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Activation of a Subset of Human NK Cells upon Contact with \textit{Plasmodium falciparum}-Infected Erythrocytes\textsuperscript{1}

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Human NK cells are the earliest source of the protective cytokine IFN-\(\gamma\) when PBMC from nonimmune donors are exposed to \textit{Plasmodium falciparum}-infected RBC (iRBC) in vitro. In this study, we show that human NK cells form stable conjugates with iRBC but not with uninfected RBC and that induction of IFN-\(\gamma\) synthesis is dependent on direct contact between the NK cell and the iRBC. NK cells respond to iRBC only in the presence of a source of IL-12/IL-18 and the subset of NK cells that preferentially respond to iRBC express high levels of the lectin-like receptor CD94/NKG2A. There is heterogeneity between donors in their ability to respond to iRBC. DNA analysis has revealed considerable heterogeneity of killer Ig-like receptor (KIR) genotype among the donor population and has identified 21 new KIR allelic variants in the donors of African and Asian descent. Importantly, we find evidence for significant associations between KIR genotype and NK responsiveness to iRBC. This emphasizes the need for large-scale population-based studies to address associations between KIR genotype and susceptibility to malaria. \textit{The Journal of Immunology}, 2003, 171: 5396–5405.

Repeated or prolonged exposure to malaria infection in humans eventually leads to the development of clinical immunity such that, despite remaining susceptible to infection, parasite replication is controlled and the infection is eliminated without the development of classical signs and symptoms. It is now generally accepted that acute infections are controlled by cellular immune mechanisms, possibly augmented by cytolytic Abs, which are absolutely dependent on the macrophage-activating cytokine IFN-\(\gamma\) (reviewed in Ref. 1). Cells of the adaptive immune response, primed either by previous malaria infection or by exposure to cross-reacting Ags, constitute an important source of IFN-\(\gamma\) (reviewed in Ref. 2). In addition, IFN-\(\gamma\) is also produced by cells of the innate immune system, notably NK cells, following exposure to malaria-infected RBC (iRBC)\textsuperscript{3} (3). Importantly, evidence from experimental infections shows that parasite growth can be modulated very early during primary human malaria infections (4), suggesting that innate immune responses may contribute to their control.

NK cells represent an important early source of IFN-\(\gamma\) during primary murine malaria infections and NK depletion leads to a more rapid increase in parasitemia and higher mortality (5, 6). Activation of NK cells, measured as enhanced lytic activity against the NK-sensitive cell line K562, has been reported in children with acute \textit{Plasmodium falciparum} infections (7) and NK cells derived from Kenyan adults are reportedly able to lyse \textit{P. falciparum} iRBC (8). We have previously shown (3) that NK cells are the earliest producers of IFN-\(\gamma\), with numbers of IFN-\(\gamma\)\textsuperscript{+} NK cells peaking between 15 and 24 h after stimulation of human PBMC with live \textit{P. falciparum}-infected erythrocytes.

In many infections, NK activation appears to occur principally in a bystander fashion, i.e., in response to production of cytokines such as IL-12 and IL-18 from monocyte-macrophages and dendritic cells (9). Although activation of human NK cells by \textit{P. falciparum} iRBC (as assessed by IFN-\(\gamma\) production) is partially dependent on IL-12 and IL-18, heterogeneity in the magnitude of the NK response among different donors cannot be explained by a lack of either IL-12 or IL-18 (3). Taking these observations together with previous reports of direct lysis of iRBC by human NK cells (8), we hypothesized that signaling via specific NK receptors might also be required for optimal activation of NK cells to produce IFN-\(\gamma\).

NK cells express both activating and inhibitory receptors. Nonpolymorphic NK receptors include the inhibitory C-type lectin CD94/NKG2 heterodimers which recognize HLA-E (10), activating NKG2D homodimers which bind stress-inducible ligands (11, 12), and activating Ig-like natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46 (13). The natural ligands for NCRs are not well defined, although recent research suggests that NKp30 may interact with ligands on dendritic cells and that NKp46 binds viral hemagglutinins (14, 15). The known ligands for the highly polymorphic killer Ig-like receptors (KIR) are all HLA class I molecules; however, it has been suggested that activating KIR, which interact with the immune tyrosine-based activating motif-containing adaptor signaling molecule DAP12 rather than carrying the inhibitory immune tyrosine-based inhibitory motif in their intracellular domain, may have evolved to recognize pathogen-specific ligands (16, 17).

In this study, we show that direct contact between NK cells and parasitized erythrocytes does occur and that it is necessary for
induction of IFN-γ synthesis. NK activation is accompanied by enhanced expression of specific regulatory receptors, indicating direct recognition of the iRBC by NK cell receptors. Furthermore, the cell donors were extremely heterogeneous both with respect to their NK response to malaria and their KIR repertoire, and a significant association was observed between the NK-iRBC response and KIR genotype. These findings emphasize the need for more extensive examination of KIR genotypes in malaria-exposed populations to explore associations among KIR genotype, NK responses to malaria, and susceptibility to malaria.

