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Cross-Talk between T Cells and NK Cells Generates Rapid Effector Responses to Plasmodium falciparum-Infected Erythrocytes

Amir Horowitz,* Kirsty C. Newman,* J. Henry Evans, † Daniel S. Korbel,* Daniel M. Davis, † and Eleanor M. Riley*

Rapid cell-mediated immune responses, characterized by production of proinflammatory cytokines, such as IFN-γ, can inhibit intraerythrocytic replication of malaria parasites and thereby prevent onset of clinical malaria. In this study, we have characterized the kinetics and cellular sources of the very early IFN-γ response to Plasmodium falciparum-infected RBCs among human PBMCs. We find that NK cells dominate the early (12–18 h) IFN-γ response, that NK cells and T cells contribute equally to the response at 24 h, and that T cells increasingly dominate the response from 48 h onward. We also find that although γδ T cells can produce IFN-γ in response to P. falciparum-infected RBCs, they are greatly outnumbered by αβ T cells, and thus, the majority of the IFN-γ+ T cells are αβ T cells and not γδ T cells; γδ T cells are, however, an important source of TNF. We have previously shown that NK cell responses to P. falciparum-infected RBCs require cytokine and contact-dependent signals from myeloid accessory cells. In this study, we demonstrate that NK cell IFN-γ responses to P. falciparum-infected RBCs are also crucially dependent on IL-2 secreted by CD4+ T cells in an MHC class II-dependent manner, indicating that the innate response to infection actually relies upon complex interactions between NK cells, T cells, and accessory cells. We conclude that activation of NK cells may be a critical function of IL-2-secreting CD4+ T cells and that standard protocols for evaluation of Ag-specific immune responses need to be adapted to include assessment of NK cell activation as well as T cell-derived IL-2. *The Journal of Immunology, 2010, 184: 6043–6052.

The optimal immune response to a malaria infection likely comprises rapid induction of inflammatory antiparasitic responses followed by equally rapid resolution of inflammation (mediated by anti-inflammatory cytokines) to prevent immunopathology (1). Rapid and robust cell-mediated immune responses can inhibit intraerythrocytic replication of malaria parasites and thereby prevent onset of clinical malaria (2). This process can be primed by ultra-low–dose infection/vaccination (3, 4) but has yet to be mimicked by subunit vaccines. Understanding the cellular and molecular pathways of this very early cellular response may allow the design of new approaches to vaccination, but there is still considerable debate over the precise sequence of events. In particular, the timing and magnitude of IFN-γ secretion are thought to be pivotal in determining the outcome of disease, and it is thus of importance to identify the major cellular sources of IFN-γ, the kinetics of its production, and the pathways by which it is induced and regulated.

IFN-γ can be produced by both innately activated cells and cells of the adaptive immune system. We have previously found that αβ T cells, γδ T cells, and NK cells all contribute to the IFN-γ response of PBMCs from malaria-naive donors cocultured for 18–24 h with live Plasmodium falciparum-infected RBCs (5), but we also found that the magnitude of the response, as well as the proportion of responding cells in each subset, varies among individuals (5, 6). In some individuals, there is little or no evidence of an NK cell response to P. falciparum-infected RBCs, whereas in other individuals, NK cells comprise ∼70% of IFN-γ–producing cells (5). We have identified variation in the strength of costimulatory signals from myeloid accessory cells (7) as well as polymorphism among NK cell regulatory receptors (8) as two components of this variation. Others, however, have concluded that γδ T cells are the major population of IFN-γ–producing lymphocytes when PBMCs from naive individuals are incubated with P. falciparum-infected RBCs (9, 10) and that NK cells make no major contribution to the innate response to P. falciparum (9), although comparison of data from different studies is complicated by substantial differences in experimental protocols.

In this study, we have carefully compared the magnitude and the timing of T cell and NK cell responses to P. falciparum-infected RBCs in a large cohort of malaria-naive donors. We find that NK cells dominate the early (12–18 h) IFN-γ response, that NK cells and T cells contribute approximately equally to the response at 24 h, and that T cells then increasingly dominate the response from 48 h onward. We also find that although γδ T cells can produce IFN-γ in response to P. falciparum-infected RBCs, they are greatly outnumbered by αβ T cells, and thus, contrary to previous reports, the majority of the IFN-γ+ T cells are αβ T cells and not γδ T cells; γδ T cells are, however, an important source of TNF. Importantly, however, we find that the NK cell IFN-γ response to P. falciparum-infected RBCs is crucially dependent on IL-2 secreted, in an MHC class II-dependent manner, by CD4+ T cells. These data corroborate our recent observations that NK cells may be recruited by Ag-specific IL-2–secreting CD4+ T cells to act as effector cells during...
the recall response to vaccine Ags (A. Horowitz, R.H. Behrens, L. Okell, A.R. Fooks, and E.M. Riley, submitted for publication) and indicate that the innate response to infection actually relies upon complex interactions between NK cells, T cells, and accessory cells.

