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Heterogeneous Human NK Cell Responses to Plasmodium falciparum-Infected Erythrocytes

Daniel S. Korbel, Kirsty C. Newman, Catarina R. Almeida, Daniel M. Davis, and Eleanor M. Riley

Human NK cells can respond rapidly to Plasmodium falciparum-infected RBC (iRBC) to produce IFN-γ. In this study, we have examined the heterogeneity of this response among malaria-naive blood donors. Cells from all donors become partially activated (up-regulating CD69, perforin, and granzyme) upon exposure to iRBC but cells from only a subset of donors become fully activated (additionally up-regulating CD25, IFN-γ, and surface expression of lysosomal-associated membrane protein 1 (LAMP-1)). Although both CD56dim and CD56bright NK cell populations can express IFN-γ in response to iRBC, CD25 and LAMP-1 are up-regulated only by CD56dim NK cells and CD69 is up-regulated to a greater extent in this subset; by contrast, perforin and granzyme A are preferentially up-regulated by CD56bright NK cells. NK cells expressing IFN-γ in response to iRBC always coexpress CD69 and CD25 but rarely LAMP-1, suggesting that individual NK cells respond to iRBC either by IFN-γ production or cytotoxicity. Furthermore, physical contact with iRBC can, in a proportion of donors, lead to NK cell cytoskeletal reorganization suggestive of functional interactions between the cells. These observations imply that individuals may vary in their ability to mount an innate immune response to malaria infection with obvious implications for disease resistance or susceptibility. The Journal of Immunology, 2005, 175: 7466–7473.

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 naive and healthy and gave fully informed consent for lysoosomal-associated membrane protein 1; IS, immunological synapse; KIR, killer cell Ig-like receptor.
their blood to be used in this study. Ethical approval was given by the London School of Hygiene and Tropical Medicine Ethics Committee, application 805.

P. falciparum culture and Ag preparation

P. falciparum parasites of the strain 3D7 were grown in O9- – human erythrocytes (National Blood Service) in RPMI 1640 (Invitrogen Life Technologies) supplemented with 25 mM HEPES (Sigma-Aldrich), 28 mM sodium bicarbonate (BDH), 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. The culture medium was changed daily and the parasitemia was determined by examination of Giemsa-stained thin blood smears. Parasite cultures were monitored to ensure that the cultures were free from Mycoplasma contamination by PCR (Stratagene). Mature schizonts were harvested from cultures of 5–8% parasitemia by centrifugation through a 60% Percoll gradient (Sigma-Aldrich).

PBMC preparation and culture

Venous blood was collected into sodium heparin (10 IU/mL blood; CP Pharmaceuticals) and diluted 1:1 in RPMI 1640 (Invitrogen Life Technologies). PBMC were isolated by density centrifugation through a Histopaque 1077 gradient (Sigma-Aldrich) and washed twice in RPMI 1640. Cells were resuspended in growth medium (RPMI 1640, 5% autologous serum, 1% 100X penicillin/streptomycin (Invitrogen Life Technologies), 2 mM l-glutamine (Invitrogen Life Technologies)), transferred to 24-well plates, and gassed with 3% O2, 4% CO2, and 93% N2 and incubated at 37°C in an atmosphere of 5% CO2, Schizont-infected (iRBC) or uninfected (uRBC) erythrocytes were added at a ratio of 3 RBC per mononuclear cell. A mixture of recombinant human IL-12 (100 ng/mL; PeproTech) and recombinant human IL-18 (100 ng/mL; MBI Fermentas) was used as a positive control.

