

# Cross-Talk with Myeloid Accessory Cells Regulates Human Natural Killer Cell Interferon- $\gamma$ Responses to Malaria

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**Data from a variety of experimental models suggest that natural killer (NK) cells require signals from accessory cells in order to respond optimally to pathogens, but the precise identity of the cells able to provide such signals depends upon the nature of the infectious organism. Here we show that the ability of human NK cells to produce interferon- $\gamma$  in response to stimulation by *Plasmodium falciparum*-infected red blood cells (iRBCs) is strictly dependent upon multiple, contact-dependent and cytokine-mediated signals derived from both monocytes and myeloid dendritic cells (mDCs). Contrary to some previous reports, we find that both monocytes and mDCs express an activated phenotype following short-term incubation with iRBCs and secrete pro-inflammatory cytokines. The magnitude of the NK cell response (and of the KIR<sup>-</sup> CD56<sup>bright</sup> NK cell population in particular) is tightly correlated with resting levels of accessory cell maturation, indicating that heterogeneity of the NK response to malaria is a reflection of deep-rooted heterogeneity in the human innate immune system. Moreover, we show that NK cells are required to maintain the maturation status of resting mDCs and monocytes, providing additional evidence for reciprocal regulation of NK cells and accessory cells. However, NK cell-derived signals are not required for activation of accessory cells by either iRBCs or bacterial lipopolysaccharide. Together, these data suggest that there may be differences in the sequence of events required for activation of NK cells by non-viral pathogens compared to the classical model of NK activation by virus-infected or major histocompatibility complex-deficient cells. These findings have far-reaching implications for the study of immunity to infection in human populations.**

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## Introduction

After years of neglect, in which the adaptive immune response to malaria was investigated in great depth with a view to rational development of antimalarial vaccines, the innate immune response to malaria has recently come under scrutiny. In part, this is driven by the realisation that innate immune responses initiate and mould the subsequent adaptive response and that understanding the innate immune response may aid the design of novel adjuvants and novel vaccine delivery systems. Furthermore, data from experiments in rodents and from clinical studies indicate that innate immune responses play a crucial role in controlling malaria infections [1] and can significantly alter the outcome of infection, especially in non-immune individuals. The key element in the cell-mediated effector response to malaria is the rapid induction of interferon (IFN)- $\gamma$  [2–6]. We have shown that human natural killer (NK) cells can be an important early source of IFN- $\gamma$  after exposure of leukocytes to *Plasmodium falciparum*-infected red blood cells (iRBCs) [7–9], although this response is extremely heterogeneous [8,9].

Emerging data suggest that signals from accessory cells such as macrophages, monocytes, and dendritic cells (DCs) are absolutely required for optimal NK cell responses to many infectious agents [10–12]. Consistent with this, we have previously demonstrated that cloned NK cells are unable to respond to iRBCs in the absence of autologous peripheral blood mononuclear cells (PBMCs) [8]. However, an essential role for accessory cells in promoting robust pro-inflamma-

tory responses to malaria is somewhat controversial given previous observations suggesting that accessory cell function is seriously impaired by exposure to malarial parasite ligands. For example, following exposure to iRBCs, both human DCs and monocytes secrete the immunosuppressive cytokine interleukin-10 (IL-10) [13,14], have an impaired ability to mature in response to lipopolysaccharides (LPS) and other ligands [13,15–17], and are unable to present antigen to T cells [13]. Moreover, DCs from children with acute malaria infection have reduced human leukocyte antigen (HLA)-DR expression [18].

Here we have assessed the precise contribution of various

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**Abbreviations:** CTG, CellTracker Green; DC, dendritic cell; FACS, fluorescence activated cell sorting; gm, growth medium alone; HLA, human leukocyte antigen; IFN, interferon; IL-[number], interleukin-[number]; iRBC, *Plasmodium falciparum*-infected red blood cell; KIR, killer cell immunoglobulin-like receptor; LPS, lipopolysaccharide; MACS, magnetic cell sorting; MFI, median fluorescence intensity; mDC, myeloid dendritic cell; MHC, major histocompatibility complex; NK, natural killer; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; TGF, transforming growth factor; TLR, toll-like receptor; uRBC, uninfected red blood cell

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## Synopsis

The outcome of infection is determined both by the ability to limit the initial phase of pathogen colonisation and by the ability to mount an effective adaptive immune response. Both of these processes are influenced by innate immune responses, of which a crucial component can be the ability of natural killer (NK) cells to secrete pro-inflammatory cytokines. Studies in both humans and mice indicate that the magnitude of the early (innate) interferon (IFN)- $\gamma$  response is a crucial determinant of the outcome of malaria infection. In this study, Newman et al. show that activation of human NK cells by *Plasmodium falciparum*-infected red blood cells to produce IFN- $\gamma$  is strictly dependent upon, and regulated by, contact-mediated and soluble (cytokine) signals from two accessory cell populations (myeloid dendritic cells and monocytes). Furthermore, the magnitude of the human NK cell IFN- $\gamma$  response to *P. falciparum*-infected red blood cells is highly correlated with levels of expression of co-stimulatory molecules on resting accessory cells. These findings suggest that it might be possible to predict the magnitude of the innate cytokine response, and possibly even susceptibility to malarial disease, from the phenotype of resting monocytes. In addition, these data contribute to the development of a new model of NK activation by non-viral pathogens in which activation of accessory cells precedes, rather than follows, NK activation.

subsets of accessory cells to NK cell activation by iRBCs. We demonstrate that cells of the myeloid lineage are not only activated by iRBCs, but also play a crucial role in controlling the magnitude of the early burst of NK cell-derived IFN- $\gamma$  via both cytokine- and contact-dependent signals. Furthermore, we provide evidence that reciprocal interactions between NK cells and myeloid accessory cells regulate levels of activation of both cell populations, with major implications for the ability of the innate immune system to recognise and respond to infection.

## 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. As we have previously shown that NK cells from only some donors make a strong IFN- $\gamma$  response to iRBCs [7–9], cells from known high-responding donors were used for the majority of experiments described here.

### *P. falciparum* Culture and Antigen Preparation

*P. falciparum* parasites (strain 3D7) were grown in O<sup>Rh-</sup> human erythrocytes (National Blood Service, <http://www.blood.co.uk>) in RPMI 1640 (Gibco, <http://www.invitrogen.com/content.cfm?pageid=11040>) supplemented with 25 mM HEPES (Sigma-Aldrich, <http://www.sigmaaldrich.com>), 28 mM sodium bicarbonate (BDH, <http://uk.vwr.com>), 20  $\mu$ g/l hypoxanthine (Sigma-Aldrich), and 10% normal human AB serum (National Blood Service). Cultures were gassed with 3% O<sub>2</sub>, 4% CO<sub>2</sub>, and 93% N<sub>2</sub> and incubated at 37 °C. The culture medium was changed daily and the parasitaemia was

determined by examination of Giemsa-stained thin blood smears. Parasite cultures were routinely shown by PCR (Stratagene, <http://www.stratagene.com>) to be free from *Mycoplasma* contamination. Mature schizonts were harvested from cultures of 5%–8% parasitaemia 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, <http://www.wockhardt.co.uk>) and PBMCs were isolated by Histopaque 1077 (Sigma-Aldrich) density gradient centrifugation as described previously [9]. Cells were resuspended at a concentration of 10<sup>6</sup> cells/ml and cultured in flat-bottomed 24-well plates for 24 h. Schizont-infected (iRBCs) or uninfected red blood cells (uRBCs) were added at a ratio of three red blood cells per mononuclear cell.

### Cell Surface and Intracellular Staining for Flow Cytometry

Surface and intracellular staining was performed as described previously [9]. The antibodies used were anti-CD3 PerCP, IgG1 PerCP, and anti-HLA-DR PerCP (all from BD Biosciences, <http://wwwbdbiosciences.com>); anti-CD11c AlexaFluor-647, IgG1 AlexaFluor-647, anti-CD56 AlexaFluor-647, IgG2a AlexaFluor-647, anti-IFN- $\gamma$  FITC, anti-CD14 FITC, IgG1 FITC, anti-CD40 R-PE, anti-CD69 R-PE, IgG2a R-PE, anti-CD80 R-PE-Cy5, and IgG1 R-PE-Cy5 (all from Serotec, <http://www.serotec.com>). Flow cytometric analyses were performed using a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences) and FlowJo analysis software (TreeStar, <http://www.treestar.com>).