Materials and Methods

P. falciparum

P. falciparum parasites of the 3D7 strain were cultured in vitro as described previously (3). Cultures were routinely screened for Mycoplasma contamination by PCR (BioWhittaker, Wokingham, U.K.) and shown to be Mycoplasma free. Mature schizont-infected erythrocytes (iRBC) were harvested by centrifugation through 60% Percoll (Sigma-Aldrich, Poole, U.K.), washed, and resuspended in culture medium. Washed uninfected erythrocytes (uRBC) were used as controls.

PBMC

Adult blood donors were recruited at the London School of Hygiene and Tropical Medicine through a volunteer blood donation system. The study was approved by the London School of Hygiene and Tropical Medicine Ethical Review Committee, and informed consent was obtained from all volunteers. PBMC were separated from heparinized venous blood on a Histopaque (Sigma-Aldrich) density gradient, washed twice in RPMI 1640, and resuspended at a concentration of 1 × 10^6 cells/ml in complete culture medium (RPMI 1640, 10% autologous plasma, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen, San Diego, CA)). Schizont-infected (iRBC) or uninfected (uRBC) erythrocytes were added to PBMC at a 3:1 ratio. PHA (1 μg/ml) was used as a positive control, as described previously (3). Cultures were incubated at 37°C in 5% CO₂ for 24 h. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added to cells for the last 3 h of the incubation.

Cell surface and intracellular staining for flow cytometry

Cells (5 × 10^5/well) were stained for surface markers and intracellular IFN-γ as described previously (3), except that for uninfected Abs (anti-NKp30, NKp44, and NKp46, and NKGD2) where cells were stained first with uninfected Ab (1 μg/100 μl of cell suspension), then with rabbit anti-mouse PE-labeled IgG1 (1 μg/100 μl of cell suspension; Sigma-Aldrich) and then stained with fluorochrome-conjugated Abs for surface CD56 and CD3 and intracellular IFN-γ as described. Isotype-matched control Abs were used for all fluorochrome-isotype combinations. The Abs used were: CD3-Tricolor, IgG1-PE, IgG2a-PE, IgG2b-PE (all from Caltag Laboratories, Towcester, U.K.); CD3-PerCP, CD56-FITC, CD56-allophycocyanin, IFN-γ-FITC, IFN-γ-PE, NK1-PE, KIR-NKAT2-PE, IgG1- FITC, IgG1-allophycocyanin, IgG1-PerCP (all from BD Biosciences, Oxford, U.K.); CD94-PE, NKGA2-PE, KARp50-3-Pe, CD158b-PE, KIRp70-PE (all from Coulter Immunotech, Marseille, France); NKGD2 (R&D Systems, Abingdon, U.K.); and NKp30, NKp44, and NKp46 (kind gifts from A. Moretta, Genoa, Italy). Stained cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences) collecting a total of 100,000 events. Data were analyzed with CellQuest software (BD Biosciences).

Cytokine ELISA

IFN-γ was detected in cell supernatants by sandwich ELISA using commercially available Abs and standards (BD Biosciences). All samples were tested in duplicate according to the manufacturer’s recommendations. 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.

Neutralization and supplementation of IL-12 and IL-18

Neutralizing goat anti-human IL-12 polyclonal Ab and mouse anti-human IL-18 polyclonal Ab (both from R&D Systems) were added in combination to PBMC stimulated with iRBC or uRBC at 0.5 and 5.0 μg/ml, respectively (optimal cytokine concentrations were determined by titration) (3). Isotype-matched control Abs (goat IgG (Sigma-Aldrich) and mouse IgG1 (R&D Systems) were used in parallel at the same concentrations. Subsequently, PBMC cultures stimulated with iRBC or uRBC were supplemented with recombinant human IL-12 and IL-18 (PeproTech, Rocky Hill, NJ) at concentrations ranging from 0.1 to 10 ng/ml. All cultures were incubated for 24 h before analysis by flow cytometry.

Generation of human NK cell clones

PBMC, from which NK cells were to be derived, were cultured for 1 wk with irradiated 721.221 cells (20,000 rad) in a 4:3 ratio in RPMI 1640 medium containing 10% human serum (AB; Sigma-Aldrich), 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 50 U/ml penicillin-streptomycin, and 50 μM 2-ME (all from Invitrogen). The cultured PBMC were stained with FITC-conjugated anti-CD3 mAb (HIT3a; BD PharMingen, Cowley, U.K.) and CD3+ cells were sorted by FACS to be one cell per well of a 96-well plate containing irradiated 10^3 allogeneic PBMC (6,000 rad) and 5 × 10^5 irradiated RPMI8866 cells (12,000 rad) in DMEM supplemented with 10% human serum, 30% Nutrient Mixture F-12 (Ham’s), 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 50 U/ml penicillin-streptomycin, 50 μM 2-ME, 100 U/ml human IL-2 (Roche, Basel, Switzerland) (referred to hereafter as DMEM plus supplements), and 1 μg/ml PHA (Sigma-Aldrich). Ninety-six-well plates containing the irradiated cells had been prepared on the previous day and incubated overnight at 37°C in 5% CO₂.