Cell surface and intracellular staining for flow cytometry

Brefeldin A (10 μg/mL; Sigma-Aldrich) was added to the cells 3 h before the end of the incubation; for time course experiments, brefeldin A was not used. Cells were washed twice in FACS buffer (1× PBS, 0.1% sodium azide (Sigma-Aldrich), and 1% PBS (Invitrogen Life Technologies)) and centrifuged for 10 min at 470 g. The cells were resuspended in FACS buffer, and fluorochrome-conjugated Abs to CD3, CD56, and CD107a were added, in the dark, at 4°C and at concentrations previously determined by titration. The cells were incubated in the dark at 4°C for 30 min, washed twice in FACS buffer, and fixed for 15 min at room temperature in fixation buffer (1× PBS and 2% paraformaldehyde (Sigma-Aldrich)). After an additional washing step, the cells were resuspended in permeabilization buffer (1× PBS, 1% saponin, and 0.1% sodium azide (Sigma-Aldrich)) along with fluorochrome-conjugated Abs to IFN-γ, perforin, or granzyme A, incubated for 30 min in the dark at 4°C, washed in FACS buffer, and resuspended in FACS buffer. Isotype control Abs were used for each staining combination. Flow cytometry analysis was performed using a BD Biosciences FACSCalibur flow cytometer and CellQuest analysis software. The following Abs were used: anti-CD56 allophycocyanin and IgG1 allophycocyanin (Beckman Coulter); anti-IFN-γ FITC, IgG1 FITC, anti-CD3 PerCP, IgG1 PerCP, anti-granzyme A PE, IgG1 PE, anti-perforin PE, IgG2b PE, and anti-CD107a PE (all BD Biosciences); and anti-CD69 PE, anti-CD25 PE, and IgG2a PE (all Caltag Medsystems).

Immunostaining of NK cells and erythrocytes for confocal microscopy

CD56+ CD3− NK cells were enriched from PBMC by magnetic bead separation (MACS) using the NK Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer’s instructions. Cells were counted, tested for viability using trypan blue, and purity confirmed by flow cytometry. Briefly, 106 NK cells and 3 × 106 iRBC were resuspended in prewarmed culture growth medium and transferred to a 96-well V-bottom plate. The cells were incubated at 37°C in 5% CO2 for 15 min and fixed for 30 min at room temperature with 15% electron microscopy grade formaldehyde (Sigma-Aldrich). The cells were spun for 10 min (400 g) and washed once with washing buffer. After 1 h of incubation in blocking solution (Dulbecco’s PBS, 5% horse serum (Sigma-Aldrich), and 3% FBS) and washing, the cells were incubated with phallolidin conjugated to Alexa Fluor 546 (1/40 in blocking solution; Molecular Probes) for 1 h at 4°C. After two washes, the cells were incubated for 10 min at 4°C with 0.1 mg/mL 4’6-diamidino-2-phenylindole (DAPI), washed once, and placed between a microscope slide and a 24 × 24 mm coverslip (thickness no. 1).

Laser scanning confocal microscopy

Confocal microscopic examination of erythrocyte/NK cell conjugates was performed under a 100× Plan Apochromat oil-immersion objective (numerical aperture, 1.4) with a Zeiss LSM 510/Axiovert 200 M inverted microscope (Zeiss) using excitation wavelengths of 405 and 543 nm. Cell-cell contacts were scanned in the yz direction every 0.4 μm through the z-plane. Maximum intensity projection-based three-dimensional reconstructions of the cell-cell contact were performed using the LSM Image Examiner software (Zeiss).

Statistical analysis

All statistical analyses were performed using Prism 4 software (GraphPad Software).

Results

The human NK cell IFN-γ response to P. falciparum iRBC varies between donors

As we have previously reported (12, 14), the human NK cell IFN-γ response to iRBC is variable, with a significant proportion of the peripheral NK cells from some donors consistently producing large amounts of IFN-γ while NK cells from other donors make no appreciable IFN-γ response. Examples of the NK cell IFN-γ response to iRBC for four individual donors used in this current study are shown (Fig. 1A). IFN-γ production from CD56bright and CD56dim cells appears to be differentially regulated. For donor 181F, neither the CD56bright nor the CD56dim NK cells produced IFN-γ, whereas for donor 178M, ~30% of each cell population stained positively for IFN-γ after 24 h. By contrast, for donor 056, the IFN-γ− cells were predominantly among the CD56dim subset while for donor EX03, the very few IFN-γ− cells seen were CD56bright. Taking together, the data for 28 donors, cells from 11 donors clearly up-regulated IFN-γ in response to iRBC (>5% of NK cells being IFN-γ+) (Fig. 1B). Importantly, the phenotype of each donor (percentage of NK cells producing IFN-γ in response to iRBC) was highly reproducible over time (Fig. 1C).