### NK Cell Purification

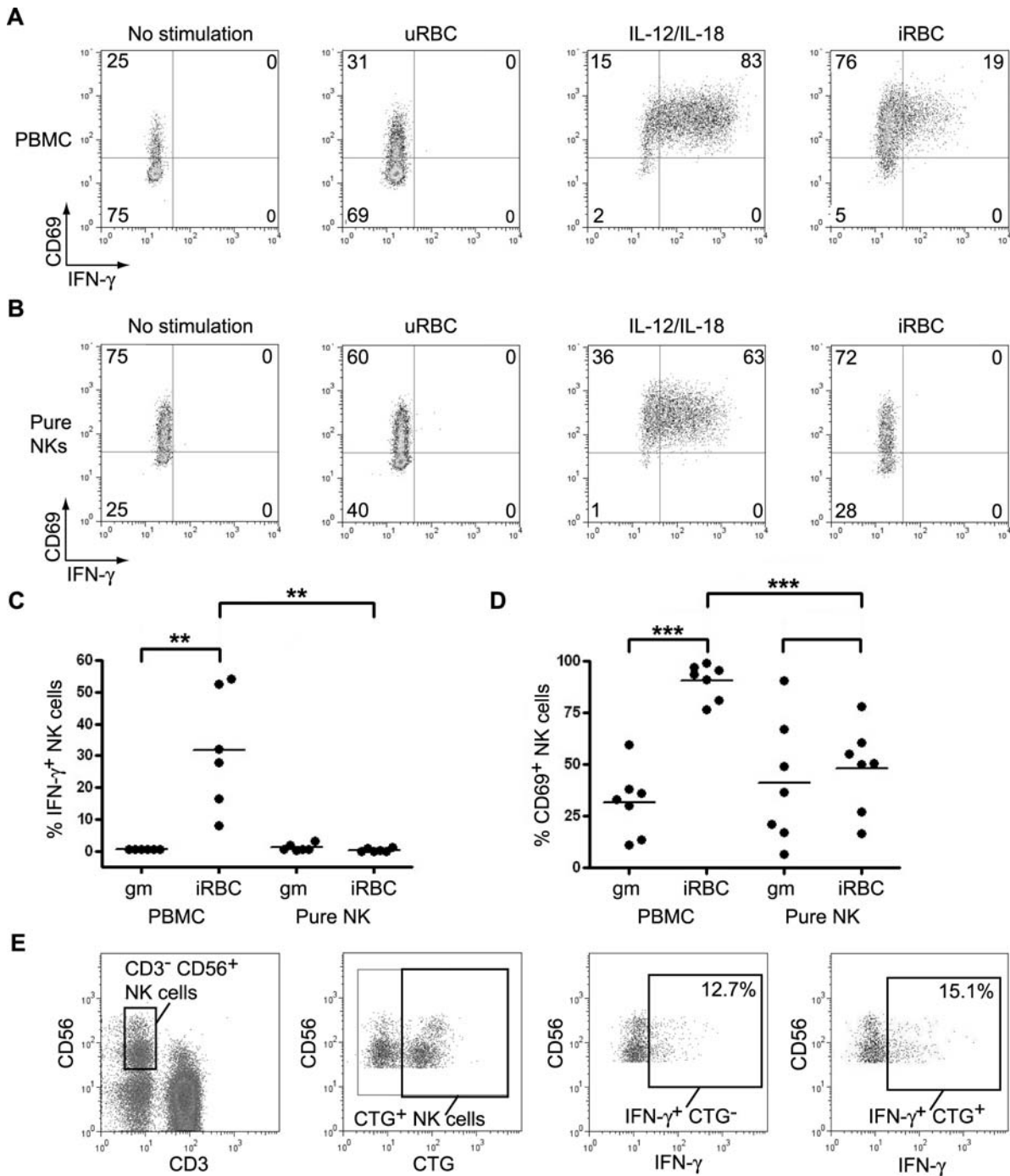
CD56<sup>+</sup> CD3<sup>-</sup> NK cells were enriched from PBMCs by magnetic cell separation (NK Cell Enrichment kit; StemCell Technologies, <http://www.stemcell.com>) according to the manufacturer's instructions and using LS separation columns (Miltenyi Biotec, <http://www.miltenyibiotec.com>). B cells, T cells, monocytes, and erythrocytes were retained in the column, and the effluent containing unlabelled NK cells was collected. NK cells were counted, tested for viability by trypan blue exclusion, and routinely checked by flow cytometry for a purity of greater than 95%.

### Depletion of Accessory Cells or NK Cells

PBMCs were depleted of different populations using monoclonal mouse antibodies to human HLA-DR (Scottish Antibody Production Unit, United Kingdom), CD14 (Serotec), or BDCA-4 (Miltenyi Biotec), and goat anti-mouse IgG MicroBeads (Miltenyi Biotec); CD19 MicroBeads (Miltenyi Biotec); biotinylated CD1c (BDCA-1) antibody and anti-biotin MicroBeads (Miltenyi Biotec); or CD56 positive selection kit (StemCell Technologies).

### Transwell Cultures

PBMCs (1  $\times$  10<sup>6</sup>) in a volume of 800  $\mu$ l of complete medium were placed in a tissue culture well. Purified NK cells (1  $\times$  10<sup>5</sup>) in a volume of 200  $\mu$ l were placed in a Transwell with a 0.4- $\mu$ m microporous polycarbonate membrane (Corning, <http://www.corning.com>) and lowered into the culture well so that all cells were submerged in culture medium. iRBCs (3  $\times$  10<sup>6</sup>, bottom well, or 0.5  $\times$  10<sup>6</sup>, top well) were added as indicated. Plates were cultured for 24 h, and NK cells from the top well



**Figure 1. NK Cells Do Not Respond to iRBCs in the Absence of Accessory Cells**

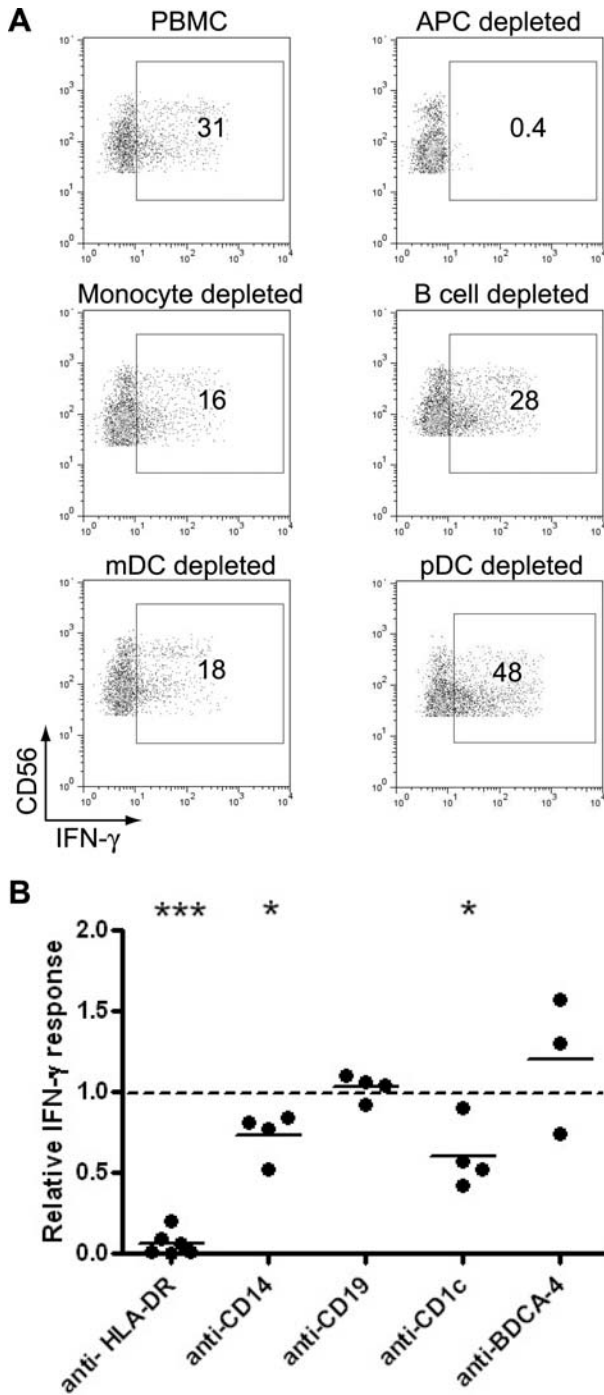
PBMCs or MACS-purified NK cells from malaria-naïve donors were cultured alone or with IL-12 and IL-18, uRBCs, or live iRBCs for 24 h. Intracellular IFN- $\gamma$  and surface expression of CD69 on CD3<sup>-</sup> CD56<sup>+</sup> NK cells were analysed by flow cytometry.

(A and B) Representative example of NK responses to uRBCs, IL-12 and IL-18, and iRBCs in PBMCs (A) and purified NK cells (B). FACS plots are gated on CD3<sup>-</sup> CD56<sup>+</sup> lymphocytes. Percentages indicate the proportion of CD3<sup>-</sup> CD56<sup>+</sup> NK cells in each gate; data are based on the collection of 100,000 total events.

(C and D) Comparison of NK responses to iRBCs in PBMCs and in purified NK cells. (C) NK cell IFN- $\gamma$  production for six donors. (D) CD69 surface expression levels for seven donors. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ;  $p$ -values are for paired  $t$  tests comparing stimulated and unstimulated NK cells from the same donor, or PBMCs and pure NK cells from the same donor.

(E) MACS-purified, CTG-labelled NK cells were added to autologous PBMCs. IFN- $\gamma$  production in CTG<sup>+</sup> (MACS-purified) and CTG<sup>-</sup> (“untouched”) NK cells was assessed by flow cytometry after 24 h of stimulation with iRBCs. A representative example of the software gating strategy to analyse IFN- $\gamma$  responses of CTG-labelled NK cells is shown. FACS plots were gated on CD3<sup>-</sup> CD56<sup>+</sup> NK cells within the lymphocyte population (first panel), and NK cells were analysed for CTG staining (second panel). CTG<sup>+</sup> CD3<sup>-</sup> CD56<sup>+</sup> NK cells (third panel) or CTG<sup>-</sup> CD3<sup>-</sup> CD56<sup>+</sup> NK cells (fourth panel) were selected and analysed for intracellular IFN- $\gamma$  production. Numbers indicate the proportion of IFN- $\gamma$ <sup>+</sup> NK cells.

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**Figure 2.** HLA-DR<sup>+</sup> Accessory Cells Provide Activating Signals to NK Cells  
 PBMCs from malaria-naïve donors were depleted of different populations of accessory cells, and NK cell responses to iRBCs were analysed after 24 h of co-culture.

(A) Representative example of NK cell IFN- $\gamma$  responses in undepleted PBMCs (top left), PBMCs depleted of all HLA-DR<sup>+</sup> antigen-presenting cells (APC) (top right), CD14<sup>+</sup> monocyte-depleted PBMCs (middle left), CD19<sup>+</sup> B cell-depleted PBMCs (middle right), CD1c<sup>+</sup> mDC-depleted PBMCs (bottom left), and BDCA-4<sup>+</sup> pDC-depleted PBMCs (bottom right). FACS plots are gated on CD3<sup>-</sup> CD56<sup>+</sup> lymphocytes. Percentages indicate the proportion of CD3<sup>-</sup> CD56<sup>+</sup> NK cells that were positive for IFN- $\gamma$ ; data are based on the collection of 100,000 total events.

