Innate Immune Response to Malaria: Rapid Induction of IFN-γ from Human NK Cells by Live Plasmodium falciparum-Infected Erythrocytes

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J Immunol 2002; 169:2956-2963; doi: 10.4049/jimmunol.169.6.2956
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Innate Immune Response to Malaria: Rapid Induction of IFN-γ from Human NK Cells by Live Plasmodium falciparum-Infected Erythrocytes

Katerina Artavanis-Tsakonas and Eleanor M. Riley

To determine the potential contribution of innate immune responses to the early proinflammatory cytokine response to Plasmodium falciparum malaria, we have examined the kinetics and cellular sources of IFN-γ production in response to human PBMC activation by intact, infected RBC (iRBC) or freeze-thaw lysates of P. falciparum schizonts. Infected erythrocytes induce a more rapid and intense IFN-γ response from malaria-naïve PBMC than do P. falciparum schizont lysates correlating with rapid IRBC activation of the CD3+CD56+ NK cell population to produce IFN-γ. IFN-γ+ NK cells are detectable within 6 h of coculture with iRBC, their numbers peaking at 24 h in most donors. There is marked heterogeneity between donors in magnitude of the NK-IFN-γ response that does not correlate with mitogen- or cytokine-induced NK activation or prior malaria exposure. The NK cell-mediated IFN-γ response is highly IL-12 dependent and appears to be partially IL-18 dependent. Exogenous rIL-12 or rIL-18 did not augment NK cell IFN-γ responses, indicating that production of IL-12 and IL-18 is not the limiting factor explaining differences in NK cell reactivity between donors or between live and dead parasites. These data indicate that NK cells may represent an important early source of IFN-γ, a cytokine that has been implicated in induction of various antiparasitic effector mechanisms. The heterogeneity of this early IFN-γ response between donors suggests a variation in their ability to mount a rapid proinflammatory cytokine response to malaria infection that may, in turn, influence their innate susceptibility to malaria infection, malaria-related morbidity, or death from malaria. The Journal of Immunology, 2002, 169: 2956–2963.

Despite more than two decades of intense research, and a number of clinical trials, there is currently no vaccine that reliably protects against blood stage malaria infections. Vaccine-related research has tended to focus on identification of target Ags of protective immunity rather than the nature of antimalarial immune effector mechanisms, and has, inevitably, concentrated on adaptive rather than innate immune responses. The innate response to malaria has, until recently, received relatively little attention. However, studies in both mice and humans have repeatedly shown that proinflammatory cytokines, specifically IL-12, IFN-γ, and TNF-α, are essential mediators of protective immunity to erythrocytic malaria (1, 2); these cytokines can derive from either the innate or adaptive arm of the immune response. Given that these cytokines play a role in both immunity and pathology of malaria (1, 2), it is important to quantify the relative contribution of these two components of the immune response to the proinflammatory response.

Resistance to rodent malaria is absolutely dependent on signals mediated by IFN-γ (1), and the difference between lethal and non-lethal infections depends on the ability of the mouse to mount an early IL-12, IFN-γ, or TNF-α response (2–5). TNF-α and IFN-γ act synergistically to optimize NO production (6), which is involved in parasite killing (7). Similarly, in humans, IFN-γ production is correlated with resistance to reinfection with Plasmodium falciparum (8, 9) and protection from clinical attacks of malaria (10), plasma TNF-α and nitrogen oxide levels are associated with resolution of fever and parasite clearance (11, 12), and plasma TNF-α and IFN-γ mediate loss of infectivity of circulating gametocytes (13). Many vaccine developers now regard IFN-γ production to be the hallmark of effector T cell function for malaria (14, 15).