**Materials and Methods**

**Blood donors**

Adult blood donors were recruited at the London School of Hygiene and Tropical Medicine through an anonymous blood donation system. All donors were malaria naïve and healthy and gave fully informed consent for their blood to be used in this study. Ethical approval was given by the London School of Hygiene and Tropical Medicine Ethics Committee (application number 805).

**PBMC preparation and culture**

Venous blood was collected into sodium heparin (10 IU/ml blood; CP Pharmaceuticals, London, U.K.), and PBMCs were isolated by Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation as described previously (11). Cells were resuspended at a concentration of 2 × 10^6 cells/ml and cultured in flat-bottom 24-well plates or U-bottom 96-well plates for periods of up to 6 d.

Recombinant IL-12 (rhIL-12; Peprotech, Rocky Hill, NJ) and rhIL-18 (MBL International, Woburn, MA) were each used at 0.1 µg/ml.

**P. falciparum parasites**

*P. falciparum* parasites (strain 3D7) were grown in O^6^-human erythrocytes (National Blood Service, London, U.K.) in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 25 nM HEPES (Sigma-Aldrich), 28 mM sodium bicarbonate (BDH Prolabo Chemicals, London, U.K.), 20 µg/ml hypoxanthine (Sigma-Aldrich), and 10% normal human AB serum (National Blood Service). Cultures were gassed with 3% O2, 4% CO2, and 93% N2 and incubated at 37°C. Parasite cultures were routinely shown to be free from *Mycoplasma/Acholeplasma* species contamination using an ELISA-based Mycoplasma Detection Kit (Roche, Basel, Switzerland) incorporating polyclonal Abs against *Mycoplasma arginini, Mycoplasma hyorhinis, Acholeplasma laidlawii*, and *Mycoplasma orale*. Highly pure (>95%) mature schizonts were harvested from cultures of 5–8% parasitemia by adherence to an LD separation column (Miltenyi Biotec, Auburn, CA). Columns were washed thoroughly with PBS to remove uninfected erythrocytes pre-elution. Schizont-infected (*P. falciparum*-infected RBC) or uninfected erythrocytes were added at a ratio of 3 RBC/mononuclear cell.

**Depletion of T cell subsets**

PBMCs were stained for CD3-expressing cells using a T cell selection kit (StemCell Technologies, Vancouver, British Columbia, Canada); CD4-expressing cells using a Dynal bead-conjugated mAb to CD4 (Invitrogen, Carlsbad, CA); CD8-expressing cells using a PE-conjugated mAb to CD8 (Caltag Laboratories, Burlingame, CA) and anti-PE MicroBeads (Miltenyi Biotec); γδ TCR-expressing cells using PE-conjugated mAb to γδ TCR (Caltag Laboratories) and anti-PE MicroBeads (Miltenyi Biotec); and αβ TCR-expressing cells using a PE-conjugated mAb to αβ TCR (BD Biosciences, San Jose, CA) and anti-PE MicroBeads (Miltenyi Biotec). Depletion of CD4 cells was carried out using a Dynal Magnetic Particle Concentrator-2 (Invitrogen). All other depletions were carried out using LD separation columns (Miltenyi Biotec) according to the manufacturer’s instructions. Postdepletion, the PBMC concentration was adjusted to 2 × 10^6, and cells were cultured with *P. falciparum*-infected RBCs at a ratio of 1:3 (as previously) and also at a ratio of 1:10 to ensure that changing the ratio of NK cells and accessory cells to *P. falciparum*-infected RBCs did not adversely affect the outcome of the experiments. No significant differences were seen between the 1:3 and 1:10 cultures, so only data from the 1:3 cultures are presented.

**Blocking experiments**

Two million PBMCs were cultured overnight in 24-well flat-bottom plates in the presence of purified blocking-neutralizing Abs or appropriate isotype control Abs for the following cytokines, cytokine receptors, and MHC molecules (all BD Biosciences): anti-IL-2 (p40p70; clone C11.5), anti–IL-18 (clone H44), anti–IL-2 (clone MQ1 17H12), anti–IFN-α [2b] (clone 7N4-1), anti-TNF (clone MAb1), anti-MHC class I (HLA-A, -B, -C; clone W6/32), and anti-MHC class II (HLA-DR, DP, DQ; clone Tü-39).