(B) NK cell IFN- $\gamma$  production after depletion of different antigen-presenting cell populations. Percentage of IFN- $\gamma$  NK cells relative to the response in undepleted PBMCs is shown; each spot represents data for a different donor. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.0001$ ;  $p$ -values are for paired  $t$  tests of the difference between depleted and undepleted cells:  
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and/or PBMCs from the bottom well were harvested, stained, and analysed by flow cytometry as previously described.

**Recombinant Cytokines and LPS**

In all experiments a combination of human recombinant IL-12 (Peprotech, <http://www.peprotech.com>) and human recombinant IL-18 (MBL International, <http://www.mblintl.com>), each at 0.1  $\mu\text{g}/10^6$  cells, was used as a positive control. Bioactive human recombinant transforming growth factor (TGF)- $\beta$  (R&D Systems, <http://www.rndsystems.com>) was used at the concentrations indicated. LPS purified from *Escherichia coli* (Sigma-Aldrich) were used at a concentration of 1  $\mu\text{g}/10^6$  cells.

**Cytokine Blocking**

The following neutralising antibodies were used to block cytokines or their respective receptors: polyclonal rabbit anti-human IL-2 (Serotec), polyclonal rabbit anti-human IL-15 (Biosource, <http://www.biosource.com>), monoclonal mouse anti-human IFN- $\alpha/\beta$  receptor chain 2 (Chemicon, <http://www.chemicon.com>), and polyclonal chicken anti-TGF- $\beta$  (R&D Systems). The antibodies or their respective isotype-matched controls were added to PBMC cultures prior to the addition of iRBCs and at the concentrations indicated.

**Quantification of Cytokine mRNA by Real-Time Quantitative PCR**

For each donor,  $10^7$  PBMCs were cultured for 24 h in the presence or absence of  $3 \times 10^7$  iRBCs. Non-adherent cells were discarded and adherent cells harvested after 15 min incubation on ice followed by scraping. Total RNA was isolated (RNeasy Micro kit; Qiagen, <http://www1.qiagen.com>) and cDNA was produced using oligo(dT)<sub>12-18mer</sub> primers and SuperScript III reverse transcriptase (Invitrogen, <http://www.invitrogen.com>) according to the manufacturer's instructions.

Real-time quantitative PCR (ABI Prism 7000; Applied Biosystems, <http://www.appliedbiosystems.com>) was carried out using QuantiTect SYBR Green (Qiagen) and the following primers:  $\beta$ -actin (5'-ACCCAGATCATGTTTGAGACCT-3' and 5'-TCCATCAGCATGCCAGTG-3'), IL-2 (5'-AGTCCCTGGGTCTTAAGTGAAAG-3' and 5'-CAAAGGCCACAGAACTGAA-3'), IL-10 (5'-TCTGGGTCTTGGTTCTCAGC-3' and 5'-TGGAGGACTTAAAGGTTACCTG-3'), IL-12 $\alpha$  (encodes p35 subunit) (5'-CTCCAGAAGGCCAGACAAAC-3' and 5'-AATGGTAAACAGGGCTCCACT-3'), IL-15 (5'-TGATGTTACCC-CAGTTGC-3' and 5'-TTCCTCACATTCTTTCATCC-3'), IL-18 (5'-CAGACCTCCAGATCGCTTC-3' and 5'-GGGTGCATTATCTCTACAGTCAGAA-3'), IL-23 (5'-GTGGGACACATGGATCTAAGAGA-3' and 5'-GGATCCTTTGCAAGCAGAAC-3'), IFN- $\alpha$  (5'-GCCATCTCTGTCCTCCATGA-3' and 5'-CATGATTTCTGCTCTGACAACCT-3'), and IFN- $\gamma$  (5'-TTCGGTAACTGACTTGAATGTCC-3' and 5'-TCTTCGACCTTGAACAGCA-3').

**TGF- $\beta$  ELISA**

The concentration of total (bioactive and latent) and spontaneously bioactive TGF- $\beta$  in culture supernatants was assessed using the Human TGF- $\beta$  1 DuoSet system (R&D Systems) according to the manufacturer's instructions.

## Luminex Bead-Based Cytokine Quantification

Multiplex quantification of cytokine concentrations in tissue culture supernatants was performed using the Luminex 100 system (Luminex, <http://www.luminexcorp.com>) with Bio-Plex upgrade (Bio-Rad Laboratories, <http://www.bio-rad.com>). Levels of IL-2, IL-10, and IL-12p70 were determined using the Beadlyte Human Multi-Cytokine Detection System 3 (Upstate, <http://www.upstate.com>) according to the manufacturer's instructions. Data analysis was performed using the Bio-Plex Manager software (Bio-Rad).

## Statistical Analysis

All statistical analyses were performed using Prism 4 software (GraphPad Software, <http://www.graphpad.com>). All  $r^2$  values given refer to a second-order polynomial fit of data shown. If data are plotted using log scale, analysis was carried out using log values. All  $t$  tests carried out were two-tailed paired  $t$  tests with 95% confidence intervals. Comparisons were made between actual data even where log axes have been used to plot data.

## Results

### Optimal NK Cell Response to iRBCs Is Accessory Cell-Dependent

We have previously shown that cloned NK cell lines are unable to respond to iRBCs in the absence of accessory cells that are present among PBMCs [8]. To confirm and extend this observation, NK cells were purified from PBMCs of seven different donors by magnetic cell sorting (MACS) and stimulated for 24 h with IL-12 and IL-18, uRBCs, or iRBCs or left unstimulated and analysed for surface expression of CD3, CD56, CD69, and intracellular IFN- $\gamma$ ; unsorted PBMCs from the same donors were stimulated and analysed in an equivalent fashion (Figure 1). In PBMC cultures, as expected, IFN- $\gamma^+$  (CD3 $^-$  CD56 $^+$ ) NK cells were detected in both cytokine-stimulated and iRBC-stimulated cultures but not in unstimulated or uRBC-stimulated cultures (Figure 1A). We deliberately selected donors who were known, from previous studies, to be relatively high responders in this assay; nevertheless, the magnitude of the IFN- $\gamma$  response to iRBCs varied between donors, with between 5% and 50% of NK cells making IFN- $\gamma$  in response to iRBCs after 24 h (Figure 1C). In complete contrast, although purified NK cells made IFN- $\gamma$  in response to cytokine stimulation (Figure 1B), no IFN- $\gamma$  was produced by purified NK cells in response to iRBCs (Figure 1B and 1C).

We have shown that up-regulation of surface CD69 expression is a marker of partial (early) activation of NK cells [9], and thus, as expected, up-regulation of CD69 was consistently seen on NK cells within the iRBC-stimulated PBMC population (Figure 1D). Surface CD69 expression tended to be higher on unstimulated purified NK cells than on unstimulated NK cells within PBMCs, presumably because of partial activation during cell separation. However, CD69 surface expression was not significantly up-regulated on purified NK cells after stimulation with iRBCs, and NK CD69 expression was significantly higher among iRBC-stimulated PBMC NK cells than among purified NK cells (Figure 1D).

Importantly, the inability of purified NK cells to respond to iRBCs is not an artefact of the NK cell purification process as MACS-purified NK cells labelled with CellTracker Green

(CTG) and added back to autologous PBMC cultures respond as well to iRBC stimulation as do "untouched" NK cells within PBMCs (Figure 1E).

Thus, these data indicate that accessory cells within PBMCs are required for both IFN- $\gamma$  and CD69 expression among iRBC-stimulated NK cells.