NK clones that had undergone expansion were split every 4–5 days in 1:1 ratio in DMEM plus supplements and were periodically monitored to be CD3+ and CD56+ by flow cytometry. Several NK clones from the same donor were mixed together to achieve a polyclonal line of pure NK cells that retained the ability to respond to iRBC and to ensure that sufficient numbers of cells from the same population were available for assay. Assays for responsiveness to iRBC were performed at least 4 days after restimulation with fresh IL-2, i.e., on “resting” NK cells.

Testing the response of purified NK cells to iRBC

NK lines were stained with the fluorescent cytoplasmic dye Cell Tracker Green (0.5 μM; Molecular Probes, Eugene, OR) for 1 h at 37°C and then incubated in 10% autologous plasma to absorb any unbound reagent. Subsequently, the NK cell lines were incubated for 24 h with autologous PBMC and iRBC or uRBC at a 1:3 ratio. Cells were then stained with CD56-FITC, CD3-Tricolor, and IFN-γ-PE and analyzed by flow cytometry as described.

Detecting erythrocyte:NK cell conjugates

PBMC (1 × 10^6 cells/ml) were incubated with 3 × 10^5/ml iRBC or uRBC for up to 3 h. Cultures were centrifuged for 1 min at 500 × g to bring cells into contact and assayed at 30-min intervals to assess conjugate formation. Cells were fixed in 4% paraformaldehyde to retain cell attachment and stained with anti-CD56-FITC and anti-CD3-Tricolor, to identify NK cells, and PE-labeled anti-human glycoporphin A (BD PharMingen), which stains only erythrocytes. NK-iRBC conjugates were scored as flow cytometry events that were CD3- and positive for CD56 and glycoporphin A. The formation of NK cell-RBC conjugates was confirmed by microscopic examination of cytospin preparations.

Transwell experiments

To assess the need for iRBC-NK contact for the induction of IFN-γ synthesis, NK IFN-γ production was compared between PBMC cultured in direct contact with iRBC or uRBC and PBMC separated from the erythrocytes by a semipermeable membrane. The 0.4-μm polycarbonate membrane (Corning, Buckinghamshire, U.K.) prevented intact RBC or free merozoites from passing from one chamber to the other but allowed free passage of soluble parasite products and monokines. PBMC (10^6 cells/ml) were placed into both the inner and outer chambers of each well of a flat-bottom 24-well tissue culture plate containing the Transwell insert. Stimulants (3 × 10^5 iRBC, 3 × 10^5 uRBC, or 1 μg/ml PHA) were added only to the outer (lower) chamber of the Transwell plate. After a 24-h incubation, PBMC were harvested separately from the inner and outer chambers, stained with anti-CD3, anti-CD56, and anti-IFN-γ Abs, and analyzed by flow cytometry.

Genomic DNA preparation and KIR genotyping

Genomic DNA was prepared from 1 × 10^5 to 2 × 10^6 PBMC using a QIAamp DNA Blood Mini kit (Qiagen, Valencia, CA), or from the RBC/granulocyte pellet following Ficoll-Paque Plus gradient separation (American Biosciences, Pisacatway, NJ) of 50 ml of anticoagulated whole blood. Generic KIR typing of genomic DNA was performed by PCR amplification with primers based on conserved regions specific to each KIR.
locus (18). For KIR allele typing, primers designed to discriminate allelenspecific polymorphisms were paired with KIR2DL1, 2DL3, 3DL1, or 3DL2 locus-specific primers, as previously described (19, 20).

RNA and cDNA preparation

Total cellular RNA was prepared from PBMC using RNA-Beet (Tel-Test, Friendswood, TX) according to the manufacturer’s guidelines, and 1 μg was used to synthesize 5′RACE cDNA using oligo(dT)18 primer and SMART II oligonucleotide primer (SMART-RACE kit; Clontech Laboratories, Palo Alto, CA).