**Cell surface and intracellular staining for flow cytometry**

Surface and intracellular staining was performed as described previously (6). The Abs/reagents used were: anti-CD3 PerCP, anti–αβ TCR-FITC, anti–γδ TCR (biotinylated), streptavidin PerCP, anti–IFN-γ-APC, anti-CD4 APC-Cy7, anti-CD8 Pacific Blue, and anti-CD3 PE-Texas Red (BD Biosciences); anti-CD56 APC (Beckman Coulter, Fullerton, CA), anti–IFN-γ FITC, anti–IL-2 APC (BD Biosciences), and anti-CD56 PE-Cy7 (BD Biosciences). All Abs were mouse monoclonals. Flow cytometric analyses were performed using a BD FACSCalibur or BD Cyan flow cytometer (BD Biosciences) and FlowJo analysis software (TreeStar, Ashland, OR). Anti-CD3 Abs were included in the intracellular staining mixes to ensure that T cells that downregulated CD3 upon stimulation were not misclassified.

**Multiplex assay**

Secreted IL-2 and TNF-α were measured, according to the manufacturer’s instructions, by cytometric bead array (CBA Th1/Th2/Th17 kiti; BD Biosciences). All samples were acquired using an FACSCount flow cytometer, and analysis was performed using FCAP Array software (Soft Flow, St. Louis Park, MN).

**Statistical analysis**

All statistical analyses were performed using Prism 5 software (GraphPad, San Diego, CA).

**Results**

**T cells and NK cells both contribute to early IFN-γ response to *P. falciparum***

We have previously demonstrated that both NK cells and T cells can produce IFN-γ following incubation for 18–24 h with *P. falciparum*-infected RBCs (5), but others have concluded that γδT cells are the only significant source of early IFN-γ (9, 10). This discrepancy may reflect, in part, heterogeneity between human blood donors in the timing and magnitude of the response and in the source of IFN-γ (6). We sought, therefore, to characterize more systematically the early IFN-γ response to *P. falciparum*-infected RBCs (Fig. 1A–E). Lymphocytes were identified as CD3^+ T cells or as CD3^+CD56^+ NK cells (Fig. 1A). After 21 h coculture with *P. falciparum*-infected RBCs, the proportion of NK cells that contained intracellular IFN-γ ranged from 0–81% with a median of 4.65%. The proportion of T cells that were positive for IFN-γ was significantly lower, ranging from 0–7% with a median of 0.82% (Fig. 1B). However, because T cells outnumber NK cells by ~10:1 among PBMCs, when we gate on all IFN-γ^+ cells (Fig. 1C), we find that T cells and NK cells contribute approximately equally to the population of IFN-γ^+ cells (Fig. 1D). On the other hand, IFN-γ staining intensity (mean fluorescence intensity [MFI]) is significantly higher for NK cells than for T cells (p < 0.0001) (Fig. 1E), indicating that NK cells are producing more IFN-γ per cell than T cells.

To determine whether NK and T cells responded to *P. falciparum*-infected RBCs with similar kinetics, we determined the proportion of all IFN-γ^+ cells that were either NK cells or T cells after culturing for periods from 6 h to 6 d (Fig. 1F). Although there is heterogeneity in the response among donors, the distinct trend was for IFN-γ^+ cells to be almost exclusively NK cells after 6 h of coculture and for NK cells to continue to dominate the IFN-γ response at 12 h. However, by 24 h, the response was evenly split between NK cells and T cells, and T cell responses gradually came to dominate the IFN-γ response over the next 5 d. This observation, that the NK cell IFN-γ response is very rapid but transient, is not unexpected but does likely explain many of the apparent discrepancies in the literature in which innate responses tend to have been assayed at a single point in time and frequently as long as 72 h postinfection of *P. falciparum*-infected RBC stimulation.

Because potent Ag-specific T cell responses to *P. falciparum*-infected RBCs might not be expected among malaria-naïve donors, we next sought to identify which subsets of T cells were...
producing IFN-γ. PBMCs from 14 donors were incubated with *P. falciparum*-infected RBCs for 21 h and gated as NK, CD3+γδTCR+, or CD3+αβTCR+ T cells; at some key time points, αβ T cells were gated as either CD4+ or CD8+ T cells (Fig. 2A). When each lymphocyte population was separately analyzed for IFN-γ expression, we again found that, on average, a higher proportion of NK cells than T cells produce IFN-γ, but among the T cell populations, a significantly higher proportion of γδTCR+ T cells were IFN-γ+ than either of the αβTCR+ T cell subsets (Fig. 2B). However, because the majority of circulating T cells are CD4+ and αβTCR+, when we gate on all IFN-γ+ cells (Fig. 2C), we find that the majority of IFN-γ+ T cells are CD4+ αβTCR+ and that CD4+ αβTCR+ T cells and NK cells contribute equally to the total IFN-γ+ population, with CD8+ αβTCR+ and γδTCR+ cells making only a minor contribution (Fig. 2D). To make sure that we had not missed the optimal timing for responses of any particular T cell subset, we repeated the kinetic analysis over 6 d (Fig. 2E) and found that αβ T cells were the major contributing T cell population throughout the response.