### Depletion of CD1c $^+$ and CD14 $^+$ Accessory Cells Inhibits the NK Cell Response

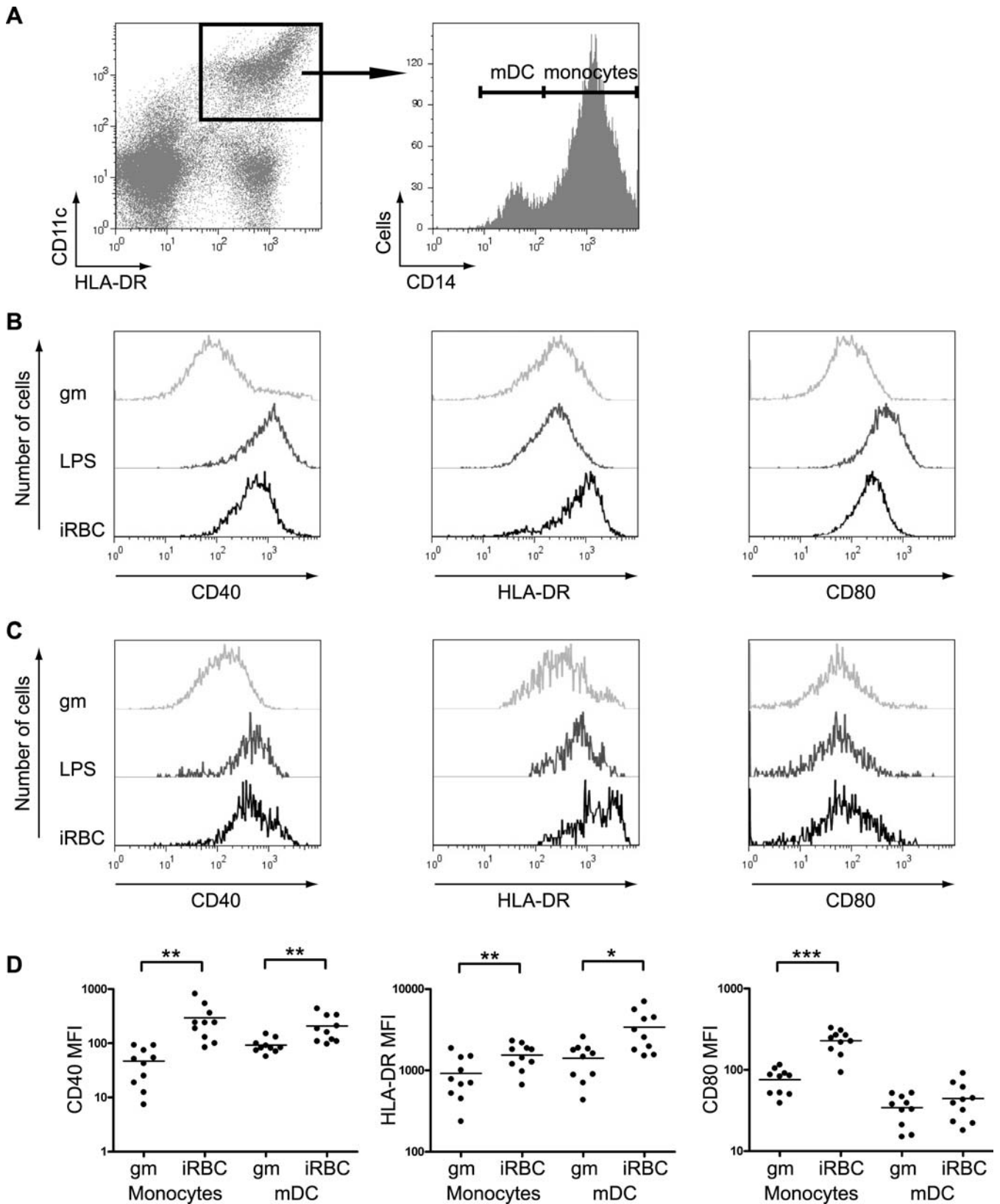
To identify the accessory cell population(s) required for NK cell activation by iRBCs, PBMCs of known high-responding donors were depleted (by MACS) of cells expressing specific cell surface markers prior to incubation with iRBCs (Figure 2). PBMCs of all the donors tested in these experiments responded to iRBC stimulation, with 5%–40% of all NK cells producing IFN- $\gamma$  after 24 h. Depletion of accessory cell populations did not affect the ability of NK cells to produce IFN- $\gamma$  following stimulation with positive control stimuli (IL-12 and IL-18; Figure S1). Depletion of HLA-DR $^+$  cells completely abolished the NK cell IFN- $\gamma$  response to iRBCs in all donors, but depletion of BDCA-4 $^+$  plasmacytoid DCs (pDCs) had no consistent effect on the NK IFN- $\gamma$  response. Likewise, depletion of CD19 $^+$  B cells did not reduce the NK cell IFN- $\gamma$  response. However, depleting PBMCs of either CD1c $^+$  myeloid DCs (mDCs) or CD14 $^+$  monocytes significantly reduced NK cell IFN- $\gamma$  responses (monocytes, paired  $t$  test,  $t = 3.63$ ,  $p = 0.036$ ; mDCs, paired  $t$  test,  $t = 3.82$ ,  $p = 0.032$ ). In the one donor where depletion using either CD14 or CD1c did not substantially reduce the IFN- $\gamma$  response (relative response, monocyte-depleted = 0.84, mDC-depleted = 0.90), a double depletion using both antibodies together was performed. In this case the IFN- $\gamma$  response was substantially reduced (relative response = 0.32).

These data indicate that the NK cell response to iRBCs is not dependent on a single population of accessory cells; rather, both mDCs and monocytes can provide activating signals for NK cells, and, in most donors, optimal NK activation requires both of these cell types to be present.

### Monocytes and mDCs Are Activated by iRBCs

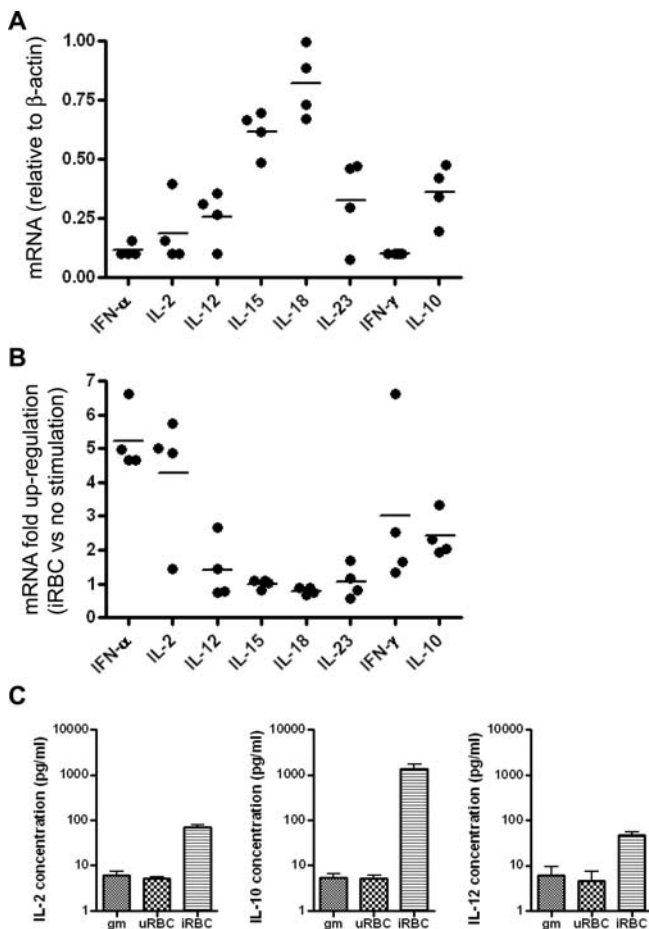
Given the numerous published reports indicating that monocyte and DC function is inhibited by exposure to iRBCs [13,14], it was important to determine whether the accessory cells required for NK cell activation were themselves activated by exposure to iRBCs. PBMCs from ten different donors were therefore stimulated with uRBCs, iRBCs, or *E. coli* LPS (or left unstimulated, growth medium alone [gm]) for 24 h, and then mDC and monocyte populations were analysed for expression of the activation markers CD40, HLA-DR, and CD80 (Figure 3; isotype control data to exclude non-specific binding of labelled antibodies by activated accessory cells are shown in Figure S2).

Significant up-regulation of CD40, CD80, and HLA-DR was observed on monocytes from all donors following 24 h incubation with iRBCs (CD40, mean fold increase of median fluorescence intensity [MFI] = 6.3, paired  $t$  test,  $t = 3.59$ ,  $p = 0.006$ ; HLA-DR, mean fold increase of MFI = 1.7, paired  $t$  test,  $t = 3.42$ ,  $p = 0.008$ ; CD80, mean fold increase of MFI = 3.0, paired  $t$  test,  $t = 7.56$ ,  $p < 0.0001$ ). Activation of mDCs was also observed, with significant up-regulation of CD40 and HLA-DR expression (CD40, mean fold increase of MFI = 2.3, paired  $t$  test,  $t = 3.60$ ,  $p = 0.006$ ; HLA-DR, mean fold increase of MFI =



**Figure 3. Accessory Cells Are Activated by iRBCs**

PBMCs from ten malaria-naïve donors were cultured with live iRBCs or *E. coli* LPS for 24 h or left unstimulated. Surface expression of HLA-DR, CD40, and CD80 on accessory cells was analysed by flow cytometry. Accessory cells were defined as HLA-DR<sup>+</sup> CD11c<sup>+</sup> cells (A, left), and CD14<sup>+</sup> monocytes and CD14<sup>-</sup> mDCs were selected for further analysis (A, right). Representative examples of surface expression levels of CD40, HLA-DR, and CD80 on HLA-DR<sup>+</sup> CD11c<sup>+</sup> CD14<sup>+</sup> monocytes (B) and HLA-DR<sup>+</sup> CD11c<sup>+</sup> CD14<sup>-</sup> mDCs (C) are shown. Data are based on the collection of 100,000 total events. Results for all ten donors are summarised in (D). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ;  $p$ -values are for paired  $t$  tests comparing stimulated and unstimulated cells from the same donor. doi:10.1371/journal.ppat.0020118.g003



**Figure 4.** Accessory Cell-Derived Cytokines Are Upregulated in Response to iRBCs

(A and B) PBMCs from four donors were stimulated with iRBCs for 24 h, and the amount of IFN- $\alpha$ , IL-2, IL-12, IL-15, IL-18, IL-23, IFN- $\gamma$ , and IL-10 mRNA in adherent cells was determined by real-time quantitative PCR. The resting levels relative to the amount of  $\beta$ -actin mRNA are shown in (A). The fold up-regulation in response to iRBC stimulation is shown in (B).

(C) Cytokine levels in tissue culture supernatants were determined for eight donors in a bead-based cytokine quantification assay. Levels of IL-2 (left), IL-10 (middle), and IL-12 (right) for no stimulation (gm), uRBC stimulation, and iRBC stimulation are shown (arithmetic mean plus standard error of the mean).

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2.4, paired *t* test,  $t = 3.16$ ,  $p = 0.012$ ). There was, however, no significant change in expression of CD80 on mDCs after 24 h co-incubation with iRBCs (mean fold increase of MFI = 1.3, paired *t* test,  $t = 2.04$ ,  $p = 0.072$ ).

These data indicate that both mDCs and monocytes are activated after 24 h co-incubation with iRBCs but that the nature of activation differs between the two accessory cell populations. Up-regulation of CD40 is more pronounced in monocytes and up-regulation of HLA-DR is more pronounced in mDCs, whilst up-regulation of CD80 occurs exclusively in monocytes.

#### Accessory Cells Express Both Pro- and Anti-Inflammatory Cytokines in Response to Activation by iRBCs

To characterise further the activation of accessory cells by iRBCs, we analysed the cytokine response profile after culturing PBMCs for 24 h with iRBCs by quantitative real-

time PCR analysis of adherent cells and by Luminex cytokine bead array analysis of culture supernatants (Figure 4).