Cloning and sequencing of new KIR alleles

The 5′RACE cDNA was amplified with the Advantage 2 PCR kit (Clontech Laboratories) using 100 μM of the universal primer mix (UPM) from the SMART-RACE kit (Clontech Laboratories) as the forward primer, and 100 μM of one of the following reverse primers: KIRc (21); R2/DS, 5′-CCTGACTGTGGTGCTCGTGGACAG A-3′; KIR2DS3/5′-TGTTCTTGGCCTTGAGAGGGG-3′; KIR2DS1/2′ specific, 5′-TG GTTCTTTGATCGAATTCTGG-3′; or KIR2DL5 specific, 5′-CCAGCT GCCTGCGATCGAAGCTA-3′. The reverse primer KIRc anneals with a segment of the sequence in the 3′ untranslated region that is conserved in all known KIR sequences, while R2/DS anneals to a sequence conserved only in the activating KIR. The remaining reverse primers are specific for different combinations of KIR genes, e.g., the KIR2DS3/5′-specific primer anneals to a segment of the sequence that is conserved in only KIR2DS3 and 2DL5. To amplify specific activating KIR genes from the cDNA, in several instances the forward UPM primer was replaced with the primers KIR2DS3/5′/2DL5-5′-CAGCACCAT GTGCGTCTGG CA-3′ or KIR2DS2-specific 5′-ATGTCGGTTAGTGGCAGCATG-3′. The UPM-KIRC primers were expected to give a KIR product of 1.6 or 1.9 kb for either 2-domain (2D) or 3-domain (3D) KIR, respectively, while the UPM-R2/DS primers produced either 1.3-kb (2D) or 1.6-kb (3D) KIR products. KIR products generated using combinations of the other KIR-specific primers were ~1.3 kb in size. The PCR protocol included an initial denaturation of 95 °C for 60 s, followed by 5 cycles of 94 °C for 20 s, 70 °C for 45 s, 72 °C for 210 s, and by 30 cycles of 94 °C for 20 s, 65 °C for 30 s, 72 °C for 210 s. A final 10-min incubation at 72 °C was performed to complete DNA synthesis. In the cases where two KIR-specific primers were used for the cDNA amplification, PCR conditions were as follows: 95 °C for 60 s, followed by 35 cycles of 94 °C for 20 s, 70 °C for 30 s, 72 °C for 210 s, followed by a final 10-min extension at 72 °C.

PCR products were gel purified using the Bio-RadQuantum Prep Freeze and Squeeze Gel Extraction Spin Columns (Bio-Rad, Hercules, CA) and subcloned using a TOPO- TA Cloning kit according to the manufacturer’s instructions (Invitrogen). The resulting products were partially sequenced using the primer NKrds, 5′-CGA AGT TCT ATG TAG ATG TG-3′ or NKR-2ds, 5′-CTG TTC TCT GTT CGT TTT CC-3′ and dye terminator automated sequencing (Applied Biosystems, Foster City, CA). Based on partial sequences, three to four representatives of each allele were selected and sequenced completely on both strands to obtain a consensus sequence. The sequence of each new allele was confirmed in genomic DNA by PCR amplification or by sequencing. Each allele (see Table II) has been named according to the conventions recently proposed by the KIR Nomenclature Committee (22).

Results

IL-12 and IL-18 are necessary but not sufficient for NK cell IFN-γ responses to P. falciparum

To determine the roles of IL-12 and IL-18 in NK cell activation by P. falciparum, both cytokines were simultaneously neutralized in PBMC/iRBC cultures from two known high responding donors and two known low responding donors (Fig. 1A). In the low responders (KAT and TH), the few NK cells that produced IFN-γ in response to iRBC were inhibited in the presence of anti-IL-12 and anti-IL-18, indicating that this response is likely due to bystander activation. For the two high responders (FMO and JET), NK IFN-γ responses were markedly reduced, although not entirely eliminated, in the presence of anti-IL-12 and anti-IL-18. However, rIL-12 and IL-18 added in combination at concentrations from 0.1 to 10 ng/ml are not sufficient for induction or enhancement of NK IFN-γ responses among PBMC from high (JET) or low/nonresponding donors (Bds, TH, and CS, in Fig. 1B) and did not induce IFN-γ secretion from other cell populations (as shown by the lack of IFN-γ in culture supernatants, as determined by ELISA, Fig. 1C). Thus, other factors must contribute to NK activation by iRBC.

NK cells can form stable conjugates with parasitized erythrocytes

Having shown that monokines are necessary but not sufficient for NK activation by iRBC, we hypothesized that a second, contact-dependent signal was required. To determine whether NK cells can bind to parasitized erythrocytes and form stable conjugates, PBMC were incubated with iRBC or uRBC, fixed, and then stained with anti-CD56, anti-CD3, and anti-glycophorin A. The mean fluorescence intensity for all glycoporphin A+ cells (i.e., all RBC) was 445 (Fig. 2C). A gate was set around all CD56+ cells and the number of events that were CD3+ (i.e., NK cells) and glycoporphin A+ (i.e., had bound RBC) was counted (Fig. 2, D and E); the MFI for RBC bound to NK cells was also 445 (Fig. 2F), indicating that NK cells had bound intact RBC. The formation of NK cell-RBC conjugates was confirmed by cytoplasmic analysis and light microscopy; individual conjugates typically contained a single RBC attached to one NK cell but occasionally up to four or five RBC were seen attached to one NK cell.

The kinetics of conjugate formation were investigated for several donors (an example is shown in Fig. 2G) by calculating the percentage of all NK cells that were conjugated to erythrocytes at each time point. Conjugates could be detected as early as 30 min after coincubation with iRBC and persisted for up to 120 min; the optimal time for detection of conjugates was 90 min (Fig. 2G). A low level of conjugate formation was detected with uRBC and this was consistent over time, suggesting ongoing transient interactions between NK cells and uRBC which does not lead to activation or IFN-γ production.
Conjugate formation with iRBC and uRBC at 90 min was determined for cells from seven donors (Fig. 2H). Although there was heterogeneity among donors in the percentage of NK cells that formed conjugates, NK cells consistently formed more conjugates with iRBC than with uRBC and this difference was highly statistically significant (Wilcoxon signed rank test, $z = 2.66, n = 9, p = 0.007$).