A recent critical review of the literature (16) concluded that control of the early peak of parasitemia in murine malaria infections was dependent on innate rather than adaptive cellular immune mechanisms, raising important questions about the role of innate immunity in control of human malaria. Enhanced NK cell activity in spleens of mice infected with irradiated Plasmodium berghei sporozoites was demonstrated many years ago (17); more recently, Plasmodium yoelii sporozoite infection has been shown to induce a rapid inflammatory response in the liver characterized by NK cell, macrophage, and T cell infiltration and IFN-γ production (18). It has been proposed that protective immunity to P. yoelii liver stages mediated by parasite-specific CD8+ T cells is dependent on the presence of IL-12 and NK cells (19), indicating an important synergy between innate and adaptive immunity in this system. There is less information regarding the role of innate immune mechanisms in controlling blood stage malaria infections; however, depletion of NK cells from Plasmodium chabaudi-infected mice results in a more severe course of infection with higher parasitemia and increased mortality (20).

Regarding the human immune response to malaria, we and others have shown that PBMC from malaria-unexposed donors can produce IFN-γ in response to stimulation by either live or dead schizont Ags (21–24). Live parasites induce proliferation of both αβ and γδ TCR+ T cells, whereas dead schizont extract activates only TCRαβ+ T cells (24). These cells have been widely assumed

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Received for publication May 20, 2002. Accepted for publication July 9, 2002.

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1 This study received financial support from the Wellcome Trust. This study was approved by the London School of Hygiene and Tropical Medicine Ethical Review Committee (submission nos. 584 and 805).

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0022-1767/02/$02.00
to be the major source of IFN-γ (23, 25). Activation of TCRαβ+ T cells has been ascribed to reactivation of a polyclonal population of memory cells primed by exposure to cross-reactive Ags (21, 26, 27), whereas the TCRγδ+ T cell response is restricted to the Vγ9Vδ2 subset (28, 29), and is induced by small, phosphorylated nonprotein Ags similar to those described for mycobacteria (30). These findings are consistent with cytokine responses to malaria Ags in unexposed donors being derived from cells of the adaptive immune system. However, Scragg et al. (31) have recently reported very early induction of TNF-α, IL-12, and IFN-γ (within 10 h) by live, parasitized erythrocytes, and Hensmann et al. (32) have shown that live parasites induce TNF-α and IFN-γ from Vγ9Vδ2 T cells and TNF-α from CD14+ monocytes, within 18 h. Cytokine induction is dependent on the presence of both monocytes and lymphocytes (31), indicating that this is not a classical endotoxin-like response as had previously been thought (33). Increased NK-like cytotoxicity has been reported during mild malaria infection (34), but appears to be depressed in children with severe disease (35). Coculture of PBMC with soluble malaria Ags has been reported to increase their cytotoxic activity against an NK-sensitive cell line (36), and CD3+CD56+ NK cells have been reported to lyse schizont-infected erythrocytes (37).

The purpose of this study therefore was to investigate the kinetics and cellular origins of IFN-γ induced by P. falciparum malaria in naive and malaria-exposed human blood donors, to determine the contribution of cells of the innate and adaptive immune system. However, Scragg et al. (31) have recently reported modest, but statistically significant levels of IFN-γ as uRBC, A mixture of human rIL-12 (rIL-12) (1 ng/ml), rIL-18 (40 ng/ml) (PeproTech, London, U.K.), and rIL-2 (2 μg/ml) (Boehringer Mannheim, Lewes, U.K.), or the mitogen PHA (1 μg/ml; Difco/Becton Dickinson, Oxford, U.K.) was used as positive controls for NK cell activation (PHA was not necessarily expected to act directly on NK cells, but to activate other cells in the PBMC culture, inducing cytokines that would lead to NK activation). To determine the effect of exogenous cytokines on the response to malaria parasites, rIL-12 or rIL-18 were added to cultures at concentrations ranging from 0.1 to 10.0 ng/ml.