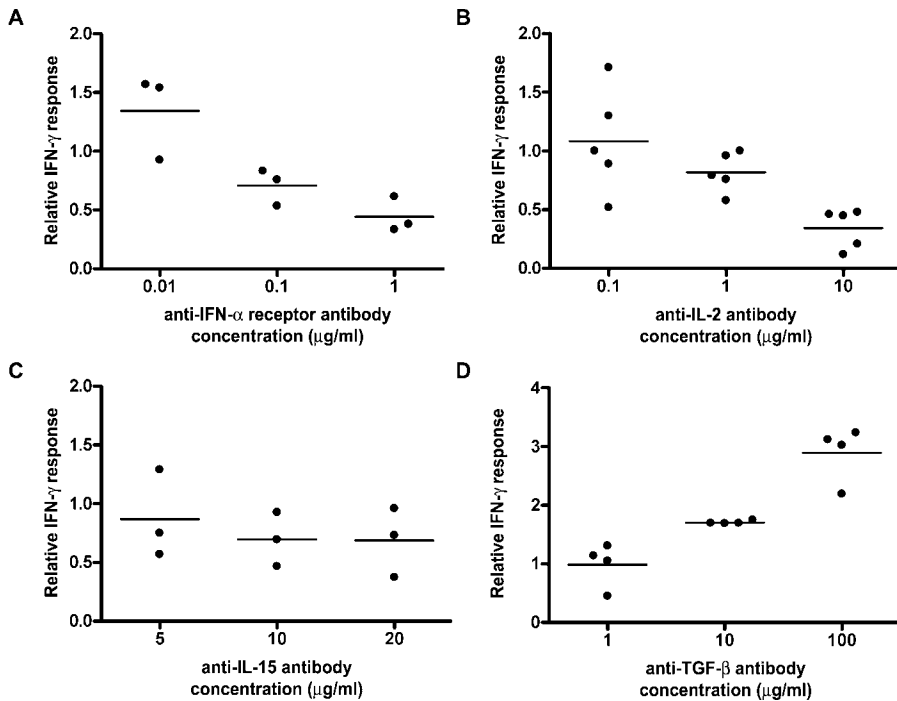
Unstimulated adherent cells expressed detectable levels of mRNA for IL-15, IL-18, and, at somewhat lower levels, IL-10, IL-12, and IL-23, indicating either constitutive expression of these cytokines or their induction during the cell separation process. Levels of IFN- $\alpha$ , IL-2, and IFN- $\gamma$  mRNA were below the limits of detection of the assay (Figure 4A). However, after iRBC stimulation there was very marked up-regulation of mRNA for IFN- $\alpha$  and IL-2 and, to a somewhat lesser extent, for IL-10 and IFN- $\gamma$  but not for IL-12, IL-15, IL-18, or IL-23 (Figure 4B). Taken together, these data indicate constitutive or induced expression of IL-12, IL-15, IL-18, IL-23, IFN- $\alpha$ , IL-2, IL-10, and IFN- $\gamma$  by adherent mononuclear cells stimulated with iRBCs. Production of IL-2, IL-10, and IL-12 in response to iRBC stimulation was confirmed at the protein level (Figure 4C); the discrepancy between mRNA and protein levels for IL-12 is most likely explained by the timing of the assays (all of which were conducted after 24 h).

#### Type 1 IFN and IL-2, but Not IL-15, Are Required for NK Cell Activation by iRBCs whilst TGF- $\beta$ Suppresses This Response

NK cell activation by pathogens is typically dependent on accessory cell-secreted cytokines [19–22], and we have shown that IL-12 and, to a somewhat lesser extent, IL-18 are required for optimal NK responses to iRBCs [7,8]. To determine whether other accessory cell-derived cytokines are also required for NK activation, the response to iRBCs was analysed in the presence or absence of neutralising antibodies to individual cytokines or their receptors. Of particular interest were those cytokines that we had found (as described above) to be specifically and strongly upregulated by iRBCs, namely IFN- $\alpha$  and IL-2. Also, since TGF- $\beta$  has recently been reported to be a powerful inhibitor of NK cell activation [23], and since we have shown that induction of TGF- $\beta$  in vivo in both human and rodent malaria infections is associated with lack of early IFN- $\gamma$  responses [24,25], it was of considerable interest to determine whether TGF- $\beta$  might regulate the NK cell IFN- $\gamma$  response to iRBCs in our in vitro assay system where platelets and mononuclear cells provide an abundant source of latent TGF- $\beta$ . Representative fluorescence activated cell sorting (FACS) plots showing the effect of blocking cytokines on IFN- $\gamma$  response to iRBCs are shown in Figure S3.

In the presence of a blocking antibody to the IFN- $\alpha$ / $\beta$  receptor, there is a significant and dose-dependent inhibition of the NK cell IFN- $\gamma$  response to iRBCs (highest antibody dose, paired *t* test,  $t = 6.42$ ,  $p = 0.023$ ) (Figure 5A) and a similar, but less marked, reduction in CD69 expression (highest antibody dose, paired *t* test,  $t = 5.26$ ,  $p = 0.034$ ) (Figure S4). Similarly, neutralisation of IL-2 markedly inhibited NK cell IFN- $\gamma$  responses in a dose-dependent manner (highest antibody dose, paired *t* test,  $t = 8.79$ ,  $p = 0.001$ ) (Figure 5B), and CD69 surface expression was also reduced, but to a lesser degree (highest antibody dose, paired *t* test,  $t = 4.43$ ,  $p = 0.021$ ) (Figure S4). On the other hand, neutralisation of IL-15 had only a minimal effect on NK cell IFN- $\gamma$  responses (highest antibody dose, paired *t* test,  $t = 1.83$ ,  $p = 0.209$ ) (Figure 5C) or CD69 expression (highest antibody dose, paired *t* test,  $t = 3.36$ ,  $p = 0.078$ ) (Figure S4).

When PBMCs were incubated with iRBCs for 24 h in the presence or absence of increasing doses of neutralising



**Figure 5.** The NK Cell Response to iRBCs Is Controlled by Type 1 IFN, IL-2, and TGF- $\beta$

PBMCs from malaria-naïve donors were stimulated for 24 h with iRBCs in the presence of increasing concentrations of neutralising antibodies to (A) the IFN- $\alpha/\beta$  receptor, (B) IL-2, (C) IL-15, or (D) TGF- $\beta$ . Percentages of IFN- $\gamma$  NK cells relative to those observed with the respective concentration of isotype-matched control antibodies are shown. Only donors known to make a strong IFN- $\gamma$  response to iRBCs were used; baseline percentages of NK cells that were IFN- $\gamma^+$  after iRBC stimulation ranged from 5% to 40% for the donors shown here. doi:10.1371/journal.ppat.0020118.g005

antibody to TGF- $\beta$ , there was a highly consistent, dose-dependent increase in the proportion of NK cells synthesising IFN- $\gamma$ , with up to a 3-fold increase in IFN- $\gamma^+$  NK cells in the presence of 100  $\mu\text{g/ml}$  antibody (paired  $t$  test,  $t = 7.98$ ,  $p = 0.004$ ) (Figure 5D) and a slight, but statistically significant, increase in CD69 expression (highest antibody dose, paired  $t$  test,  $t = 4.00$ ,  $p = 0.028$ ) (Figure S4). Conversely, addition of recombinant human TGF- $\beta$  led to a very marked, dose-dependent suppression of the NK cell IFN- $\gamma$  response to iRBCs (highest dose, 1 ng/ml; paired  $t$  test,  $t = 12.8$ ,  $p = 0.001$ ; unpublished data).

### Optimal NK Cell Response to iRBCs Requires Contact between NK Cells and Accessory Cells

For some microbial stimuli it has been reported that optimal NK cell activation requires contact with accessory cells [12,22,26,27]. We thus sought to determine whether such contact-dependent signals were also required for NK activation by iRBCs. Purified NK cells (in the upper chamber of a Transwell culture system) were separated from PBMCs (in the lower chamber) by a microporous membrane (0.4- $\mu\text{m}$  diameter), which allowed diffusion of small molecules including cytokines, but not migration of leukocytes, iRBCs, or extracellular malarial merozoites. When a soluble positive control stimulus (phytohaemagglutinin) was added to the lower well, NK cells in the inner (top) well responded normally, indicating that diffusion of soluble signals was not impeded (Figure S5). When iRBCs were added to the lower chamber, NK cells within the PBMC population synthesised IFN- $\gamma$  (Figure 6A, PBMC control) and upregulated CD69 (Figure 6B, PBMC control). However, addition of iRBCs to the

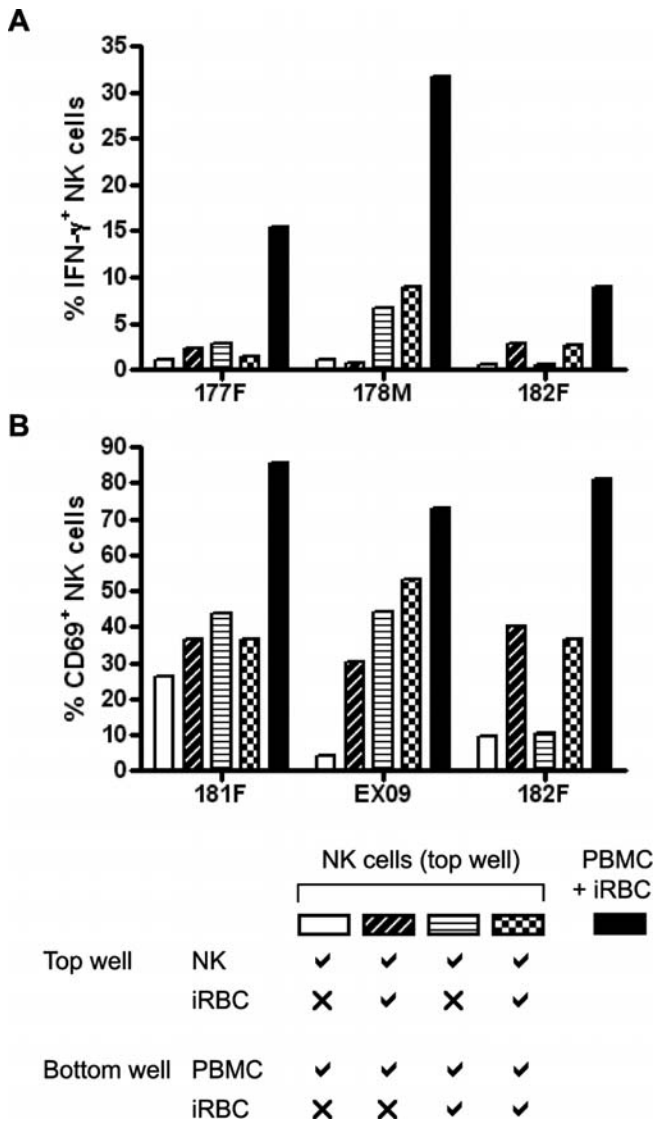
upper well, the lower well, or both the upper and lower wells together was insufficient to induce optimal IFN- $\gamma$  production among the NK cells in the upper well, suggesting that contact-mediated interactions between NK cells and iRBCs and/or between accessory cells and iRBCs are insufficient for full NK activation and that direct cell-cell contact between the NK cells and the accessory cells is required.