**NK cell and T cell responses to P. falciparum-infected RBCs are both cytokine and MHC class II dependent**

We have previously shown that NK cell responses to *P. falciparum*-infected RBCs are absolutely dependent upon myeloid cell-derived IL-12 (12) and partially dependent upon IL-18, IFN-α, and IL-2 (7). Moreover, we, and others, have previously shown that memory CD4+ T cells from malaria-naive individuals are able to respond to *P. falciparum*-infected RBCs in a classical MHC class II-restricted manner (13–15), and it is now generally accepted that these cells have been primed by cross-reacting Ags (13, 16). To confirm and extend these observations, PBMCs from 10 malaria-naive donors (selected to represent a range of NK responses from nonresponders to high responders) were cultured with *P. falciparum*-infected RBCs for 24 h in the presence of blocking or neutralizing Abs to IL-12, IL-18, IL-2, IFN-αR, MHC class I, MHC class II, or the relevant isotype control Abs. A representative data set for one donor is shown in Fig. 3; data for all 10 donors are shown in Fig. 4. As expected, NK cell IFN-γ
FIGURE 2. IFN-γ response of NK cells and T cell subsets. PBMCs were incubated with *P. falciparum*-infected RBC for periods of 6 h to 6 d and analyzed by flow cytometry. A. Representative flow cytometry data from one donor demonstrating gating strategy for NK cells (CD56+ CD3− lymphocytes) and T cells (all CD3+ lymphocytes) (I), which are then further classified as TCR-γδ+ or TCR-αβ+ T cells (II) and then all TCR-αβ+ T cells are identified as being CD4+ or CD8+ T cells. Each population of lymphocytes can then be gated for detection of intracellular IFN-γ after 24 h coculture with *P. falciparum*-infected RBC (III, right column) or without (III, left column; GM). B. The percentage of all NK cells or all γδ+ T cells, CD4+ T cells, or CD8+ T cells expressing intracellular IFN-γ after 24 h coculture with *P. falciparum*-infected RBCs. *n* = 14 naive donors. C. Gating strategy for identification of all IFN-γ+ lymphocytes and their subsequent identification as either NK cells or γδ+ T cells, CD4+ T cells or CD8+ T cells. D. The percentage of IFN-γ+ cells that are NK cells or γδ+ T cells, CD4+ T cells or CD8+ T cells per 100,000 lymphocytes after 24 h coculture with *P. falciparum*-infected RBCs. *n* = 14 naive donors. E. The percentage of all IFN-γ+ cells that are either NK cells or T cells after 6, 12, or 24 h or 2, 4, or 6 d coculture with *P. falciparum*-infected RBCs. Numbers on x-axis identify individual blood donors. *B, D, and E*. Horizontal lines represent medians. The *p* values are for two-tailed paired Wilcoxon tests with 95% CIs comparing NK cells with T cells from the same donor. GM, growth medium.
responses were markedly and significantly diminished by anti–IL-12, anti–IL-2, and anti–IFN-αR, although the effect of anti–IL-18 was less obvious (Fig. 4 A–D). Also, in confirmation of a previous study (8), blocking Ab to MHC class I had no effect on the NK cell response (Fig. 4 E). Somewhat unexpectedly, however, NK cell responses to P. falciparum-infected RBCs were markedly reduced in the presence of MHC class II-blocking Ab (Fig. 4 F).

As expected, given the previously published data (7, 8, 12–16), the preponderance of CD4+ T cells in the IFN-γ–responding population, and the small but noticeable contribution of CD8+ T cells, total T cell IFN-γ responses were markedly diminished in the presence of anti-MHC class II Abs (Fig. 4 F) and significantly, but less markedly, diminished by MHC class I blockade (Fig. 4 E). Total T cell responses were also significantly inhibited by neutralizing Abs to IL-12, IL-18, and IL-2, and there was a marginally significant effect of anti–IFN-αR (Fig. 4 A–D), indicating that T cells are also dependent upon accessory cell-derived cytokines.