In view of reports of differing function between CD56$^{\text{bright}}$ and CD56$^{\text{dim}}$ NK cell subsets, we analyzed the two populations separately. The majority of the NK cells were CD56$^{\text{dim}}$ but a distinct subpopulation was CD56$^{\text{bright}}$ (see Fig. 2D). The percentage of CD56$^{\text{bright}}$ NK cells forming conjugates was consistently higher than the percentage of CD56$^{\text{dim}}$ NK cells forming conjugates (mean 18.9% vs 14.9%) and this difference was statistically significant (paired $t$ test, $t = 2.45, p = 0.03$); however, the difference is small and unlikely to be biologically relevant.

Contact between NK cells and iRBC is necessary but not sufficient for NK cell IFN-$\gamma$ responses to $P.$ falciparum

Having shown that IL-12 and IL-18 are not sufficient for optimal NK cell activation and that NK cells form stable conjugates with iRBC, we hypothesized that contact between NK cells and iRBC was essential for full activation to IFN-$\gamma$ production. Thus, PBMC were incubated with iRBC or uRBC using a Transwell system which allowed for the simultaneous assaying of IFN-$\gamma$ production by NK cells exposed to all parasite-derived or parasite-induced products, including intact iRBC, and NK cells exposed only to soluble parasite components and cytokines derived from cells in the PBMC preparation. Cells from six donors were tested (Fig. 3); cells from the inner (no contact) and the outer (contact) wells were harvested and stained for CD56, CD3, and IFN-$\gamma$. PHA was used as a positive control; uRBC and unstimulated cultures were used as negative controls.

Cells from one of the six donors did not respond to iRBC but made a strong response to the positive control stimulus PHA (Fig. 3C). Cells from the other five donors responded to PHA and iRBC but the percentage of NK cells staining for intracellular IFN-$\gamma$ was significantly lower among PBMC cultured without direct contact with iRBC (inner wells) than among PBMC in the outer well (direct contact with iRBC; Wilcoxon signed rank test, $z = 2.2, n = 6, p = 0.03$). Importantly, no difference in response was observed between inner wells and outer wells for PHA-stimulated cells, indicating free circulation of soluble stimuli within the cultures (Wilcoxon signed rank test, $z = 1.2, n = 6, p = 0.2$).

Having shown that both IL-12/IL-18 and contact between NK cells and iRBC are essential for optimal NK activation, we hypothesized that purified NK cells, cultured with iRBC in the absence of monokine-producing cells, would fail to make IFN-$\gamma$ but that addition of monokine-producing cells to NK lines would restore their responsiveness. Thus, oligoclonal NK cell lines (made by combining several NK cell clones from a single donor) were incubated with iRBC or uRBC in the presence or absence of autologous PBMC (in a 1:1 ratio) for 24 h before staining for IFN-$\gamma$. The cloned NK cells were stained with Cell Tracker Green to allow them to be differentiated from NK cells in the added PBMC. An example of the data obtained for one donor is shown in Fig. 4,
A–F); a summary of the data from three different donors is shown in Fig. 4G. As expected, IFN-γ-producing NK cells were detected among PBMC cultured with iRBC but not uRBC (Fig. 4, A and B). However, in the absence of monokine-producing cells, cloned NK cells (Cell Tracker +) were unable to make IFN-γ (Fig. 4, C and D). When unlabeled PBMC were added back to the NK line, cloned NK cells were now able to make IFN-γ (Cell Tracker +, IFN-γ +; Fig. 4, E and F). Similar results were observed for all cell lines tested (Fig. 4G). Although the percentage of NK cells making IFN-γ was lower in the NK cell lines than in the comparable PBMC population, cloned NK cells consistently produced IFN-γ only in the presence of PBMC.