### Flow cytometry

Brefeldin A (10 μg/ml; Sigma-Aldrich) was added to cell cultures for the last 3 h of incubation. After the appropriate incubation period, supernatants were collected from each culture and stored at −70°C for cytokine analysis by ELISA. Cells were washed twice with FACS buffer (1× PBS, 0.1% NaN3, 0.1% BSA), resuspended in FACS buffer at a concentration of 5 × 10⁶ cells/100 μl, and stained for 40 min at 4°C with appropriate combinations of labeled Abs. Cells were washed twice with FACS buffer, fixed in 2% paraformaldehyde in PBS for 15 min at room temperature in the dark, and washed once with FACS buffer. Cells were resuspended in 100 μl paraformaldehyde fixation buffer (1× PBS, 1% saponin, 0.1% sodium azide) with anti-cytokine Ab, incubated in the dark at 4°C for 30 min, and washed with FACS buffer. Finally, cells were suspended in 300 μl FACS buffer and analyzed in a FACScan flow cytometer (BD Biosciences). Data analysis was performed with CellQuest software (BD Biosciences). A total of 100,000 events was collected from each sample. The following mAbs were used: CD3 Tricolor (TRI), CD8 FITC, IgG1 TRI (all CalTag Laboratories, Burlingame, CA); TCRβ FITC, TCRγδ FITC, CD56 FITC, CD4 FITC, IgG1 FITC, IgG2 FITC, Vγ9 PE, IFN-γ PE, TNF-α PE, and IgG1 PE (all BD Biosciences); V602 FITC and Vγ9 FITC (Serotec, Oxford, U.K.).

### Cytokine ELISA

IFN-γ and IL-12 (p40 and p70) were detected in cell supernatants by sandwich ELISA using commercially available reagents; all samples were tested in duplicate according to the manufacturer’s recommendations. IFN-γ Abs and standard were purchased from BD Biosciences; IL-12 Abs and standard were from R&D Systems (Abingdon, U.K.). Where samples gave values above the top of the standard curve, supernatants were retested at 1/10 or 1/100 dilutions in RPMI 1640, and cytokine levels were recalculated.

### IL-12 and IL-18 neutralization

Neutralizing goat anti-human IL-12 polyclonal Ab (R&D Systems) was added to PBMC cultures at concentrations ranging from 0.5 to 10 μg/ml. An isotype-matched control Ab (goat IgG; Sigma-Aldrich) was used at the same concentrations. Neutralizing mouse polyclonal anti-human IL-18 or a mouse IgG1 control Ab (both R&D Systems) were used at concentrations from 0.1 to 5.0 μg/ml.

### Results

**IFN-γ response to P. falciparum by cells from malaria-naive donors**

We examined the kinetics of IFN-γ secretion from PBMC of five European, malaria-naive donors in response to iRBC or rPfSL, measuring IFN-γ concentration in culture supernatants by ELISA (Fig. 1). urBRC induced minimal IFN-γ production over a period of 6 days. Lysed parasites induced modest, but statistically significant IFN-γ responses in cells from all donors. Cells from three donors produced IFN-γ from about day 4; cells from one donor made only a modest and transient response; and cells from one other donor made high levels of IFN-γ within 24 h. In contrast, live parasites rapidly induced high levels of IFN-γ production from cells from all donors, with IFN-γ concentrations in excess of 10,000 pg/ml by this point.
NK cells, NK-T, or H9253/H9254 presented triggering of a population of innately activated cells such as PfSL. Shown are the total number of IFN-3 NK cells.

Background staining, 42% of the IFN-3 after 24-h incubation of PBMC with 3 10^6 lysed infected erythrocytes are shown in Fig. 2. For cells incubated with iRBC, IFN-3 of cells, of which the most numerous are CD56 TCR-1 and CD56 TCR+ T cells. To determine the source of IFN-3 we incubated PBMC with iRBC, PfSL, or uRBC and harvested cells after periods of between 3 and 72 h for analysis of cell surface phenotype and intracellular IFN-3.