**NK cell responses to P. falciparum-infected RBCs are dependent upon T cell help**

The observation that NK cell responses to P. falciparum-infected RBCs were highly dependent upon both IL-2 and MHC class II raised the possibility that, as previously described for human NK cell responses to influenza (17, 18), NK cells require signals (such as IL-2) from Ag-specific CD4+ T cells to respond optimally to P. falciparum-infected RBCs. To test this hypothesis, we depleted PBMCs of various lymphocyte populations and tested the remaining PBMCs for their ability to make IFN-γ in 24-h cultures (Fig. 5). NK cells among CD3-depleted PBMCs made strong responses to rhIL-12 plus IL-18 (Fig. 5 A), indicating that these two cytokines (at optimal concentrations) are sufficient for NK cell activation. Conversely, NK cells among CD3-depleted PBMCs were completely unable to make IFN-γ in response to P. falciparum-infected RBCs (Fig. 5 B), but NK cells among CD20 (B cell)-depleted PBMCs made a robust IFN-γ response to P. falciparum-infected RBCs (Fig. 5 C). To determine which subset(s) of T cells provide help to NK cells, we depleted PBMCs of just CD4+ T cells, just CD8+ T cells, all αβ TCR+ T cells, or all γδ TCR+ T cells and cultured the remaining PBMCs with P. falciparum-infected RBCs for 24 h (Fig. 5 D–G). It was clear that the NK cell response to P. falciparum-infected RBCs was only completely ablated when all T cells were removed but that removal of any T cell subset significantly reduced the NK cell IFN-γ response. Finally, to rule out any nonspecific effects of magnetic bead treatment, unlabeled PBMCs were incubated with anti-PE
IL-2 production in response to \textit{P. falciparum}-infected RBCs and to identify the major source(s) of this cytokine (Fig. 6). IL-2 was detected in culture supernatants within 3 h of PBMC-\textit{P. falciparum}-infected RBC coculture, peaked at 4 h, and remained detectable for at least 24 h (Fig. 6A). In parallel cultures, intracellular IL-2 in NK cells and T cell subsets was analyzed by flow cytometry (Fig. 6B–D). All IL-2+ cells were gated (Fig. 6B) and counted (Fig. 6C). IFN-γ+ cells were first detected at 3 h postinitiation of coculture, and their numbers peaked at 12 h; the discrepancy in timing between the accumulation of soluble IL-2 in culture medium (Fig. 6A) and the peak of IL-2–secreting cells (Fig. 6C) presumably reflects the balance between secretion and consumption of IL-2 in the culture. At all time points, the vast majority (>80%) of IL-2–producing cells were CD4+ T cells with only minor contributions from CD8+ T cells at 6 and 12 h and from γδT cells and NK cells at 3 h (Fig. 6D). Interestingly, IL-2 responses were much less heterogeneous among the various donors than were IFN-γ responses.

γδ T cells produce TNF in response to \textit{P. falciparum}-infected RBCs, but TNF is not required for NK cell or T cell IFN-γ responses

Because γδ T cells seemed to be necessary for an optimal NK response to \textit{P. falciparum}-infected RBCs (Fig. 5) but did not seem to be significant producers of IL-2 (Fig. 6), we considered whether other cytokines produced by γδ T cells might contribute to NK cell activation. As Hensmann et al. (10) have previously shown that γδ T cells represent a significant source of intracellular TNF during the early (18 h) response to \textit{P. falciparum}-infected RBCs, and as Robinson et al. (19) concluded (from T cell subset depletion assays) that γδ T cells are a significant source of TNF, we considered the possibility that γδ T cell-derived TNF might potentiate NK cell responses. TNF was detected in supernatants within 4 h of PBMC-\textit{P. falciparum}-infected RBC coculture and peaked at ∼12 h (Fig. 7A). In parallel, PBMCs from eight malaria-naive donors were incubated with \textit{P. falciparum}-infected RBCs for up to 24 h and then stained for intracellular TNF and surface markers to identify macrophages (CD14+ CD68+) and γδ T cells (Fig. 7B). When gating on all macrophages (Fig. 7D), TNF+ macrophages were detectable within 2 h of \textit{P. falciparum}-infected RBC coculture, the response peaking (with >35% of all macrophages being TNF+) at 4 h and declining to barely detectable levels by 24 h. In comparison, TNF+ γδ T cells were detected from ∼5 to 6 h postinitiation of \textit{P. falciparum}-infected RBC stimulation, and the response was maximal (with ∼30% of all γδ T cells being TNF+) at 12–24 h. However, we found no evidence that TNF was required for induction of an early IFN-γ response, because when we stimulated PBMCs from the same donors with \textit{P. falciparum}-infected RBCs in the presence of neutralizing Abs to TNF, the NK cell and T cell IFN-γ responses were unaffected (Fig. 7E).