Since both CD56dim and CD56bright cells appeared to be able to respond to iRBC by making IFN-γ, but previous studies had suggested that CD56dim and CD56bright cells varied in their ability to make this cytokine, we compared the IFN-γ response among CD3− CD56bright and CD3− CD56dim NK cells within individual donors. In accordance with previous studies (15), a significantly higher proportion of CD56bright cells than CD56dim cells made IFN-γ in response to stimulation with high doses of IL-12 and IL-18 (mean 63.6 vs 42.9%, t = 6.90, df = 27, p < 0.0001). By contrast, there was no significant difference in the proportion of CD56 bright and CD56dim cells responding to iRBC (Fig. 1B) but, since CD56dim cells outnumber CD56bright cells by at least 15:1 in peripheral blood, significant numbers of IFN-γ− NK cells were seen only in donors in whom the CD56dim population was able to respond.

CD69 surface expression is universally up-regulated on human NK cells in response to iRBC

To determine whether or not NK cells from IFN-γ− low responders were completely nonresponsive to iRBC, we examined expression of the early activation marker CD69 after coculture with iRBC. The C-type lectin-like glycoprotein CD69 is known to be a sensitive and very early marker of leukocyte activation (16, 17). It is encoded within the NK gene complex (18), is rapidly expressed on the cell surface from preformed intracellular stores in a RNA and peptide synthesis-independent manner (19), and its cross-linking by Ab triggers exocytosis of cytotoxic granules (20).

Representative examples of changes in CD69 expression on NK cells after 24 h of coculture with iRBC, uRBC, or IL-12/18 for one IFN-γ−-low-responding donor (Fig. 2A) and one high-responding...
donor (Fig. 2B) are shown. Following incubation of PBMC for 24 h in growth medium alone, the proportion of NK cells expressing CD69 varied between 10 and 50%, presumably reflecting differing levels of ex vivo activation (Fig. 2C). Stimulation with high doses of IL-12 and IL-18 induced CD69 expression on virtually all NK cells (examples are shown in Fig. 2, A and B). CD69 expression levels did not increase after incubation with uninfected erythrocytes; indeed, taking the data from all 30 donors tested the proportion levels did not increase after incubation with uninfected erythrocytes. PBMC from IFN-γ-naive donors were stimulated with live P. falciparum iRBC or uRBC for 24 h. The production of IFN-γ from malaria-naive donors were stimulated with live erythrocytes is heterogeneous between donors but stable over time. PBMC collection of 50,000 total events. Representative plots for one IFN-γ responders (A) and one IFN-γ low responder (B) are shown. C, The percentage of NK cells expressing CD69 in response to growth medium (GM) alone, uRBC, or iRBC was calculated for 30 donors. Each dot represents a single donor. D, Comparison of CD69 expression between IFN-γ high-responding and low-responding donors in response to iRBC. The percentage of NK cells expressing CD69 in response to growth medium (GM) alone, uRBC, or iRBC was calculated for 30 donors. Each dot represents a single donor. CD69 expression is shown for all CD3 CD56dim NK cells (left panel), CD3 CD56bright cells (middle panel), and CD3 CD56dim NK cells from individual donors.

FIGURE 1. The NK cell IFN-γ response to P. falciparum-infected erythrocytes is heterogeneous between donors but stable over time. PBMC from malaria-naive donors were stimulated with live P. falciparum iRBC or uRBC for 24 h. The percentage of NK cells expressing CD69 varied between donors and the fold increase in median fluorescence intensity (MFI) (Fig. 2, C and E). Importantly, there were also significant differences in the degree of CD69 up-regulation between donors. Both the percentage of NK cells expressing CD69 and the fold increase in MFI for CD69 expression were significantly higher in individuals classified as IFN-γ high responders (>5% of NK cells able to make IFN-γ after coculture with iRBC) than in low responders (Fig. 2D). This higher expression of CD69 in high IFN-γ responders than in low IFN-γ responders was seen in both the CD56dim and CD56bright NK cell
IFN-γ may be universal but the other may be restricted to the subgroup of two signals required for NK activation by iRBC (21), one signal may be universal but the other may be restricted to the subgroup of IFN-γ-producing donors.