Table I. Rapid IFN-γ induction in NK cells by P. falciparum

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<tr>
<th>Donor</th>
<th>Ethnicity</th>
<th>uRBC</th>
<th>PfSL</th>
<th>IRBC</th>
<th>Total</th>
<th>NK</th>
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<td>1315 ± 278</td>
<td>1853 ± 344</td>
<td>256 ± 94</td>
<td>612 ± 183</td>
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Malaria exposed

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<th>PfSL</th>
<th>IRBC</th>
<th>Total</th>
<th>NK</th>
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<td>773</td>
<td>1112</td>
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<td>684 ± 90</td>
<td>786 ± 133</td>
<td>973 ± 243</td>
<td>156 ± 63</td>
<td>217 ± 86</td>
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The early (24 h) IFN-γ response to P. falciparum. IFN-γ+ cells were detected by flow cytometry after 24-h coculture of PBMC with malaria-infected iRBC or uRBC or PfSL. Shown are the total number of IFN-γ+ cells/100,000 events and the number of CD3+ CD56− IFN-γ+ cells. Percentages refer to the proportion of IFN-γ+ cells that are NK cells.

day 4 and concentrations of up to 200,000 pg/ml by day 6. Differences between PfSL-induced and iRBC-induced IFN-γ concentrations were statistically significant at all time points (Wilcoxon signed rank test, Z = −2.023, n = 5, p = 0.043). Thus, the IFN-γ response to iRBC was more rapid, and maximal concentrations were 10-fold higher than the response to PfSL.

Cellular source of early IFN-γ production

We hypothesized that the rapid IFN-γ response to iRBC represented triggering of a population of innately activated cells such as NK cells, NK-T, or γδ T cells. To determine the source of IFN-γ production during the first 3 days of exposure of naive PBMC to malaria Ags, we incubated PBMC with iRBC, PfSL, or uRBC and harvested cells after periods of between 3 and 72 h for analysis of cell surface phenotype and intracellular IFN-γ by flow cytometry. IFN-γ+ cells were gated and analyzed for expression of CD3, TCRβ, TCRγδ, and CD56. As an example, data for one donor after 24-h incubation of PBMC with 3 10^6 iRBC or uRBC or 3 10^6 lysed infected erythrocytes are shown in Fig. 2. For cells incubated with iRBC, IFN-γ was derived from a mixed population of cells, of which the most numerous are CD56− CD3− NK cells. After subtraction of the background counts for the isotype control Ab and uRBC control, 62% of the iRBC-induced IFN-γ+ cells were CD3− CD56− NK cells; the remainder were CD3+ T cells. After 24-h incubation with PfSL, the number of cells staining for IFN-γ was almost 4-fold lower than for iRBC; after subtraction of background staining, 42% of the IFN-γ+ cells were found to be NK cells.

To determine the optimum parasite concentration for NK cell activation, a dose-response analysis was conducted (Fig. 3, a and b). Optimal NK cell IFN-γ production was observed at either 10^6 or 10^7 iRBC/10^6 PBMC. For additional experiments, intact and lysed parasites were used at a final concentration of 3 10^6 parasites/10^6 PBMC in a volume of 1 ml; control cultures contained 3 10^6 uRBC.

To determine the kinetics of the NK cell response to malaria parasites, the proportion of NK cells making IFN-γ was examined over a period of 72 h (data from two representative donors are shown in Fig. 3, c and d). iRBC-induced IFN-γ was detected within NK cells as early as 6 h and peaked between 15 and 24 h in all donors. The kinetics of the iRBC-induced response was similar to that of PHA-mediated NK activation. Twenty-four-hour cultures were thus used to detect NK cell IFN-γ production in all future assays.

As others (31, 32) have reported early production of TNF-α from PBMCs and as NK cells have been reported to make TNF-α (38), we also stained NK cells for intracellular TNF-α. As can be seen in Fig. 3, e and f, a small percentage of NK cells could be induced to express TNF-α after incubation with PHA for 15–24 h, but few, if any, NK cell were induced to secrete TNF-α by incubation with iRBC (note the difference in the scale on the y-axis for IFN-γ and TNF-α).