\textit{T cell help for NK cell activation by \textit{P. falciparum}-infected RBCs does not require NK cell-T cell contact}

We have shown that T cell-derived signals are required for NK cell activation by \textit{P. falciparum}-infected RBCs. One of these signals is clearly IL-2, emanating principally from CD4+ αβ T cells, but T cell depletion studies suggest that there may also be an as yet undefined role for γδ T cells in NK cell activation. We have previously shown that direct contact between NK cells and myeloid accessory cells is required for optimal NK cell responses to \textit{P. falciparum}-infected RBCs (7), and we therefore considered the possibility that T cells might also provide contact-dependent signals to NK cells (Fig. 8). Intact PBMCs and CD3-depleted PBMCs were cultured in separate compartments of a transwell plate separated by a semipermeable membrane; intact PBMCs in the lower chamber

\textbf{FIGURE 4.} NK cell and T cell responses to \textit{P. falciparum}-infected RBCs are both cytokine and MHC class II dependent. PBMCs from 10 donors were cultured with \textit{P. falciparum}-infected RBCs for 24 h in the presence of blocking Abs to cytokines, cytokine receptors, MHC molecules, or with isotype-matched control Abs and the percentages of NK cells (\textit{left columns}) and T cells (\textit{right columns}) expressing intracellular IFN-γ determined by flow cytometry. The \(p\) values are for two-tailed paired Wilcoxon test with 95% CI. A, Anti–IL-12. B, Anti–IL-18. C, Anti–IFN-γ. D, Anti–IFN-αR. E, Anti–MHC class I. F, Anti–MHC class II.

\textbf{CD4+ T cells produce IL-2 within 12 h of coculture with \textit{P. falciparum}-infected RBCs}

Because NK cells require both T cell help and IL-2 to respond to \textit{P. falciparum}-infected RBCs, it seemed likely that T cells are an essential source of IL-2. We sought to determine the kinetics of microbeads and passed through the magnetic column (Fig. 5H); a robust IFN-γ response was seen.

\textbf{NK cells require both T cell help and IL-2 to respond to \textit{P. falciparum}-infected RBCs}

We have shown that T cell-derived signals are required for NK cell activation by \textit{P. falciparum}-infected RBCs. One of these signals is clearly IL-2, emanating principally from CD4+ αβ T cells, but T cell depletion studies suggest that there may also be an as yet undefined role for γδ T cells in NK cell activation. We have previously shown that direct contact between NK cells and myeloid accessory cells is required for optimal NK cell responses to \textit{P. falciparum}-infected RBCs (7), and we therefore considered the possibility that T cells might also provide contact-dependent signals to NK cells (Fig. 8). Intact PBMCs and CD3-depleted PBMCs were cultured in separate compartments of a transwell plate separated by a semipermeable membrane; intact PBMCs in the lower chamber
and CD3-depleted PBMCs in the upper chamber. When *P. falciparum*-infected RBCs were added to the CD3-depleted (upper) PBMC compartment but not to the intact PBMCs (lower compartment), NK cells in neither compartment produced IFN-γ, suggesting that *P. falciparum*-infected RBCs need to be in the same compartment as the T cells in order for NK cells to become activated, consistent with a need for APCs to take up Ag and present it to T cells in an MHC class II-dependent manner. However, if *P. falciparum*-infected RBCs were added to both compartments, NK cells in both the intact PBMC population and in the CD3-depleted NK cell population were able to respond fully, indicating that soluble T cell-derived signals passing through the semipermeable membrane were sufficient to activate the NK cells. To determine if this soluble signal is IL-2, we added neutralizing anti–IL-2 Ab to the CD3-depleted PBMCs in the upper chamber and added *P. falciparum*-infected RBCs to both compartments. In this case, the NK cell IFN-γ response was significantly inhibited in both compartments.

**Discussion**

Although there is a general consensus that a rapid, cell-mediated, inflammatory response is required to contain the initial stages of human blood-stage malaria infections, there has been considerable debate over many years as to the key cellular effectors of this response (2). We have therefore conducted a detailed kinetic analysis of all the likely sources of IFN-γ and find that the very early (<24 h) IFN-γ response to *P. falciparum*-infected RBCs is dominated, in most PBMC donors, by NK cells, that NK cells and T cells contribute more or less equally to the response at 24 h, and that T cells come to dominate the response from 48 h onward. This rapid NK cell response to *P. falciparum*-infected RBCs among PBMCs from

**FIGURE 5.** NK cell responses to *P. falciparum*-infected RBCs are dependent upon CD4+ T cell help. Intact PBMCs or PBMCs depleted of B cells or various T cell subsets were incubated for 24 h with 100 ng/ml each of rhIL-12 plus rhIL-18 (A) or with *P. falciparum*-infected RBC (B–H), and the percentages of NK cells staining for intracellular IFN-γ were measured by flow cytometry. PBMC were depleted of all CD3+ cells (A, B), CD20+ B cells (C), αβ T cells (D), CD4+ T cells (E), CD8+ T cells (F), or γδ T cells (G), or incubated with anti-PE microbeads (without Ab) (H) and passed through the magnetic column. The p values are for two-tailed paired Wilcoxon tests, 95% CI, for 15 donors.