Association between IFN-γ production and NK receptor expression

The requirement for cell-cell contact for NK cell activation by iRBC indicates the existence of a specific receptor-ligand interaction between the NK cell and iRBC. We hypothesized that the differences between donors in their ability to form conjugates with iRBC (Fig. 3) or to mount an IFN-γ response to P. falciparum (Fig. 4) hinged on the differential expression of specific NK receptors. We therefore compared the levels of NK receptor expression by IFN-γ-producing and nonproducing NK cells (both within a single donor and between donors) using four-color flow cytometry. PBMC were cultured with iRBC or uRBC for 24 h and NK cells (CD56 +, CD3−) were analyzed for expression of IFN-γ and a panel of KIR and lectin-like receptors. Receptor expression levels (calculated as MFI) on IFN-γ + and IFN-γ − NK cells were compared to determine whether receptor expression was associated with activation, and receptor expression on all NK cells (taking IFN-γ + and IFN-γ − together) was compared to determine the overall effect of Ag stimulation, regardless of cytokine production. Cells from seven donors (five responders and two low/nonresponders) were tested. No consistent differences in expression of KIR were observed between IFN-γ + and IFN-γ − NK cells (data not shown); however, this does not preclude variable expression of individual KIR genes or of specific alleles of individual KIR genes, between responding and nonresponding cells, as the Abs used do not distinguish between KIR alleles and only two of the available anti-KIR Abs detect the product of a single gene (the other Abs recognize epitopes that are common to a number of different KIR). Similarly, no consistent differences were observed between IFN-γ + and IFN-γ − NK cells in expression of the nonpolymorphic NCR, NKp30, NKp44, or NKp46 or the activating C-type lectin receptor NKG2D.

By contrast, consistent differences were observed between IFN-γ + and IFN-γ − NK cells in expression of both components of the regulatory CD94/NKG2A receptor (Table I). In the five donors whose NK cells are able to respond to iRBC, high levels of both NKG2A and CD94 were expressed more commonly on iRBC-stimulated NK cells than on uRBC-stimulated controls; little difference in CD94 or NKG2A expression was observed in the two nonresponding donors (CS and TH). Taking all seven donors together, the increased expression of CD94/NKG2A on iRBC-stimulated cells was statistically significant (Table 1). Importantly, among the responding donors, CD94 and NKG2A expression was higher in IFN-γ − NK cells than in IFN-γ + NK cells; again, this difference was statistically significant, suggesting either that cells expressing high levels of CD94/NKG2A preferentially respond to iRBC or that CD94/NKG2A is up-regulated on activated cells.

KIR genotype

KIR genotype, including high resolution allele typing for the genes KIR2DL1, 2DL3, 3DL1, and 3DL2, was determined for 27 donors of European, Asian, and African descent for whom the IFN-γ NK cell response to iRBC had been measured (Fig. 5). At the locus level, 15 distinct KIR genotypes were identified among the donors. Although all of the genotypes had been described previously in various ethnic groups, three had not been observed previously in individuals of African descent (23, 24). The number of distinct genotypes was much greater when allelic polymorphisms were also considered. For example, 9 of the 27 donors were homozygous for the simplest arrangement of KIR genes and hence were identical at the locus level. However, each of these nine donors had a distinct KIR genotype when allelic polymorphisms at several of the loci were examined. Therefore, this analysis revealed marked KIR diversity in the donors at both the locus and allelic level.

Identification and characterization of new KIR alleles

Novel patterns of reactivity with primers designed to identify distinct allelic variants suggested that several individuals carried novel KIR alleles. Sequence analysis of cDNA clones from 11 donors of African and Asian descent identified 21 new KIR alleles, representing 8 different KIR genes (Fig. 5). These comprised five variants of KIR2DL1, one of 2DL2, two of 2DS2, two of 2DS4, two of 2DS5, five of 3DL1, one of 3DL2, and three new variants of a chimeric 3DL1/2 gene (Table II). Seven of the new variants differed from known KIR alleles by synonymous nucleotide changes in the coding regions or by nucleotide differences in the noncoding 5′ and 3′ untranslated regions. The remaining 14 alleles differed by nonsynonymous nucleotide changes in the coding regions and encoded novel proteins. The majority of the nucleotide changes were at known polymorphic sites and were identical in several donors, others were confirmed in genomic DNA by PCR amplification or sequencing. Each of the novel allele sequences has been submitted to the KIR Nomenclature Committee, and 16 have received official names (Table II). The remaining five are awaiting assignment of official allele names pending further sequence analysis (22).
Several new variants of a previously described chimera containing the extracellular D0-D1-D2 domains of KIR3DL1 and the stem-transmembrane-cytoplasmic tail of 3DL2, so-called KIR3DL1/2v (19), were identified (Table II). Each variant had the same chimeric structure as the original KIR3DL1/2v, but differed by several nucleotide substitutions, primarily in the region encoding the cytoplasmic tail and in the 3′ untranslated region. In addition, several new variants of the activating receptor KIR2DS4 were discovered. KIR2DS4 is unique in that alleles either encode a membrane-associated form of the protein or a deletion variant that potentially encodes a secreted protein lacking the D2, stem, transmembrane, and cytoplasmic regions (25, 26). We have identified new variants of both types of 2DS4 alleles, demonstrating that more than one allele encoding the deletion variant exists in the human population (Table II).

### Association between KIR genotype and NK responsiveness to iRBC

NK responses to iRBC were arbitrarily categorized as high (≥10%), low (1.0–9.9%), or absent (<1.0%) (Fig. 5). Given the known levels of KIR diversity within human populations, we did not anticipate that any robust associations would be observed between KIR genotype and NK responsiveness to iRBC in this relatively small study. However, the data are sufficient to discount some relatively simple hypotheses. For example, since KIR haplotypes can be broadly divided into two subtypes—group A that carries either one (2DS4) short-tailed, activating KIR and group B that carries two or more activating KIR—it was possible to test for an association between NK-iRBC responses and the number of activating KIR. There was no significant difference in the proportion of individuals carrying B haplotypes among the three levels of NK-iRBC responders (28.5, 33.3, and 25.0% in high, low, and nonresponders, respectively) and no correlation between the number of activating KIR expressed by any donor and the magnitude of the NK-iRBC response ($r^2 = 0.03$).