To determine whether rapid induction of IFN-γ from NK cells by iRBC was a universal phenomenon, we looked at the 24-h IFN-γ response in a cohort of 30 human blood donors (Table I). The number of IFN-γ+ cells/100,000 events was calculated for IFN-γ− and IFN-γ− NK cells.
PBMC cultured with uRBC, iRBC, or PfSL, and the number (%) of the IFN-γ/H9253/H11001 cells that were NK cells (i.e., CD3/H11002/H9253, CD56/H11001, IFN-γ/H9253/H11001) is also shown. In both the malaria-unexposed and malaria-exposed donors, there is marked heterogeneity in the number of IFN-γ/H9253/H11001 cells in both PfSL and iRBC cultures. In the malaria-unexposed donors, iRBC induced significantly higher numbers of PBMC to produce IFN-γ/H9253/H11001 than did PfSL (Wilcoxon signed rank test, Z = 2.308, p = 0.021), but this difference was not significant for the malaria-exposed donors (Z = 1.177, p = 0.239). Similarly, in unexposed donors, iRBC induced significantly more NK cells (in terms of both absolute numbers and percentages) to produce IFN-γ/H9253/H11001 than did PfSL (Z = 2.591, p = 0.009) and, again, this difference was not significant for the malaria-exposed donors (Z = 1.255, p = 0.209). The total number of IFN-γ/H9253/H11001 cells, and particularly the number of IFN-γ/H9253/H11001 NK cells, was somewhat lower in the malaria-exposed donors than in the malaria-unexposed donors, but there was considerable heterogeneity within the malaria-exposed group and these differences were not statistically significant (Student’s t test, t ≤ 1.88, p ≥ 0.07 for all comparisons). Perhaps the most noticeable trend in the data was the marked variation between donors in the numbers of IFN-γ/H9253/H11001 NK cells that were induced by iRBC (ranging from 2,940 cells/100,000 events to less than 20).

Taken together, these data suggest that: 1) the ability to mount a rapid IFN-γ response to malaria parasites is partially dependent upon the ability of NK cells to respond to malaria parasites; 2) the difference in the magnitude of the response to iRBC and PfSL is due in large part to the ability of iRBC to activate NK cells; and 3) individuals vary in their ability to make an NK cell response to malaria parasites.

**Heterogeneity of the human NK cell response to malaria parasites**

Variation between individuals in their T cell response to specific Ags is commonplace and relatively easily explained by differences in T cell repertoire, MHC genotype, and prior exposure to Ag. Substantial variation in the NK cell response to a given stimulus was less expected and less easy to explain, as this represents an innate response in which prior exposure to the pathogen is not expected to augment the response. Indeed, NK cells from malaria-exposed donors were somewhat less likely to make IFN-γ than cells from naive donors (Table I and Fig. 4). One possibility is that the heterogeneity represents inherent differences between donors in the ease with which their NK cells are activated to produce...
IFN-γ. However, we found no obvious correlation between the response to iRBC and the response to other stimuli such as recombinant cytokines or (as shown in Fig. 4) PHA.

Is NK cell activation dependent on IL-12 or IL-18?

As activation of NK cells is known to be at least partially IL-12 and IL-18 dependent in many systems (39–41), we hypothesized that the difference between donors in their ability to make an NK cell response to malaria might be due to differences in their ability to make IL-12 or IL-18 in response to iRBC activation.

To determine whether NK cell responses to malaria were indeed IL-12 or IL-18 dependent, we incubated PBMC with iRBC or uRBC for 24 h in the presence or absence of increasing concentrations of neutralizing Ab to human IL-12 or IL-18 (Fig. 5). The percentage of IFN-γ− NK cells was markedly reduced in the presence of 0.5 μg/ml anti-human IL-12, but not by equivalent concentrations of an isotype-matched Ab (Fig. 5a); NK cell responses were not further reduced by increasing doses of Ab. Anti-IL-12 at a concentration of 0.5 μg/ml consistently reduced the percentage of IFN-γ− NK cells by between 50 and 100% (Fig. 5b). In a comparable experiment, anti-IL-18 Ab had rather variable effects on the proportion of NK cells that made IFN-γ in response to iRBC. In titration experiments, anti-IL-18 Ab inhibited NK activation by up to 50% at high concentrations (5.0 μg/ml) (Fig. 5c) and was able to partially inhibit induction of IFN-γ in NK cells of some donors (e.g., 094M and FMO), but not others. (e.g., 053M) (Fig. 5d).