**Up-regulation of CD25 on NK cells following incubation with iRBC**

To explore further these differences in NK responses between donors and within the CD56dim and CD56bright NK populations within donors, we examined the expression of an alternative marker of activation, the high-affinity IL-2Rα/CD25, which reflects the proliferative capacity of NK cells (22). PBMC were incubated for 24 h with iRBC, uRBC, and IL-12/18 or no stimulation and the levels of CD25 were assessed by flow cytometry; representative examples of responses to iRBC in an IFN-γ low (Fig. 3A)- and high (Fig. 3B)-responding donor are shown. For resting NK cells, cultured in growth medium alone, the proportion of CD56bright cells expressing CD25 was significantly higher than the proportion of CD25−/CD56dim cells (mean = 19 and 6%, respectively, n = 10, p = 0.01; Fig. 3C). After stimulation with IL-12/18, the proportion of NK cells expressing CD25 reached >95% in all donors tested while no up-regulation was observed in response to uRBC (data not shown).

Expression of CD25 was up-regulated in response to iRBC in NK cells from all donors; however, the degree of up-regulation differed between donors and significant up-regulation of CD25 expression was restricted to the CD56dim population with no significant change in expression of CD25 in the CD56bright population (Fig. 3C). Interestingly, the degree of up-regulation of CD25 was significantly correlated with the ability to produce IFN-γ (r² = 0.86, n = 10, p = 0.0001; Fig. 3D).

Thus, the heterogeneity of the NK response to iRBC can be seen at the level of IFN-γ production and expression of both CD69 and CD25, and induction of these markers of NK activation appears to be linked.

**Up-regulation of markers of cytotoxic activity in NK cells following incubation with iRBC**

Since it has previously been reported that human NK cells can be cytotoxic for iRBC (23) and since CD69, which has been linked to the cytotoxic potential of NK cells (22), was heterogeneously expressed, we wanted to determine whether expression of other markers of cytotoxic activity were also differentially expressed among donors. Two major components of NK cell cytotoxic granules are the membrane-disrupting protein perforin and the serine protease granzyme A, which mediates caspase-independent apoptosis (24). A more recently described marker of cytotoxic activity is the lysosomal-associated membrane protein 1 (LAMP-1 or CD107a) which is expressed on the NK cell surface following cytotoxic granule exocytosis induced by exposure to MHC-deficient target cells (25).

Thus, PBMC were incubated for 24 h with iRBC, uRBC, and IL-12/18 or no stimulation, and NK cell expression of LAMP-1 or perforin and granzyme A was assessed by surface and intracellular staining, respectively (Fig. 4).

In contrast to a previous report (26), freshly isolated NK were not found to be positive for perforin (Fig. 4A) and granzyme A was expressed only at low levels (Fig. 4B). However, in agreement with the previous report (26), both perforin and granzyme A were spontaneously up-regulated during in vitro culture; after 24 h in culture in the absence of any exogenous stimuli, close to 100% of

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**FIGURE 3.** Expression of CD25 on NK cells in response to live P. falciparum-infected erythrocytes. PBMC were cultured with or without iRBC for 24 h and expression of CD25 was assessed by surface staining and flow cytometric analysis. Representative plots for an IFN-γ low-responding (A) and an IFN-γ high-responding donor (B) are shown. (Donors were designated as high responders if ≥5% of their NK cells expressed IFN-γ after 24-h PBMC incubation with iRBC.) FACS plots are gated on CD3− lymphocytes. Percentages indicate the proportion of CD3− CD56dim or CD3− CD56bright cells that were positive for CD25; data are based on the collection of 50,000 total events. C, Expression of CD25 on the CD56dim and CD56bright NK cell subsets for 10 donors after 24-h incubation with growth medium (GM; i.e., no stimulation) or iRBC. Each dot represents a single donor. D, Correlation between the increase in CD25 expression on NK cells with the proportion of IFN-γ+ NK cells for 10 donors after incubation with iRBC. *, p < 0.05; ***, p < 0.01.