**FIGURE 6.** Rapid IL-2 production by CD4+ T cells after coculture with *P. falciparum*-infected RBCs. PBMCs were incubated with *P. falciparum*-infected RBCs for up to 24 h. A, Median concentration of IL-2 in culture supernatants, determined by cytometric bead array, over time. n = 5 donors. B, Gating strategy for identification and phenotypic characterization of IL-2–producing cells. C, The total number of IL-2+ lymphocytes at each time point. n = 10 donors. D, The proportion of all IL-2+ lymphocytes that are either NK cells or are γδ+ T cells, CD4+ T cells, or CD8+ T cells when comparing responses over 24 h. Data are presented for 10 donors at all time points except at 12 h for which data from 14 donors are shown. Horizontal lines indicate median values.
malaria-naive donors is in full agreement with both of our own earlier studies (5) and others (20). D’Ombrain et al. (9) concluded that NK cells do not contribute significantly to the innate IFN-γ response to P. falciparum-infected RBCs; however, their studies did not include the very early time points (12–18 h) at which we observe maximal NK cell responses, suggesting that an NK response may have been present in their donors but was missed, although it is surprising that D’Ombrain et al. (9) report so few IFN-γ+ NK cells at 24 h. Although Hensmann et al. (10) stained for CD3, CD56, and IFN-γ after 18 h coculture with P. falciparum-infected RBCs, they studied only four donors and did not explicitly report the NK cell IFN-γ response; from the data presented in their paper, it is possible that between 10% and 40% of IFN-γ+ cells in their donors could have been NK cells, which is well within the range that we have observed in our much larger donor panel.

More problematic is our observation that the vast majority of IFN-γ-producing T cells (at all time points from 6 h to 6 d of P. falciparum-infected RBC coculture) are TCR αβ+ and CD4+; this finding is in direct contradiction to the findings of both Hensmann et al. (10) and D’Ombrain et al. (9), who suggested that γδ+ T cells are the major producers of IFN-γ in response to P. falciparum-infected RBC stimulation. The reason for these conflicting results is unclear. It is possible that there are technical explanations. The three studies used different P. falciparum clones, which might differ in their production of the phosphorylated nonpeptidic Ags, which are believed to be the ligands for Vγ9Vδ2 T cells (21), although our observations are consistent with the production of IFN-γ in response to P. falciparum-infected RBC stimulation. We have previously observed that γδ T cells require quite considerable amounts of IL-2 to proliferate in response to P. falciparum-infected RBCs (22); we do not routinely add exogenous IL-2 or other costimulatory agents to our cultures, which might explain the lack of γδ T cell IFN-γ responses in our experiments, but again the γδ T cell TNF response suggests that this is not the explanation.
Lastly, we have taken great care to avoid misclassification of lymphocyte populations by 1) using simultaneous seven-color analysis of CD3, CD56, αβTCR, γδTCR, CD4, CD8, and IFN-γ expression; 2) inclusion of anti-CD3 in the intracellular mix to identify activated T cells that may have downregulated surface expression of CD3; and 3) using a biotinylated anti-γδTCR Ab that does not suffer from the tendency that we have observed of some supposedly γδTCR-specific Abs to stain both CD3+ and αβ TCR+ cells. We note with some concern that the majority of the IFN-γ+ γδTCR+ cells in the study by D’Ombrain et al. (9) also express NK cell markers, raising interesting questions as to the true identity of these cells. Alternatively, the differences seen in the various studies may reflect genuine heterogeneity in γδT cell responses among individuals or populations, reflecting the known inter- and intrapopulation diversity in numbers and TCR repertoires of γδ T cells (23).

The strong αβTCR+ CD4+ T cell IFN-γ response that we observed after 2–6 d of P. falciparum-infected RBC activation is highly consistent with previous reports in naive humans (13, 14, 24), with evidence for cross-reactive priming of malaria-reactive T cells by a diverse array of commonly encountered micro-organisms (13) and with data from murine infection models, indicating that αβ T cells can play a crucial role in the IFN-γ response to primary infections (25–27). It is difficult to predict whether such cross-reactive responses would be beneficial should these individuals become infected with malaria; naive adults show very variable responses to primary malaria infections with some making very rapid, and perfect responses to malaria; naive adults show very variable responses to primary infections (25–27). It is difficult to predict whether such cross-reactive responses would be beneficial should these individuals become infected with malaria; naive adults show very variable responses to primary malaria infections with some making very rapid, and perfect responses to malaria; naive adults show very variable responses to primary malaria infections with some making very rapid, and partial protective, proinflammatory responses, whereas others make little or no inflammatory response and develop rapidly escalating parasitemia (28, 29).