As the NK cell response to iRBC was clearly IL-12 dependent, we wondered whether the difference between high and low responder donors was due to differences in IL-12 induction from macrophages or dendritic cells in the PBMC culture. Absolute levels of IL-12 p40 or p70 in culture supernatants at 24 h were very low (frequently undetectable) and did not differ between antigenic stimuli or between donors (data not shown). More importantly, addition of rIL-12 at concentrations from 0.1 to 10.0 ng/ml to PBMC cultured with either PfSL or iRBC had no consistent effect on either the concentration of IFN-γ in supernatants or the percentage of IFN-γ− NK cells after 24 h (data not shown). Also, addition of exogenous rIL-12 did not enhance the response of low responding donors and did not enhance PfSL-induced responses to the level of iRBC-induced responses (data not shown). Thus, although IL-12 plays an essential role in the activation of NK cells to make IFN-γ, lack of IL-12 is insufficient to explain the differences in IFN-γ responses between individuals or between live and dead parasites.

In a comparable experiment, addition of rIL-18 to 24-h PBMC cultures with PfSL or iRBC had no effect on the NK cell response to PfSL, although low doses of rIL-18 (0.1 or 1.0 ng/ml) did marginally enhance the NK response to iRBC in most donors (data not shown).

Discussion

The main aim of immunological research on malaria over the past 20 years has been vaccine development and has thus, by necessity, focused on adaptive immune responses. The innate response to malaria has received relatively little attention. However, growing awareness of the protective role of innate immune mechanisms in their own right and their role in induction of adaptive immunity
we have therefore conducted a detailed study of the kinetics of IFN-γ/H9253 proliferating cells have been identified as early as 6 h after stimulation with iRBC (uRBC values subtracted) (a) or PHA (growth medium values subtracted) (b) is shown. CD56+ cells were selected by gating on side scatter and CD56+; the gated cells were then analyzed for IFN-γ and CD3 expression. The proportion of CD3+CD56+ cells that were positive for IFN-γ is shown; data are based on collection of 100,000 events. Data from 18 individual donors are shown. All donors are malaria naive except for those marked ♦ who have varying levels of prior malaria exposure. *, No IFN-γ+ NK cells detectable. Comparison of NK responses to iRBC and a recombinant cytokine (IL-2/12/18) mixture (n = 11) showed a similar lack of correlation (data not shown).

FIGURE 4. Heterogeneity of the human NK cell response to iRBC. The percentage of NK (CD3+CD56+) cells staining for intracellular IFN-γ after 24-h coculture with iRBC (uRBC values subtracted) (a) or PHA (growth medium values subtracted) (b) is shown. CD56+ cells were selected by gating on side scatter and CD56+; the gated cells were then analyzed for IFN-γ and CD3 expression. The proportion of CD3+CD56+ cells that were positive for IFN-γ is shown; data are based on collection of 100,000 events. Data from 18 individual donors are shown. All donors are malaria naive except for those marked ♦ who have varying levels of prior malaria exposure. *, No IFN-γ+ NK cells detectable. Comparison of NK responses to iRBC and a recombinant cytokine (IL-2/12/18) mixture (n = 11) showed a similar lack of correlation (data not shown).

suggests that studies of innate immunity to malaria are warranted. Rapid induction of monokines and lymphokines (such as IL-12, IFN-γ, and TNF-α) may enable the infected host effectively to control the exponential replication of blood stage parasites until the adaptive immune response can take over. This might be of most benefit during a primary infection, but, given the extent of antigenic polymorphism in malaria, innate responses may also be required to control reinfections of variant genotype until novel adaptive responses can be generated.