**FIGURE 4.** Expression of perforin, granzyme A, and LAMP-1 on NK cells in response to P. falciparum iRBC. PBMC were cultured with or without iRBC for 24 h and expression of perforin (A), granzyme A (B), and LAMP-1 (C, CD107a) was assessed by intracellular staining followed by flow cytometric analysis. All plots are gated on CD3− lymphocytes; data are based on the collection of 50,000 total events. Numbers indicate the MFI of staining of the CD56bright and CD56dim subsets of NK cells, respectively. Data from a representative experiment is shown in the first three panels. A and B, Right-hand panels show the fold increase in NK cell MFI for perforin and granzyme A or the CD56bright and CD56dim subsets of NK cells, respectively. Data from a representative experiment is shown in the first three panels. A and B, Right-hand panels show the fold increase in NK cell MFI for perforin and granzyme A or the CD56bright and CD56dim subsets of NK cells, respectively. Data from a representative experiment is shown in the first three panels. A and B, Right-hand panels show the fold increase in NK cell MFI for perforin and granzyme A or the CD56bright and CD56dim subsets of NK cells, respectively. Data from a representative experiment is shown in the first three panels. A and B, Right-hand panels show the fold increase in NK cell MFI for perforin and granzyme A or the CD56bright and CD56dim subsets of NK cells, respectively. Data from a representative experiment is shown in the first three panels.
NK cells stained positively for both granzyme and perforin and both markers were especially highly expressed in CD56<sup>dim</sup> cells (Fig. 4, A and B). Interestingly, this spontaneous up-regulation of perforin was slightly, but statistically significantly, inhibited in both CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells cultured with uRBC (paired t test, MFI for uRBC vs growth medium alone, t > 2.43, df = 7, p < 0.05 in both cases).

After coculture with iRBC for 24 h, the levels of NK expression of both perforin and granzyme (expressed as fold increase in MFI) were modestly increased when compared with PBMC cultured alone; this increase was particularly pronounced for the CD56<sup>bright</sup> population (Fig. 4. A and B, right panels). The degree of up-regulation of intracellular perforin was similar in all donors tested while up-regulation of granzyme A was more variable between donors. However, variation in granzyme A up-regulation was not correlated with IFN-γ responses in either CD56<sup>bright</sup> or CD56<sup>dim</sup> cells (r² < 0.25, p > 0.2 in each case).

By contrast, there was no spontaneous increase in surface expression of LAMP-1 during in vitro culture (Fig. 4C) and, accordingly, incubation with uRBC did not affect levels of LAMP-1 expression (data not shown). LAMP-1 expression was not significantly enhanced by stimulation with IL-12/18 (data not shown). Surface expression of LAMP-1 was, however, up-regulated in response to iRBC in NK cells from some donors and, where LAMP-1 up-regulation was observed, it was confined exclusively to the CD56<sup>dim</sup> subset of NK cells (Fig. 4C). Moreover, the degree of up-regulation of LAMP-1 was highly correlated with the frequency of IFN-γ-producing cells (r² = 0.90, n = 13, p < 0.0001).

Are markers of NK cell activation coexpressed in single cells?

The data presented so far indicate that some markers of NK activation (notably CD69, perforin, and granzyme A) are up-regulated in cells from all donors and in both CD56<sup>bright</sup> and CD56<sup>dim</sup> NK subsets, whereas other markers (IFN-γ, CD25, and LAMP-1) are up-regulated only in a subset of donors and, in the case of CD25 and LAMP-1, only in CD56<sup>dim</sup> cells. To determine whether up-regulation of IFN-γ occurs in the same cells that express CD25, LAMP-1, and, and very high levels of CD69, cells were incubated with iRBC for 24 h and stained for CD3, CD56, intracellular IFN-γ, and either CD69, CD25, or LAMP-1. Representative plots are shown in Fig. 5.

IFN-γ<sup>+</sup> cells were all CD69<sup>high</sup> and the CD69 MFI of the IFN-γ<sup>+</sup> cells was higher than the CD69 MFI of the IFN-γ<sup>-</sup> cells (Fig. 5A). The vast majority of IFN-γ<sup>-</sup> were also CD25<sup>+</sup> and the MFI was higher for IFN-γ<sup>-</sup> cells than for IFN-γ<sup>-</sup> cells; however, not all CD25<sup>+</sup> cells expressed IFN-γ, at least at the 24-h time point examined. By contrast, although a very few cells were found to be double positive, IFN-γ and LAMP-1 tended to be expressed in a mutually exclusive manner.

Kinetics of NK cell activation in response to iRBC

To determine the kinetics of NK cell activation by iRBC and to check that data obtained after 24 h of coculture are a true representation of the potential responsiveness of individual donors, 24-h time-course experiments were conducted with cells from both IFN-γ high and low responders. Replicate wells containing PBMC plus iRBC were cultured for up to 24 h and, at each time point, cells were collected and stained for CD3, CD56, and either IFN-γ, CD69, LAMP-1, or CD25 (Fig. 6). As we had found previously (12), IFN-γ is not detectable in iRBC-activated NK cells at 6 h but increases rapidly to maximal levels between 12 and 24 h (Fig. 6A). The kinetics of CD25 expression were indistinguishable from those of IFN-γ (Fig. 6B), suggesting that these two markers are indeed coinduced. By contrast, CD69 up-regulation begins as early as 2 h after initiation of the coculture, reaches 50% of the maximal level within 3 h, and is maximal at 6–12 h (Fig. 6C). LAMP-1 up-regulation shows an intermediate profile, being significantly above baseline at 6 h but reaching maximal levels between 12 and 24 h (Fig. 6D).