Contact between NK cells and accessory cells was also required for optimal up-regulation of CD69, although partial up-regulation of CD69 was observed in some cases when purified NK cells were in contact with iRBCs and/or when accessory cells were in contact with iRBCs. The up-regulation of CD69 by purified NK cells in the Transwell system (Figure 6), but not in regular cultures of purified NK cells (Figure 1), is presumably due to the presence of soluble, cytokine-mediated signals derived from PBMCs in the lower well.

### NK Cell-Derived Signals Maintain the Maturation Status of Accessory Cells but Are Not Required for Accessory Cell Responses to iRBCs or LPS

In recent years it has become apparent that interactions between NK cells and DCs can be bidirectional. NK cells, especially those activated by prior exposure to IL-2, enhance DC maturation, inflammatory cytokine secretion, and antigen presentation in allogeneic situations and when exposed to virus-infected cells [28–30]. On the other hand, the degree of reciprocity between NK cells and monocytes, and the potential for NK cells to augment accessory cell responses to non-viral pathogens, has not to our knowledge been investigated. We therefore examined whether the interactions that we had observed to be crucial for the activation of





**Figure 6.** Contact between NK Cells and Accessory Cells Is Necessary for an Optimal Response to iRBCs

Purified NK cells and autologous PBMCs were co-cultured in Transwell plates, separated by a microporous membrane. The presence of iRBCs in the respective compartments, purified NK cells in the top well and PBMCs in the bottom well are indicated in the key to the figure. Intracellular production of IFN- $\gamma$  (A) and surface expression levels of CD69 (B) on NK cells in the top well were analysed by flow cytometry. The black bars represent NK cell responses for PBMCs stimulated with iRBCs. All other bars represent values for purified NK cells (upper well). Percentages indicate the proportion of CD3<sup>-</sup> CD56<sup>+</sup> NK cells that were positive for IFN- $\gamma$ ; data are based on the collection of 100,000 total events. doi:10.1371/journal.ppat.0020118.g006

NK cells by iRBCs led to reciprocal activation of either monocytes or mDCs.

Whole PBMCs or PBMCs depleted of NK cells were cultured for 24 h without stimulation (gm) or with LPS or iRBCs. After 24 h, cells were stained for HLA-DR, CD14, either CD40 or CD80, and either CD11c or CD11b (Figure 7A). Both mDCs and monocytes expressed significantly lower levels of activation markers when cultured (without additional stimulation) in the absence of NK cells (Figure 7B–7D), confirming a previous report of a role for NK cells in

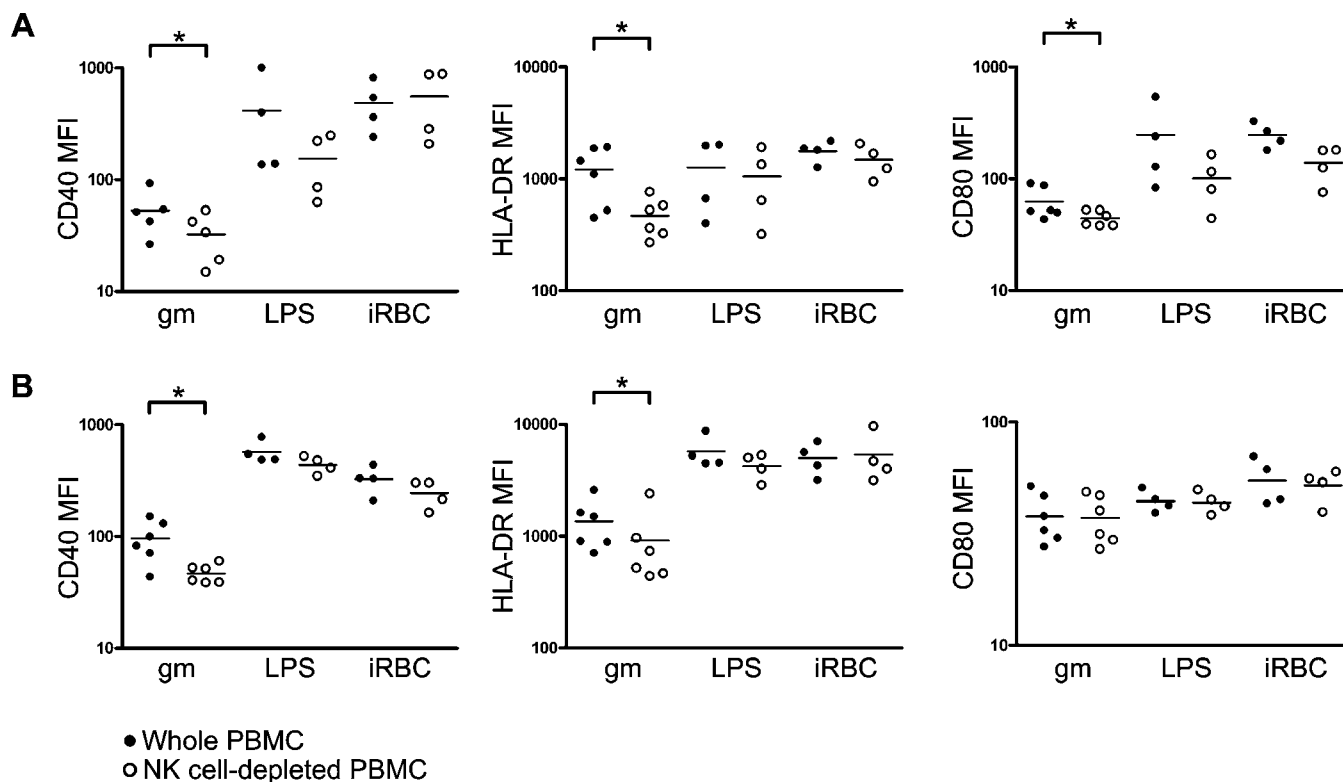
promoting DC maturation [29] and extending this observation to monocytes. On the other hand, when cells were cultured with either LPS or iRBCs, there was no significant effect of the presence or absence of NK cells on expression levels of any of the activation markers in either mDCs or monocytes (Figure 7B–7D). Thus, while NK cells play a role in maturation of both monocytes and mDCs in resting conditions, the response of either of these cell types to LPS or iRBC stimulation is largely NK cell-independent. These data suggest that, at this early time point, the major inducers of myeloid cell activation are the pathogens themselves, rather than NK-derived IFN- $\gamma$ .

**Degree of Monocyte Activation Correlates with Magnitude of NK Cell IFN- $\gamma$  Response**

We have previously shown that human NK cell responses to iRBCs are heterogeneous [8,9]. Here, we noticed a considerable degree of heterogeneity of accessory cell surface marker expression (Figure 3D) and of cytokine production (Figure 4) among donors, in terms of both resting levels and responses to iRBCs. Given the absolute dependence of the NK cell response to iRBCs on accessory cells, we sought to determine whether heterogeneity of NK cell responses correlated with heterogeneity of accessory cell responses. Since NK cell IFN- $\gamma$  responses can vary between CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells—presumably because of differential expression of NK cell receptors including the killer cell immunoglobulin-like receptors (KIRs), which are expressed by CD56<sup>dim</sup> but not CD56<sup>bright</sup> NK cells—we analysed the correlation between accessory cell and NK cell responses for both of these NK cell populations.

Highly significant correlations were observed between iRBC-induced concentrations of IL-12 (Figure 8A) and IL-2 (Figure 8B) and the magnitude of the NK cell IFN- $\gamma$  response. A particularly close correlation was observed between IL-12 concentrations and the proportion of IFN- $\gamma$ <sup>+</sup> CD56<sup>bright</sup> (KIR<sup>-</sup>) cells, whilst, for several donors (see arrows in Figure 8), a significant discrepancy was seen in the response of the CD56<sup>dim</sup> population. Somewhat surprisingly, there was no correlation between iRBC-induced up-regulation of HLA-DR, CD40, or CD80 on either monocytes or mDCs and NK IFN- $\gamma$  production (unpublished data). However, resting levels of expression of both CD40 and HLA-DR were tightly correlated with the NK cell IFN- $\gamma$  response (Figure 8C and 8D). For resting levels of CD40 expression there was again a much closer correlation with the IFN- $\gamma$  response of CD56<sup>bright</sup> NK cells than with that of CD56<sup>dim</sup> NK cells. One plausible interpretation of these data is that the NK cell IFN- $\gamma$  response is very sensitive to accessory cell CD40 expression and IL-12 production, but that in CD56<sup>dim</sup> cells this response is moderated by KIR/major histocompatibility complex (MHC) class I-mediated signals.