However, rather to our surprise, a significant association was observed between NK-iRBC responses and an allele of 3DL2. Of the 23 donors for whom 3DL2 alleles have been typed, 8 donors carried at least one copy of the *002 allele; of these, 5 of 7 high responders, but only 3 of 16 low/nonresponders, carried *002 at this locus ($\chi^2 = 6.36, df = 1, p = 0.025$).
Discussion

Taken together, the data reported here demonstrate that two separate signals are required for optimal NK cell activation by *P. falciparum iRBC*; one signal is cytokine mediated and the other is delivered following direct contact between the NK cell and iRBC. Furthermore, our data suggest that contact leads to formation of stable NK-iRBC conjugates and that interactions between ligands on the iRBC and specific cell surface receptors of the NK cell lead to activation and IFN-γ production.

The requirement for IL-12 and IL-18 for optimal NK activation is not surprising because these cytokines have been shown to mediate bystander activation of NK cells in response to a number of different infectious organisms (9). Less predictable, however, was that the nonresponsiveness of cells from some donors could not be overcome by adding exogenous cytokines, indicating that differential responsiveness of NK cells from different donors is not due to differences in their propensity to produce monokines in response to malaria Ags. In support of this conclusion, we have shown that

Table 1. Up-regulation of NKG2A and CD94 on iRBC-stimulated NK cells

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<tr>
<th></th>
<th>% Positive NK Cells</th>
<th>MFI of NK Cells</th>
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<tbody>
<tr>
<td></td>
<td>IFNγ⁺</td>
<td>IFNγ⁻</td>
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<tr>
<td>NK2A</td>
<td></td>
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<tr>
<td>CS</td>
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<td>TH</td>
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<tr>
<td>FMO</td>
<td>82.9</td>
<td>53.9</td>
</tr>
<tr>
<td>JR</td>
<td>56.5</td>
<td>48.5</td>
</tr>
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<td>77.5</td>
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<td>88.4</td>
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<td>053M</td>
<td>87.7</td>
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<tr>
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<td>2.37</td>
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<tr>
<td>p</td>
<td>0.04</td>
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CD94

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<tr>
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<th>% Positive NK Cells</th>
<th>MFI of NK Cells</th>
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<tbody>
<tr>
<td></td>
<td>IFNγ⁺</td>
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<td>p</td>
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* Numbers represent the percentage of NK cells expressing NK2A (top) or CD94 (bottom) and the corresponding MFI values. For iRBC-stimulated cells, values are shown for IFN-γ⁺ and IFN-γ⁻ NK cells separately and for all NK cells. For uRBC-stimulated cells (where numbers of IFN-γ⁺ cells were extremely low), values are shown for all NK cells only. Initials indicate cell donor.

FIGURE 5. KIR genotype and higher resolution allele typing for 27 donors. Donors have been divided into three groups based on the level of their NK response to iRBC (percentage of IFN-γ⁺ NK cells after 24-h iRBC stimulation, uRBC values have been subtracted). An open box indicates the presence of a KIR gene, whereas a shaded box indicates its absence. Allelic subtypes, where known, are indicated by open boxes with text. Open boxes without text indicate that the KIR allele was not determined. In heterozygous individuals, allele names are separated by a semicolon. Alleles not discriminated by subtyping are listed together, e.g., KIR2DL3*002/6.
iRBC induce IL-12 production within 18 h from PBMC of all donors tested to date (M. Walther and E. M. Riley, unpublished data). Studies are underway to identify the source of this IL-12 and the molecular basis for its induction.

Direct contact between the NK cell and iRBC was demonstrated microscopically and by detection of glycophorin A-stained RBC-NK conjugates by flow cytometry. Although it is possible that NK cells could have taken up glycophorin A at the synapse (27), we think this is unlikely as, in that case, the amount of glycoporphin A that NK cells could have taken up at the synapse would be expected to be very low, giving (27), we think this is unlikely as, in that case, the amount of glycoporphin A that NK cells could have taken up at the synapse would be expected to be very low, giving (27), we think this is unlikely as, in that case, the amount of glycoporphin A that NK cells could have taken up at the synapse would be expected to be very low, giving

The need for direct contact between the NK cell and iRBC for NK cell activation explains our previous observation that intact iRBC but not freshly lysed iRBC are able to activate NK cells and offers an alternative explanation for the differential responsiveness observed between donors (3), namely, that NK cells from different donors may vary in their expression of activating or inhibitory NK cell receptors.