The nature of the contact between human NK cells and P. falciparum-infected erythrocytes

We have previously shown that direct contact between NK cells and iRBC and/or accessory cells present in the PBMC population is essential for optimal induction of IFN-γ production (14). We have also shown that, during coculture of iRBC and PBMC, a significant fraction of CD56<sup>+</sup> CD3<sup>−</sup> NK cells acquire surface expression of the erythrocyte-specific marker glycoporphin A (14), indicating either the formation of stable conjugates between NK cells and iRBC or transfer of iRBC membrane components to NK cells.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Coexpression of CD69 and CD25 but not LAMP-1 with IFN-γ in iRBC-stimulated NK cells. Four-color FACS staining was conducted to allow comparison of the expression of CD69, CD25, or LAMP-1 with IFN-γ in NK cells following 24 h of PBMC culture with iRBC. All plots are gated on CD3<sup>+</sup> CD56<sup>+</sup> NK cells. Cells were stained for intracellular IFN-γ and surface expression of either CD69 (A), CD25 (B), or LAMP-1 (C). One representative experiment of 33 (CD69) or 4 (CD25 and LAMP-1) is shown.

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Kinetics of NK cell activation in response to iRBC. The expression of IFN-γ (A), CD25 (B), CD69 (C), and LAMP-1 (D) in response to iRBC was assessed in replicate wells of PBMC from three or four individual donors at 0, 1, 2, 3, 6, 12, 18, or 24 h. The proportion of NK cells staining for each marker was calculated as described previously. Closed circles indicate IFN-γ high-responding donors, and open circles IFN-γ low-responding donors. Donors were designated as high responders if >5% of their NK cells expressed IFN-γ after 24-h PBMC incubation with iRBC.
Functional interaction (either activating or inhibitory) between NK cells and their targets requires the formation of an immunological synapse (IS); one of the first events in the assembly of the IS is rearrangement of the cytoskeleton to facilitate clustering of lipid rafts, surface receptors, and signaling molecules (reviewed in Ref. 27). Thus, to determine whether NK cells make direct functional contacts with iRBC, NK cells and iRBC were cocultured for 15 min, fixed, stained with DAPI (DNA) and fluorochrome-conjugated phalloidin (F-actin), and visualized by laser scanning confocal microscopy to look for alterations in the NK cell cytoskeleton that might be indicative of IS formation.

Cells from eight donors were examined. Representative images are shown of NK cells from two donors (Fig. 7, A and B). We observed that NK cells from all donors tested could form conjugates with iRBC and that iRBC:NK cell conjugate formation was a very frequent event, with 40–50% of NK cells making stable contacts with iRBC at any one time (Fig. 7A). However, clustering of F-actin at the contact site (which is indicative of cytoskeletal reorganization) was observed in only four donors, three IFN-γ high responders and one low responder (compare Fig. 7A, no clustering, with the example of F-actin clustering shown in Fig. 7B), and, in those donors, clustering of F-actin was seen in only 10–20% of contacts. Clustering of F-actin was confirmed by scanning the contact sites in the xy direction throughout the z-plane (Fig. 7C) and by projected three-dimensional reconstruction (Fig. 7D).

**FIGURE 7.** NK cells form stable contacts with *P. falciparum*-infected erythrocytes. A total of 3 × 10^6 iRBC and 10^6 MACS-purified NK cells from an IFN-γ low-responding (A) and an IFN-γ high-responding donor (B) were cocultured for 15 min at 37°C, fixed at room temperature, and stained with phalloidin conjugated to the fluorochrome Alexa Fluor 546 as a probe for F-actin (green); nuclei were stained with DAPI (blue). The cells were visualized by laser scanning confocal microscopy. The transmitted light image is shown in the left panel and the fluorescence image at 405 and 543 nm is shown in the right panel. Scale bars in A and B represent 5 μm. C, Representative scans through the z-plane of the cell-cell contact shown in B. F-actin fluorescence intensity is false color-coded, the highest intensity being cyan. D, Three-dimensional reconstruction of the cell-cell contact shown in B using a maximum intensity projection based on scans through the z-plane every 0.4 μm. F-actin fluorescence intensity is false color-coded and nuclei were stained with DAPI (blue). DIC, differential interference contrast.