Our observation that TGF- $\beta$  is able to markedly suppress NK cell IFN- $\gamma$  responses (Figure 5) led us to consider whether the heterogeneity of NK cell responses was due to differential production and/or activation of TGF- $\beta$ . There was no consistent increase or decrease in the concentration of bioactive TGF- $\beta$  in the supernatants of iRBC-stimulated PBMCs; a modest increase in bioactive TGF- $\beta$  concentration was observed for two donors, but these were, in fact, the donors with the highest NK cell IFN- $\gamma$  responses to iRBCs, suggesting that suppression by TGF- $\beta$  does not explain the



**Figure 7.** NK Cells Are Required to Maintain Maturation Status of Accessory Cells

Whole PBMCs (closed circles) or NK cell-depleted PBMCs (open circles) were cultured alone or with *E. coli* LPS or iRBCs for 24 h. The MFIs of CD40, HLA-DR, and CD80 surface staining on (A) monocytes and (B) mDCs were determined by flow cytometric analysis. \*,  $p < 0.05$ ;  $p$ -values are for paired  $t$  tests comparing whole PBMCs with NK cell-depleted PBMCs from the same donor.

doi:10.1371/journal.ppat.0020118.g007

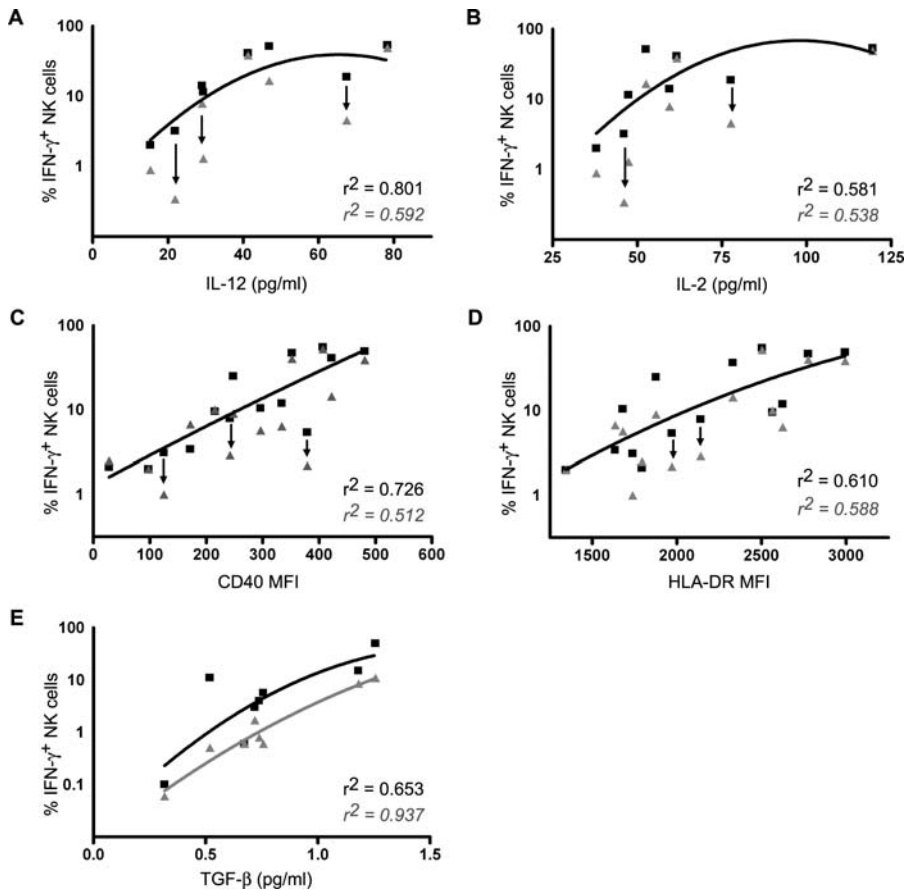
heterogeneity of NK cell IFN- $\gamma$  response. Rather, regulation of bioactive TGF- $\beta$  appears to be positively correlated with NK cell IFN- $\gamma$  expression (Figure 8E), and furthermore, the correlation with CD56<sup>dim</sup> NK cells was higher than that with CD56<sup>bright</sup> NK cells. This suggests that TGF- $\beta$  is not causal in NK cell activation, but rather that the total amount of IFN- $\gamma$  produced determines the amount of TGF- $\beta$ . Thus, activation may lead to induction of negative feedback mechanisms in order to prevent excessive pro-inflammatory responses.

## Discussion

By carefully dissecting the requirements for activation of human NK cells by iRBCs, we have revealed that the early NK cell-derived IFN- $\gamma$  response is absolutely dependent upon contact with, and cytokine production by, accessory cells. We have identified the requisite accessory cells as monocytes and mDCs and have shown that both cell types become highly activated during the first 24 h of exposure to iRBCs. Furthermore, we have demonstrated that whilst NK cells provide essential signals that maintain the maturation status of resting monocytes and mDCs, NK cells are not required for these same cells to respond fully to iRBCs. Finally, we have shown that the capacity of NK cells to secrete IFN- $\gamma$  is highly correlated with both the resting phenotype of the accessory cells and the concentration of iRBC-induced monokines. Thus, functional heterogeneity of monocytes and mDCs among human populations appears to play a crucial role in

determining the magnitude of the innate immune response to iRBCs.

A wealth of evidence suggests that, after interaction with malarial parasites, both monocytes and mDCs secrete immunosuppressive cytokines and have an impaired ability to respond to a variety of stimulatory ligands [13–17,31]. These data have led some to conclude that monocyte and DC function is universally impaired by contact with malarial parasites and that this underlies an apparent suppression of immune responses seen during malaria infections. However, there have been several reports of activation of monocytes and DCs by *Plasmodium*-derived ligands [32–34]. Studies of rodent malaria infections have begun to resolve this debate, showing that in the first 24 h of infection, splenic DCs are activated, secrete pro-inflammatory cytokines including IL-12 and tumour necrosis factor- $\alpha$ , and present antigen to T cells, but that they subsequently become refractory to re-stimulation and secrete anti-inflammatory cytokines such as IL-10 [35]. This sequence of events allows infection to be controlled whilst simultaneously avoiding immune-mediated pathology [25]. Here we show that human monocytes and mDCs are highly activated after 24 h of co-incubation with iRBCs. The myeloid cell response occurred in the presence or absence of NK cells, indicating that direct interaction with parasites—as opposed to NK-derived IFN- $\gamma$ —drives the initial activation of these cells. Furthermore, this activation initiates and regulates the crucial early burst of IFN- $\gamma$  from NK cells. However, our observation that NK activation goes hand in hand with IL-10 and TGF- $\beta$  production, and that TGF- $\beta$  can suppress



**Figure 8.** NK Cell IFN- $\gamma$  Response to iRBCs Correlates with Accessory Cell Activation

NK cell IFN- $\gamma$  responses in PBMCs from eight malaria-naïve donors were analysed after 24 h of co-culture with iRBCs. The concentration in tissue culture supernatants of IL-12p70 and IL-2 was determined in a bead-based cytokine assay, and that of bioactive TGF- $\beta$  by ELISA. The fluorescence intensities of CD40 and HLA-DR surface staining on CD14<sup>+</sup> monocytes and the percentage of IFN- $\gamma$ <sup>+</sup> CD56<sup>bright</sup> NK cells were determined by flow cytometric analysis. Shown are the percentages of IFN- $\gamma$ <sup>+</sup> CD56<sup>bright</sup> NK cells (black squares) and IFN- $\gamma$ <sup>+</sup> CD56<sup>dim</sup> NK cells (grey triangles), along with the correlation of the percentage of IFN- $\gamma$ <sup>+</sup> CD56<sup>bright</sup> NK cells (black line) with the concentration of IL-12p70 and IL-2 in culture supernatants (A and B), with the MFI of CD40 and HLA-DR on resting monocytes (C and D), and with the up-regulation of bioactive TGF- $\beta$  in culture supernatants (E). The correlation of TGF- $\beta$  in culture supernatants with IFN- $\gamma$ <sup>+</sup> CD56<sup>dim</sup> NK cells is also shown (E, grey line). Correlation coefficients are indicated for CD56<sup>bright</sup> (upper values) and CD56<sup>dim</sup> (lower values). Correlations were calculated using a second-order polynomial fit algorithm. Black arrows indicate examples of donors where IFN- $\gamma$  response is highly suppressed in the CD56<sup>dim</sup> NK cells. Flow cytometric data are based on the collection of 100,000 total events. doi:10.1371/journal.ppat.0020118.g008

NK IFN- $\gamma$  production, is consistent with the notion that pro-inflammatory responses to malaria are carefully regulated by homeostatic mechanisms to prevent immune-mediated pathology.

Our observations regarding the accessory cell requirements for NK cell activation by malaria parasites are in partial agreement with a previous report invoking a role for monocytes in this response [36]; however, we show that mDCs play an equally important role. The fact that NK activation is suboptimal but not absent in the presence of only one or the other accessory cell population suggests that although there is some redundancy in their roles, they also provide complementary signals. Our data are consistent with reports of leukocyte activation by malarial glycosylphosphatidylinositol signalling through toll-like receptor (TLR) 2 and TLR4 [37], which are both expressed on monocytes and mDCs but not pDCs [38,39], although a role for other receptors cannot be ruled out. The reported activation of pDCs by a malarial hemozoin-TLR9 pathway [40,41] does not appear to be required for NK IFN- $\gamma$  production, consistent with

previous reports that NK cytotoxic activity but not IFN- $\gamma$  secretion is dependent on pDC-derived signals [42,43]. In fact, depletion of pDCs increased the IFN- $\gamma$  response of NK cells in two out of three donors, suggesting that pDCs may have a net anti-inflammatory effect in this system, perhaps mediated by either IL-10 or TGF- $\beta$ . Further experiments are required to test this possibility.