Our working hypothesis is that inhibitory ligands for NK cells may exist on normal RBC but that neoantigens expressed on the surface of iRBC provide an activating signal. The nature of the activating or inhibitory ligand(s) is still obscure, but obvious candidates include...
normal RBC components that are abnormally exposed on the surface of parasitized erythrocytes (28) or various parasite-encoded ligands (belonging to the clonally variant families of P. falciparum erythrocyte membrane protein 1, Rifin, and subtelomeric open reading frame proteins) (29) that are inserted into the erythrocyte surface membrane. To date, the only pathogen-derived ligands known to be recognized by NK cells are influenza hemagglutinin (15) and CMV-encoded MHC homologues, the UL18 gene product of human CMV (30) and m144 and m157 from murine CMV. Differential binding of m157 to activating lectin-like Ly49H NK cell receptors is causally linked to CMV resistance and susceptibility in mice (31, 32).

Although no consistent differences in expression levels of KIR or activating C-type lectin receptors were observed between responding and nonresponding NK cells, expression of both CD94 and NKG2A was higher on activated (i.e., IFN-γ−) NK cells than on mononactivated (IFN-γ−) cells following stimulation with iRBC, and higher on cells stimulated with iRBC than on resting cells or cells cultured with uRBC. As NKG2A forms heterodimers with CD94, the parallel increased expression of NKG2A and CD94 suggests that the CD94:NKG2A lectin-type receptor is expressed at higher levels on responding NK cells than on nonresponding NK cells. CD94:NKG2A has been identified as an inhibitory receptor, rescuing target cells from lysis by binding of nonclassical MHC class I molecules (17, 33). CD94/NKG2A expression on NK cells can be induced by cytokines, particularly IL-15 (34), and, in T cells, cross-linking of TCR by interaction with peptide-MHC is required for CD94/NKG2A expression (35). In this case, NK activation by iRBC, CD94/NKG2A is expressed at higher levels on IFN-γ− cells than on IFN-γ+ cells and thus it is likely that interactions between NK receptors and target cell ligands does in some way modify CD94/NKG2A expression. Expression of an inhibitory receptor on IFN-γ− NK cells suggests that CD94:NKG2A may serve a homeostatic regulatory function, preventing the potentially pathological effects of persistent NK activation, as has been described for other leukocyte populations (reviewed in Ref. 36). In accordance with this hypothesis, preliminary data indicate that blocking of CD94:NKG2A with Ab enhances the NK response to both iRBC and PHA (D. Korbel and E. M. Riley, unpublished data).

Our experiments give some indication of heterogeneity of the iRBC response among clones within a donor. In the presence of autologous PBMC, oligoclonal NK cell lines (derived from individuals previously categorized as high responders) were able to respond to iRBC, but the percentage of IFN-γ− NK cells was always lower in the oligoclonal line than in the parent PBMC population. One explanation for this finding is that the oligoclonal cell lines lack one or more of the dominant IFN-γ-producing NK clones present in the PBMC population. Since individual NK clones may vary in their expression of inhibitory and activating receptors (37), the balance of receptor expression among NK clones may influence their response to iRBC.

Consistent with our hypothesis that variation in NK receptor expression might be associated with NK-iRBC responses, analysis of KIR genotypes in our panel of donors revealed marked diversity in the presence or absence of genes for certain inhibitory and activating receptors; all 27 donors had distinct KIR genotypes and a number of new KIR alleles was identified. The majority of the KIR allelic variants described to date have been identified in individuals of European descent. Previous analysis of single African-American and Asian-Indian individuals indicated that novel alleles remained to be found in these populations (21). This prediction was borne out in this study, where we identified 21 new alleles from only 8 donors of African and 3 of Asian descent. These results emphasize the need to further characterize KIR in both the African and Asian populations, as such information will be necessary for studies to correlate KIR genotype with malaria and other diseases.

Rather to our surprise, given the relatively small numbers of donors studied, we have found a significant association between a 3DL2 allele expressed by individual donors and the likelihood of making a strong NK response to P. falciparum iRBC. Although it is hard to envisage a mechanism by which an inhibitory receptor might be causally associated with NK activation, it is possible that this 3DL2 allele might be in linkage disequilibrium with a specific allele at an activating gene locus. High-resolution genotyping of a much larger population is required to determine the significance of these observations.

In summary, we have shown that activation of human NK cells by P. falciparum iRBC to produce an early burst of IFN-γ requires two signals, one dependent upon contact between the NK cell and iRBC and the other cytokine mediated (and likely dependent upon interactions between iRBC and dendritic cells or monocyte-macrophages). NK cell activation by iRBC correlates with high levels of expression of the regulatory CD94:NKG2A lectin-like receptor. Studies are underway to identify both the NK receptor and the ligand on the iRBC that mediates NK activation. Importantly, however, the study provides evidence for an association between KIR genotype and NK-iRBC responses. Given that NK cells act very early during the immune response to infection, our data raise the intriguing possibility that KIR genotype may influence the susceptibility to infections such as malaria.

Acknowledgments

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References