**Discussion**

The data presented here firmly establish that *P. falciparum*-infected erythrocytes can induce IFN-γ production in human NK cells from malaria-naive donors but, while being highly reproducible within donors, the proportion of peripheral NK cells that are able to respond in this way differs between donors. Among IFN-γ low responders, NK cell activation appears to be only partial and is characterized by moderate up-regulation of CD69, perforin, and granzyme in both the CD56<sup>bright</sup> and CD56<sup>dim</sup> NK subsets. By contrast, among high responders, IFN-γ production is accompanied by maximal up-regulation of CD69 and selective up-regulation of CD25 and LAMP-1 in the CD56<sup>dim</sup> NK cell subset. That the response to iRBC can be such a stable phenotype within an individual suggests that it is hardwired and possibly genetically regulated; that the response varies so greatly within a population suggests that the genes involved may be polymorphic. Furthermore, the fact that only a subset of the NK repertoire is able to respond to iRBC even in high responding donors suggests that the relevant gene (or genes) may be variably expressed among different NK cell clones.

Our previous studies have shown that iRBC-mediated induction of IFN-γ production by NK cells is dependent on both accessory cell-dependent, cytokine-mediated signals and a signal mediated by direct contact between NK cells and iRBC and/or accessory...
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cells within PBMC (12, 14), but the exact nature of the interactions between these three cell types remained unclear (21). We have shown that IL-12 and IL-18 are required for optimal NK activation by iRBC ((12, 14); preliminary data suggest that IFN-α, but not IL-15, is also required and that TGF-β can down-modulate the NK IFN-γ response but, to date, we have no evidence for systematic variation in these cytokine-mediated effects between IFN-γ-high responder or low-responder individuals (K. C. Newman, D. S. Korbel, K. Cowgill, and E. M. Riley, unpublished data). Experiments are ongoing to determine the primary source of these cytokines and to identify the key accessory cells required for optimal NK activation by iRBC.

Our direct imaging data demonstrate that stable contacts occur between iRBC and NK cells and suggest that NK activation may be related to cytoskeletal reorganization at the contact sites, as has been reported for activating IS formation at the contact site between NK cells and EBV-transformed B cells (28). Stable contacts between NK cells and iRBC were abundantly seen in cultures from both IFN-γ-high- and low-responding donors, suggesting that NK cells from all donors are able to recognize and bind iRBC. However, cytoskeletal reorganization (clustering of F-actin) was observed only in some NK cells from some donors, suggesting that iRBC recognition does not always lead to functional consequences. More extensive imaging studies are underway to determine whether this cytoskeletal reorganization reflects formation of a true activating immune synapse, to identify the signals required for its induction, and to determine the downstream consequences in terms of IFN-γ production and/or cytotoxicity.

Currently available data indicate that the contact-mediated signal may be required for full activation of NK cells. Thus, freeze-thaw lysates of iRBC, which are very unlikely to form immune synapses with either NK cells or accessory cells but are efficient inducers of proinflammatory cytokines from monocyte/macrophages (29), induce significantly less IFN-γ production (12) and CD69 up-regulation (K. C. Newman, D. S. Korbel, and E. M. Riley, manuscript in preparation) than do intact iRBC. In Transwell experiments in which NK cells receive soluble but not contact-mediated signals, CD69 is up-regulated (K. C. Newman, D. S. Korbel, and E. M. Riley, manuscript in preparation) but IFN-γ is not (14). Thus, it is conceivable that contact between NK cells and iRBC results in immune synapse formation only in a subset of donors and that this leads to full NK cell activation, including up-regulation of CD25 and either IFN-γ production or exocytosis of cytotoxic granules. In this model, partial activation could be explained by bystander effects mediated by cytokines. In support of this, CD69 up-regulation on NK cells occurs within the first 6 h of coculture of PBMC with iRBC, which corresponds with the secretion of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, and IL-12 (Ref. 29 and M. Walther, et al., manuscript in preparation). Alternatively, given that contact appears to occur between NK cells and iRBC in all donors, it may be that contact mediates partial NK cell activation while the accessory cell-dependent signal may differ between donors and underlie the heterogeneity of response. Further studies are ongoing to dissect the contributions of different signals to the observed differences in response.