It has long been known that T cells from malaria-naive donors can proliferate and secrete cytokines in response to P. falciparum Ags. Responses tend to peak after 6–7 days activation in vitro and the proliferating cells have been identified as either TCRαβ+ T cells, which respond in a classical MHC-restricted manner to both live and dead parasite Ags, or TCRγδ+ T cells that respond preferentially to live parasites (23, 24); both cell types have also been shown to secrete IFN-γ (21, 23, 25). Recently, it has been suggested that live P. falciparum can induce rapid (within 12–24 h) IFN-γ and TNF-α responses (31) and that TCRγδ+ T cells may contribute to the early IFN-γ response (32). We have also recently demonstrated an important role for IL-12 in malaria-induced IFN-γ production (42). We have therefore conducted a detailed study of the kinetics of IFN-γ production by P. falciparum-activated PBMC.

The first interesting observation was that live parasites induce a much stronger and much more rapid IFN-γ response than do dead parasites. Differences between live and dead parasites have been noticed previously, particularly in their ability to activate TCRγδ+ T cells (24, 32). We have now extended this observation to include preferential induction of IFN-γ production in NK cells by live parasitized erythrocytes. The requirement for live parasites within intact RBC for induction of the innate response may be due to a need for direct contact between the parasitized red cell and the leukocyte (be it a lymphocyte or an accessory cell), or may be due to instability of the parasite-derived ligands that interact with cell surface receptors.

The nature of the malarial ligands that might be involved in induction of the innate response is largely unknown. Scragg et al. (31) have shown that early cytokine induction by P. falciparum is not due to the presence of classical endotoxins: levels of IL-12 and IL-10 induced by malaria Ags are orders of magnitude lower than the levels induced by binding of bacterial LPS to CD14, and a CD14+ monocyte-like cell line that responds to a wide variety of bacterial endotoxins by secretion of TNF-α fails to respond to P. falciparum (31). Malarial GPIs have been shown to activate macrophages and vascular endothelium (33, 43). GPIs from Trypanosoma cruzi have been shown to induce IL-12 via binding to Toll-like receptor 2 (44), but nothing is currently known of malarial ligands for Toll-like receptors. P. falciparum-derived, phosphorylated, nonprotein moieties similar to those isolated from mycobacteria have been shown to be ligands for TCRγδ+ T cells (30) and, due to their highly lipophilic nature, are likely to have a rather short t1/2 in solution. Finally, malarial GPIs have also been reported to activate murine CD1d-restricted NK-T cells (45), although these cells then produce IL-4 rather than IFN-γ, but at least one study has failed to reproduce the findings (46).

In this study, we have shown that CD3+CD56+ NK cells are major contributors to the early IFN-γ response to malaria parasites. IFN-γ+ NK cells can be identified as early as 6 h after stimulation in some donors, and the peak of the NK IFN-γ response occurs between 15 and 24 h. Preliminary studies in our laboratory indicate...
that the activated NK cells undergo apoptosis from ~24 h (K. Artavanis-Tsakonas and E. Riley, unpublished observation). In the majority of donors, NK cells are the first population to become IFN-γ⁺, with γδ T cells becoming IFN-γ⁺ after 48–72 h and αβ T cells beginning to make IFN-γ after 4–6 days (data not shown).

We were not surprised to find that the NK cell response was highly IL-12 dependent; this is widely reported for NK cells (39), and has been shown for NK responses to a number of pathogens (40, 41). We considered the possibility that differences between donors, and between Ag preparations, in the magnitude of the IFN-γ response might be due to differences in IL-12 induction. However, addition of rIL-12 did not significantly enhance the response of individual donors and did not increase the response to PfSL to levels seen for iRBC.

The requirement for IL-18 was less clear, with some donors showing a reduction in NK cell responses to iRBC in the presence of anti-IL-12 Ab and cells from other donors being unaffected. In contrast, low doses of rIL-18 did marginally enhance the proportion of NK cells that could be induced to produce IFN-γ in response to iRBC in most donors, indicating that IL-18 can indeed augment the NK response to malaria parasites. Taking the IL-12 and IL-18 data together, it appears that while monokines, produced for example by dendritic cells or macrophages in the PBMC cultures, are required to optimize the NK cell response to P. falciparum, these monokines are not sufficient on their own to induce the levels of IFN-γ production obtained with malaria-infected RBC. A lack of IL-12 or IL-18 in the culture medium is insufficient to explain the very low levels of NK cell activation induced by parasite lysates or the very low responses to iRBC in some donors. However, the influence of other cytokines (e.g., IL-2 and IL-15) and the ability of an individual’s macrophages or dendritic cells to respond to malaria Ags in other ways require further investigation.