The data presented here reveal that accessory cell-dependent activation of NK cells by iRBCs is multifactorial and, as in other systems, involves multiple cytokines (IL-2, IL-12, IL-18, and IFN- $\alpha$ ) [44–46] as well as direct contact between NK cells and accessory cells [12,22,26,27]. Whether accessory cells are the source of IL-2, as recently reported for human pDCs and mDCs [47], is not clear, and we cannot rule out that IL-2 is produced by a small contaminating population of rapidly responding T cells. Contact-dependent signals might include CD40–CD40L signalling [12], delivery of IL-12 from mDCs via an immune synapse [48], or binding of MHC–peptide complexes by activating KIR [49]. We believe that indirect, accessory cell-mediated signals are likely to be the major

**Table 1.** Accession Numbers for Genes and Proteins

Protein	Ensembl Family/Gene ID <sup>a</sup>	UniProt ID <sup>b</sup>
IL-2	ENSG00000109471	P60568
IL-10	ENSG00000136634	P22301
IL-12p35	ENSG00000168811	P29459
IL-12p40	ENSG00000113302	P29460
IL-15	ENSG00000164136	P40933
IL-18	ENSG00000150782	Q14116
IL-23	ENSG00000110944	Q6NZ80
IFN- $\alpha$	ENSF00000000349	—
IFN- $\gamma$	ENSG00000111537	P01579
TGF- $\beta$	ENSF00000000951	—
CSF-1	ENSG00000184371	P09603
CD1c	ENSG00000158481	P29017
CD3	ENSF00000004297	P04234/P09693
CD11c	ENSG00000140678	P20702
CD11b	ENSG00000169896	P11215
CD14	ENSG00000170458	P08571
CD19	ENSG00000177455	P15391
CD40	ENSG00000101017	P25942
CD40L	ENSG00000102245	P29965
CD56	ENSG00000149294	P13591
CD69	ENSG00000110848	Q07108
CD80	ENSG00000121594	P33681
CD94	ENSG00000134539	Q13241
BDCA-4	ENSG00000099250	Q14786
TLR2	ENSG00000137462	O60603
TLR4	ENSG00000136869	O00206
TLR9	ENSG00000173366	Q9NR96
NKG2A	ENSG00000134545	P26715
HLA-DR	— <sup>c</sup>	—
KIR	— <sup>d</sup>	—

<sup>a</sup><http://www.ensembl.org>.

<sup>b</sup><http://www.ebi.uniprot.org>.

<sup>c</sup>HLA database, <http://www.ebi.ac.uk/imgt/hla>.

<sup>d</sup>KIR database, <http://www.ebi.ac.uk/ipd/kir>.

CSF1, colony stimulating factor 1.

doi:10.1371/journal.ppat.0020118.t001

mechanism of NK cell activation by iRBCs, but we cannot rule out that the NK cell receives additional signals via direct interaction with iRBCs. Stable contacts between iRBCs and NK cells have been reported by ourselves and by others [9,36], and we have observed reorganisation of the actin cytoskeleton at the point of iRBC–NK contact [9]; the functional consequence of these interactions are, however, not yet fully understood.

Our observation that NK cells are not required for optimal activation of monocytes and mDCs by pathogen-derived ligands (iRBCs and LPS) runs somewhat counter to previous reports that NK cells augment DC maturation, cytokine secretion, and antigen presentation [28–30]. One plausible explanation for this discrepancy is that the sequence of events leading to activation of NK cells and accessory cells depends on the nature of the stimulus. In the case of virally infected or neoplastic cells, altered expression of MHC or MHC-like ligands on the target cell will lead to direct activation of NK cells either through engagement of natural cytotoxicity receptors or changes in the balance of activating and inhibitory signals delivered by KIR receptors (reviewed in [50]). In this case, amplification of the innate response and induction of adaptive immunity would be facilitated if NK cells were able to provide activating signals to DCs and other antigen-presenting cells, which are much less sensitive to

changes in MHC expression. On the other hand, in the case of bacterial and protozoal pathogens, which do not grossly alter MHC class I expression or induce MHC-related stress ligands on host cells, direct activation of NK cells is unlikely. Rather, DCs and monocytes are likely to be directly activated by binding of pathogen-associated molecular patterns to TLRs and other pattern recognition receptors and may not require additional signals from NK cells.

Given the importance of the innate response to malaria in determining the outcome of disease (reviewed in [1]), dissecting the basis for the heterogeneity of the human NK cell response to iRBCs could be crucial in understanding disease susceptibility. The close correlations shown here between accessory cell maturation, accessory cell responses to iRBCs, and induction of IFN- $\gamma$  production by NK cells suggest that heterogeneity of NK cell responses to iRBCs [8,9] is a reflection of deeply rooted heterogeneity of innate responses within the human population. Ongoing studies of the immunogenetics of susceptibility to human malaria should begin to identify the underlying basis for this heterogeneity, but linking genotype to immunological phenotype may not be straightforward. Multiple genes, including various IFN regulatory factor proteins and components of the colony stimulating factor 1 signalling pathway, influence the maturation status of accessory cells [51–53]. Moreover, the much stronger correlation of accessory cell status with activation of CD56<sup>bright</sup> (KIR<sup>−</sup>) NK cells than with activation of CD56<sup>dim</sup> (KIR<sup>+</sup>) NK cells suggests that NK cell activation by pathogen-induced accessory cell signals may be modulated by NK cell receptor expression. Since KIR genes, along with MHC genes, are some of the most highly polymorphic loci in the human genome [54], dissecting the genetic basis of diversity in the human innate response is a daunting task. Nevertheless, as CD56<sup>dim</sup> cells are the major population of NK cells in peripheral blood and spleen [55], our data suggest that NK cell receptor expression is likely to have a significant influence on overall NK cell responsiveness and thus potentially affect the severity of malarial disease and other systemic infections. Despite the difficulties associated with carrying them out, population-based studies of disease risk and diversity in innate response genes could have far-reaching implications, and we have recently begun such studies in malaria-endemic populations. Since the antigen-presenting and co-stimulatory functions of accessory cells are pivotal for induction of adaptive immunity, and the magnitude of the early IFN- $\gamma$  response affects adaptive responses [56], heterogeneity of expression of CD40, HLA-DR, and NK cell IFN- $\gamma$  is likely to have a profound impact on the generation of acquired immune responses.

## Supporting Information

**Figure S1.** Depletion of HLA-DR-Positive Cells Does Not Affect the Ability of NK Cells to Produce IFN- $\gamma$  following Stimulation with IL-12 and IL-18

(A) Representative example of NK cell response to IL-12 and IL-18 when cultured in the context of whole PBMCs or PBMCs depleted of HLA-DR-positive cells.

(B) Data from five donors showing proportion of NK cells positive for intracellular IFN- $\gamma$  following stimulation with IL-12 and IL-18 in the context of whole PBMCs or PBMCs depleted of HLA-DR-positive cells. There was no significant difference between responses (paired *t* test, *t* = 1.43, *p* = 0.226).

Found at doi:10.1371/journal.ppat.0020118.sg001 (1.3 MB TIF).

**Figure S2.** Staining with Isotype-Matched Control Antibodies Is Not Affected by Stimulation of Accessory Cells

Representative histograms showing staining of monocytes with (A) IgG1 and (B) IgG2a antibodies following culture of PBMCs with no stimulation (gm), LPS, or iRBCs. (C) Pooled data for monocytes stained for IgG1 following culture with indicated stimuli. (D) Pooled data for monocytes stained for IgG2a following culture with indicated stimuli. No significant increase in staining intensity of either antibody was observed after stimulation with *E. coli* LPS or iRBCs.

Found at doi:10.1371/journal.ppat.0020118.sg002 (385 KB TIF).

**Figure S3.** Effect of Blocking Antibodies on the NK Cell Response to iRBCs

Representative FACS plots showing the effect on the NK cell response to iRBCs of blocking with monoclonal antibodies of the following specificities: (A) anti-IL-2, (B) anti-TGF- $\beta$ , and (C) anti-IFN- $\alpha$  receptor.

Found at doi:10.1371/journal.ppat.0020118.sg003 (1.4 MB TIF).

**Figure S4.** Up-Regulation of CD69 on NK Cells in Response to iRBCs Is Controlled by Type 1 IFN, IL-2, and TGF- $\beta$ 

PBMCs from malaria-naïve donors were stimulated for 24 h with iRBCs in the presence of increasing concentrations of neutralising antibodies to (A) the IFN- $\alpha/\beta$  receptor, (B) IL-2, (C) IL-15, or (D) TGF- $\beta$ . MFI of CD69 on NK cells relative to that observed with the equivalent concentration of isotype-matched control antibody is shown.

Found at doi:10.1371/journal.ppat.0020118.sg004 (826 KB TIF).

**Figure S5.** Diffusion of Soluble Factors through Transwell Membrane (A) Experimental setup. PBMCs were added to both the inner and

outer compartments of a Transwell. Phytohaemagglutinin (PHA) (a mitogen) was added to the outer compartment, and the cells were cultured for 24 h at 37 °C. Cells were aspirated and stained for the surface markers CD56 and CD3 and intracellular IFN- $\gamma$ .

(B) Proportion of IFN- $\gamma$ + NK cells amongst PBMCs from outer and inner compartments following 24 h culture (six donors). There is no significant difference in the response of cells aspirated from the outer or inner well (paired *t* test, *t* = 1.08, *p* = 0.331).

Found at doi:10.1371/journal.ppat.0020118.sg005 (275 KB TIF).

**Accession Numbers**

Online database accession and ID numbers for genes and proteins mentioned throughout this article can be found in Table 1.

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**Author contributions.** KCN, DSK, and EMR conceived and designed the experiments. KCN, DSK, and JCH performed the experiments. KCN, DSK, and EMR analyzed the data and wrote the paper.

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**Competing interests.** The authors have declared that no competing interests exist.

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