A consistent finding in this study was that partial activation was seen in both CD56bright and CD56dim NK cells while full activation was preferentially seen in CD56dim cells, and indeed LAMP-1 and CD25 were only up-regulated in the CD56dim population. CD56dim NK cells are the major population found in the peripheral blood while CD56bright NK cells are the major population in the secondary lymphoid tissue (30). CD56bright cells have been reported to lack perforin and to be superior to CD56dim cells in their ability to secrete IFN-γ (31), and, consequently, it has been suggested that the major effector function of CD56dim NK cells is cytotoxicity while CD56bright NK cells act mainly by secretion of cytokines (26, 31). Our results do not entirely support this notion. Thus, although CD56bright cells could produce IFN-γ in response to iRBC and CD56dim cells were the only ones to express LAMP-1 on their surface, CD56dim cells were just as likely as CD56bright cells to produce IFN-γ while perforin and granzyme A were up-regulated to a greater extent in CD56bright than CD56dim cells. Thus, our data suggest that fully iRBC-activated CD56dim cells sequentially up-regulate CD69 and CD25 and at the same time either differentiate into IFN-γ-producing cells or begin to express LAMP-1. This dichotomy of effector function within the CD56dim subset is in line with data indicating distinct intracellular signaling pathways leading either to cytokine secretion or cytotoxicity (32, 33).

Two recent studies have suggested that both intact iRBC and schizont extracts can induce cytokine secretion from human and mouse accessory cells in a TLR9-dependent manner (34, 35), but the nature of the receptors involved in direct iRBC recognition is unclear. Despite their lack of MHC class I expression, uninfected RBC clearly do not activate NK cells even when, as here, they derive from unrelated donors. Indeed, our observation that they significantly reduce baseline levels of NK activation suggests that iRBC may express alternative ligands for inhibitory NK cell receptors. If so, then reduced expression of this ligand on iRBC, or modification of its structure such that it no longer binds effectively to its receptor, might explain the ability of iRBC to activate NK cells. Alternatively, iRBC may express on their surface a novel ligand for an activating receptor. In either case, that only a subset of NK cells from a subset of individuals respond to iRBC might be explained by polymorphism or variable expression of the receptor. Our previously published data showing an association between NK cell IFN-γ responsiveness and killer cell Ig-like receptor (KIR) genotype (14), combined with our finding here that full activation of NK cells is essentially restricted to the CD56dim subset, which, unlike the CD56bright subset, expresses KIR receptors at high levels (26), suggests that KIR gene expression may influence the NK response to iRBC. The balance of expression of activating and inhibitory KIRs and the affinity of binding of individual KIR variants to polymorphic MHC class I ligands influence the ease of activation of NK cells (36), and heterogeneous KIR gene expression among NK clones has been shown to affect their activation and effector function (37). Since erythrocytes express few, if any, surface MHC molecules, any moderation of NK activation would likely be moderated through “trans” KIR-MHC interactions with other cells, e.g., cytokine-producing accessory cells.

The impact of differential NK cell activation on susceptibility or resistance to clinical malaria is not yet known. Expression of CD25 reflects the proliferative capacity of NK cells (22) and up-regulation of this marker by iRBC may lead to rapid expansion of the NK cell population early in a malaria infection, affecting the magnitude of the subsequent NK cell response. The notion that the rapid availability of IFN-γ from NK cells may limit the initial expansion of the parasite population (and thus be host protective, as in murine malaria (8, 9)) is strongly supported by our observation that, during experimentally induced human malaria infections, growth of blood-stage parasites is directly correlated with the extent of the early proinflammatory cytokine response (38). Whether NK cells might also contribute to killing of iRBC via direct cytotoxicity is less apparent, although up-regulation of markers such as LAMP-1...
in high-responding cells is highly suggestive of this. Clearly, clinical studies of the relationship among NK cell phenotype, NK receptor genotype, and outcome of malaria infection are required to determine how NK cell responses to iRBC contribute to host resistance to malaria in humans.

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Disclosures

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