidanz, Letter, Immunol. Lett. **32:**273–274, 1992). Such an expansion can also be observed when PBMC from naïve donors are exposed to *P. falciparum* preparations for 6 to 8 days in vitro (3, 12, 13). The expanded, or activated, T-cell populations express mRNA for TNF and IFN-γ (13, 35) and can inhibit parasite growth in vitro (10, 35). Some investigators have reported that αβ T cells also respond to PFE stimulation by expansion and cytokine secretion after 6 to 8 days (7, 9, 28, 35). There has been debate about which experimental conditions and parasite preparations favor αβ over γδ T-cell expansion; although it is not clear whether this might be influenced by mycoplasma contamination, such contamination is a potential confounder that has to be borne in mind. Our data indicate that the initial IFN-γ response involves primarily intact parasitized erythrocytes acting (directly or indirectly) on γδ T cells,

TABLE 3. Quantitative analysis of intracellular-staining results for five experiments with PBMC of three different donors[a]

Preincubation erythrocytes and region	% Double-positive cells stimulated by:			
	PFE		URBC	
	Intact	Lysed	Intact	Lysed
PFE				
SLR $\alpha\beta^+$	0.18 ± 0.07 (0.04–0.35)	0.29 ± 0.13 (0.00–0.75)	0.01 ± 0.004 (0.00–0.02)	0.01 ± 0.004 (0.00–0.02)
SLR $\gamma\delta^+$	1.28 ± 0.26 (0.84–2.32)	1.52 ± 0.24 (1.11–2.44)	0.17 ± 0.02 (0.09–0.22)	0.13 ± 0.02 (0.08–0.18)
BLR $\alpha\beta^+$	0.23 ± 0.02 (0.18–0.25)	0.25 ± 0.02 (0.18–0.28)	0.08 ± 0.02 (0.02–0.12)	0.06 ± 0.01 (0.04–0.09)
BLR $\gamma\delta^+$	0.31 ± 0.08 (0.11–0.5)	0.90 ± 0.06 (0.73–1.12)	0.10 ± 0.02 (0.02–0.13)	0.16 ± 0.01 (0.13–0.18)
URBC				
SLR $\alpha\beta^+$	0.04 ± 0.01 (0.02–0.08)	0.09 ± 0.01 (0.07–0.11)	ND	ND
SLR $\gamma\delta^+$	0.13 ± 0.02 (0.09–0.21)	0.14 ± 0.03 (0.10–0.24)	0.01 ± 0.01 (0.00–0.03)	0.02 ± 0.01 (0.00–0.03)
BLR $\alpha\beta^+$	ND	ND	ND	ND
BLR $\gamma\delta^+$	0.13 ± 0.02 (0.08–0.13)	0.21 ± 0.03 (0.14–0.31)	ND	0.08 ± 0.01 (0.06–0.12)

[a] Cells were preincubated for 8 days with either intact PFE or URBC and subsequently stimulated with intact or lysed PFE or URBC. The numbers represent the number of IFN-γ^+ and surface marker double-positive cells as percentages of the total cells in the respective gated region (SLR, small lymphocyte region; BLR, blasting lymphocyte region) with the standard errors and (in parentheses) the total range of measured responses (Fig. 7). ND not detectable.

whereas after a week there is a greater contribution from lysed parasites, acting on both the αβ and γδ T-cell populations.

The mechanism of this priming process deserves further investigation. Previous work suggests that γδ T-cell expansion in vitro requires the presence of $CD4^+$ cells but that this can be circumvented by the addition of feeder cells, IL-2, or IL-15 to the cultures (11, 13). Intriguingly, Waterfall and colleagues have noted that mycoplasma-free intact parasitized erythrocytes are considerably more effective than lysed parasites at inducing γδ T-cell expansion in vitro but that lysed parasites become effective if IL-2 is added (38). In the experimental system described here, the addition of inhibitory anti-IL-2 antibodies at the same time as priming with intact parasitized erythrocytes had little effect on the IFN-γ response to rechallenge with parasite lysates 8 days later (unpublished observations), but this is a complex problem that requires more detailed appraisal.

We conclude from these findings that the cellular origin of cytokines that cause classical malaria symptoms, such as fever, is considerably more complex than has generally been appreciated. Previously it was thought that monocytes and macrophages are major sources of pyrogenic cytokines, such as TNF, in the early phase of infection and that this results from direct stimulation by parasite toxins (2, 19, 31). However, the current evidence indicates that malaria parasites do not effectively stimulate TNF production from human monocytes without the participation of $CD3^+$ cells (32) and that $\gamma\delta^+$ T cells are potentially an abundant source of both TNF and IFN-γ in the earliest phase of infection (4, 13, 26, 38). The importance of intact parasitized erythrocytes in stimulating this early cytokine response might seem anomalous in view of the clinical evidence that schizont rupture (i.e., lysis of parasitized erythrocytes) is the primary stimulus for the outpouring of cytokines that causes malaria fever paroxysms. However, it is important to note that malaria fever does not generally occur until several days after parasites have entered the blood stream. Previously it was believed that this simply reflected the need for parasite numbers to reach a certain threshold density in order to evoke the level of cytokine response necessary to cause fever. The current data suggest that the situation is more complex and that initial exposure to parasitized erythrocytes at the earliest stage of erythrocytic infection may play an important role in priming both $\alpha\beta^+$ and $\gamma\delta^+$ T cells to release proinflammatory cytokines when massive schizont rupture occurs. Better understanding of this multistage pathway for proinflammatory cytokine induction could be of considerable importance in devising effective immunological strategies for the prevention and treatment of severe malaria.

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