In addition to NK activation by cytokines, our data raise the possibility that ligands expressed on the intact iRBC are specifically recognized, either by the NK cell itself or by an APC population. Preliminary data from our laboratory indicate that iRBC can bind to NK cells and that contact between the NK cell and the iRBC is required for optimal IFN-γ induction (K. Artavanis-Tsakonas and E. Riley, unpublished data). Also, there is a report in the literature that human NK cells can lyse parasite-infected RBC (37), indicating that direct cell:cell contact occurs. Thus, optimal activation of NK cells may require integration of two or more signals transduced through different receptors.

It is clear that there is considerable variation between individual donors in the magnitude of their NK cell IFN-γ response. This heterogeneity does not appear to be due to inherent differences in the case of NK activation or differences in IL-12 or IL-18 production. The difference between malaria-exposed and malaria-unexposed donors is intriguing. Although the numbers are quite small, and the difference not quite statistically significant ($t = 1.878, p = 0.07$), the numbers of NK cells making IFN-γ were lower in iRBC-stimulated cultures from the exposed than the unexposed donors. At this stage, we cannot say whether this represents down-regulation of NK cell responses by acquired immune responses or is due to genetic differences between the groups (10 of the 12 exposed donors were Africans compared with only 1 of 18 in the unexposed group). A possibility is that genetic variation in NK cell receptor expression, particularly of activating killer cell Ig-like receptors (47), might influence the response to malaria.

Importantly, our data suggest that the NK cells are a significant source of IFN-γ in the first few hours of a malaria infection, and thus may be an important component of the innate defense against malarial parasitemia. Surprisingly, this innate response is not universal among human blood donors, raising interesting questions.

**FIGURE 5.** Effect of neutralizing Ab to IL-12 and IL-18 on IFN-γ production from NK cells after 24-h coculture with iRBC. PBMCs (10⁵/ml) were cultured with 3 × 10⁶ iRBC, or 3 × 10⁶ freeze-thawed schizonts (PfSL) for 24 h in the presence of neutralizing Ab to human IL-12 (a and b) or IL-18 (c and d) or control, isotype-matched Abs. Control cultures were incubated with 3 × 10⁶ uRBC; uRBC values have been subtracted from iRBC and PfSL values. CD56⁺ cells were selected by gating on side scatter and CD56⁺; the gated cells were then analyzed for IFN-γ and CD3 expression. The proportion of CD3⁺ CD56⁺ cells that were positive for IFN-γ is shown; data are based on collection of 100,000 events. Optimal anti-cytokine Ab concentrations were determined by titration (a and c); data for five donors at this optimal concentration are shown (b and d).
about the functional importance of the innate response to malaria infection. Two opposing hypotheses can be proposed: rapid IFN-γ production may be associated with efficient induction of IFN-γ-mediated effector mechanisms and enhanced ability to control malaria infections. Alternatively, rapid innate production of IFN-γ may predispose to overproduction of inflammatory cytokines and increased risk of severe malaria. Studies in malaria endemic areas will be required to determine which, if either, of these scenarios is correct.

Acknowledgments

We thank Claire Swales for assistance with culturing and mycoplasma testing of malaria parasites and Elizabeth King for assistance with cytokine measurements. We also thank Greg Bancroft, Dan Davis, Christian Engwerda, Paul Kaye, and Peter Parham for advice and useful discussions. Finally, we thank Katie Flanagan, Tom Doherty, and Richard Jennings at the Hospital for Tropical Diseases, and Carolyne Stanley for assistance with collecting clinical samples.

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