A somewhat unexpected finding of this study was the absolute dependency of NK cells on CD4+ T cell-derived IL-2 for their response to P. falciparum-infected RBCs. In hindsight, this should not have been a surprise. We have previously shown that NK cell responses to P. falciparum-infected RBCs depend upon cytokine and contact-mediated signals from myeloid cells (7), and we did notice in those experiments that NK responses were diminished by anti–IL-2 and that adding purified adherent accessory cells to isolated NK cells did not fully restore the NK response seen in intact PBMCs (7). Moreover, T cell and IL-2 dependency has previously been reported for NK cell activation by influenza virus (18), and, in mice, NK cell cytotoxicity against some tumor cells is αβTCR+ T cell dependent (30, 31), but the possibility that this is IL-2 mediated remains to be formally tested. However, although it has long been taken for granted that Ag-specific T cells mediate some of their antimicrobial effects by activation of innate cells, such as macrophages, the possibility that Ag-specific T cells might interact with other cells of the innate response, in particular with NK cells, has largely been ignored.

Our current hypothesis is that, on exposure to cognate Ag presented by MHC class II, TCR αβ T cells secrete IL-2, which, in combination with essential signals from myeloid accessory cells, activates NK cells. The preponderance of CD4+ T cells among the IL-2+ cell population is consistent with the very marked effect on the NK cell response of MHC class II blockade. The transwell experiments indicate that the IL-2 is secreted from the T cells and diffuses in solution toward the NK cells; there is no apparent requirement for contact between the T cell and the NK cell or for the focal secretion of IL-2 into an immune synapse. However, it is likely that T cells also provide other NK cell-potentiating signals. For example, although we found no evidence that P. falciparum-infected RBC-activated γδ T cells produced significant amounts of IL-2, depletion of γδ T cells from PBMCs substantially reduced the NK cell IFN-γ response. We do not believe that this is simply due to a reduction in the proportion of T cells in the cultures because γδ T cells represent a very small proportion of the total T cell pool and an equally small proportion of the IL-2+ T cells. We have not yet identified the γδ T cell contribution to the NK response, but it does not appear to be TNF.

Our data suggest that full reactivation of IFN-γ-producing T cells also requires IL-2–mediated signals because the peak of the T cell IL-2 response preceded the peak of the T cell IFN-γ response, and neutralization of IL-2 markedly reduced IFN-γ responses of T cells as well as NK cells. However, NK cell IFN-γ responses peaked within 6 h of the peak IL-2 response, whereas T cell IFN-γ responses did not peak until ∼36 h after the peak IL-2 response, suggesting that T cells may require higher concentrations of IL-2 or more sustained IL-2 signaling to become fully activated than do NK cells.

Our observations imply that enhancing T cell responses to malaria (i.e., by vaccination) should also enhance the NK cell response. Given its speed (which precedes the bulk of the T cell IFN-γ response by hours or days), the NK IFN-γ response may represent an important determinant of vaccine efficacy, and the ability of vaccine-induced T cells to support NK cell effector function might be an important biomarker of an effective T cell response to the vaccine. Finally, our demonstration that NK cell responses to P. falciparum-infected RBCs are so highly dependent on T cell IL-2 may necessitate reconsideration of data based simply on T cell depletion of PBMCs; the possibility that the IFN-γ–producing cells might be NK cells, and that the main role of T cells might be to secrete IL-2, needs to be considered, and IFN-γ ELISA, cytometric bead array, or ELISPOT data need to be confirmed by intracellular cytokine staining and flow cytometry.

Acknowledgments

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Disclosures

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References


9. D’Ombrain, M. C., D. S. Hansen, K. M. Simpson, and L. Schofield. 2007. Heterogeneous human NK cell responses to malaria (i.e., by vaccination) should also enhance the NK cell response. Given its speed (which precedes the bulk of the T cell IFN-γ response by hours or days), the NK IFN-γ response may represent an important determinant of vaccine efficacy, and the ability of vaccine-induced T cells to support NK cell effector function might be an important biomarker of an effective T cell response to the vaccine. Finally, our demonstration that NK cell responses to P. falciparum-infected RBCs are so highly dependent on T cell IL-2 may necessitate reconsideration of data based simply on T cell depletion of PBMCs; the possibility that the IFN-γ–producing cells might be NK cells, and that the main role of T cells might be to secrete IL-2, needs to be considered, and IFN-γ ELISA, cytometric bead array, or ELISPOT data need to be confirmed by intracellular cytokine staining and flow cytometry.

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