**Plasmodium falciparum-Infected Erythrocytes and IL-12/IL-18 Induce Diverse Transcriptomes in Human NK Cells: IFN-α/β Pathway versus TREM Signaling**

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**Abstract**

The protective immunity of natural killer (NK) cells against malarial infections is thought to be due to early production of type II interferon (IFN) and possibly direct NK cell cytotoxicity. To better understand this mechanism, a microarray analysis was conducted on NK cells from healthy donors PBMCs that were co-cultured with *P. falciparum* 3D7-infected erythrocytes. A very similar pattern of gene expression was observed among all donors for each treatment in three replicas. Parasites particularly modulated genes involved in IFN-α/β signaling as well as molecules involved in the activation of interferon regulatory factors, pathways known to play a role in the antimicrobial immune response. This pattern of transcription was entirely different from that shown by NK cells treated with IL-12 and IL-18, in which IFN-γ and TREM-1-related genes were over-expressed. These results suggest that *P. falciparum* parasites and the cytokines IL-12 and IL-18 have diverse imprints on the transcriptome of human primary NK cells. IFN-α-related genes are the prominent molecules induced by parasites on NK cells and arise as candidate biomarkers that merit to be further investigated as potential new tools in malaria control.

**Introduction**

Infections caused by malaria parasites, especially by the species *Plasmodium falciparum*, remain a serious world health concern. The innate and adaptive arms of the immune system are involved in immunity to malaria, however, the engaged macrophages, dendritic cells, γδ T cells, natural killer (NK) cells, and NKT cells fail to fully eliminate the infection [1,2].

Characterized by cytotoxicity and cytokine secretion, NK cells play a critical role as the front line of defence against pathogens and tumor cells. Within the setting of malaria, several studies have elucidated the interactions between NK cells, infected erythrocytes (iRBCs) and other immune cells leading to specific NK responses to *P. falciparum* [3–5]. Experiments performed with NK cells derived from malaria-naïve or infected individuals showed that these cells have cytolytic activity against *P. falciparum*-iRBCs that is possibly mediated by FAS and Granzyme B [6,7]. The DBL-alpha domain of *P. falciparum*-infected erythrocyte membrane protein 1 (PFEMP1) was identified as the molecule through which NK cells recognize iRBCs [8]. MYD88-associated IL-18R in NK cells was shown to be the major indirect sensor for *P. falciparum* infection [9].

Experimental evidence suggested that, in addition to their up-regulation of CD69 and CD25 after contact with iRBCs, NK are one of the first cells to produce IFN-γ in response to *P. falciparum* infection [3,5]. This event was described to be dependent on cross-talk with accessory cells either via direct or indirect interactions. The possible bidirectional interplay between ICAM and LFA-1 on NK cells and macrophages was shown to be important for NK cell up-regulation of CD69 and IFN-γ secretion [10]. Indirectly, the production of cytokines by accessory cells, especially IL-12, IL-18, IFN-alpha and IL-2, was shown to boost NK cell activation and IFN-γ release in response to iRBCs [11].

However, the magnitude of IFN-γ release by NK cells is known to be heterogeneous among individuals, possibly influencing susceptibility to disease [5]. In this line, qualitative and quantitative differences in NK subsets found in malaria patients were linked to the severity of the disease [3]. In addition, correlations between KIR genotype and NK cell responsiveness to iRBCs have been reported [4].

Microarray techniques have been widely used for research as well as for diagnostic purposes. Therefore, applications pertinent to host-microorganism interactions may be a good predictor of the biological processes thereby involved. In this study, Affymetrix oligonucleotide microarrays were used to examine the gene expression profile of primary NK cells from three healthy donors that were co-cultured with *P. falciparum* parasites. This pattern of gene expression was compared to the same NK cells following stimulation with IL-12+IL-18.

The response of NK cells to malaria has been the topic of several studies over the previous few years, but there is still a lack of information regarding the impact of *P. falciparum* on NK cells at a transcriptional level. A greater understanding of the NK cell mechanisms of sensing and responding to iRBCs is needed seeking...
the advantages of NK cell-targeted vaccines development against malaria.

Materials and Methods

Ethics statement

The three healthy individuals who served as NK cell donors are themselves authors of this study. Therefore, acquisition of verbal informed consent was considered sufficient by the ethics committee for the study approval. Verbal consent was obtained in the presence of a witness unrelated to the study, who has attested to its voluntary character in a signed document. The study was approved by the Ethics Committee of the University of Tubingen, Germany.

P. falciparum culture

The P. falciparum laboratory strain 3D7 was maintained in continuous culture as described elsewhere [12] and frequently tested for mycoplasma contamination by PCR prior to cocultivation with NK cells. Parasites were constantly synchronized with 5% sorbitol. Mature schizont-iRBCs were harvested by magnetic cell sorting with LD columns (MACS; Miltenyi Biotec, Berg, Gladbach, Germany). Schizonts’ purity (≥90%) and red blood cell integrity were confirmed by Giemsa stain.

PBMCs preparation

Venous blood was collected and immediately processed. Three healthy adults (donors E, K and V) with no prior exposure to Plasmodium parasites were used in this study. Samples were collected into 9 ml ammonium heparin tubes (161 U. heparin/ml blood; S. Monovette) and diluted 1:1 with RPMI 1640 (Sigma Aldrich). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Ficoll Paque TM plus (GE Healthcare). The cells were washed twice with 2% FBS in RPMI 1640; resuspended in culture medium (RPMI 1640) containing 5% autologous serum, 1% 100× PenStrep (Invirotigen) and 2 mM L-Glutamine (Invitrogen); transferred to 24-well flat-bottomed plates (Nunc); and cultured as described below.

PBMC/parasite co-incubation

Freshly isolated mononuclear cells from donors E and K were incubated under four different conditions: in culture medium alone (CM or untreated); with a mixture of IL-12 and IL-18 (Peprotech and MBL, respectively; 200 ng/10^6 cells each); or with schizont-iRBCs or with uninfected erythrocytes (uRBCs) at a ratio of three RBCs for each mononuclear cell. PBMCs from donor V were incubated under two different conditions: with iRBCs and with culture medium. Co-cultures were maintained at 37°C and 5% CO2 for 24 hours. After the incubation, NK cells were isolated from PBMCs, checked for purity by FACS, and subjected to RNA extraction as described below. The experiment was repeated three times (1–3 weeks apart) for each one of the three donors.

To evaluate the activation pattern of each donor’s NK cells, PBMCs were likewise incubated with schizont-iRBCs (1 PBMC : 3 iRBC), with iRBCs together with human IFN-alpha2b (Miltenyi Biotec; 500 U/10^6 cells), or with a mixture of IL-12 and IL-18 (200 ng/10^6 cells each) and also kept in culture medium alone. After 24 hours at 37°C and 5% CO2, cells were harvested, iRBCs were lysed and PBMCs were stained for flow cytometry.

Cell surface and intracellular staining for flow cytometry

The following antibodies were used for flow cytometric staining: CD56-FITC, CD3-PE, CD3-APC, CD69-PE, intracellular IFN-γ, 7AAD and the appropriate isotype controls (all from BD biosciences). Extracellular staining of cells was performed according to the manufacturer’s instructions. For intracellular staining of IFN-γ, Brefeldin A solution (Biolegend) was added 4 hours before the end of the incubation period and cells were fixed and permeabilized with Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) according to the manual instructions.

Isolation of NK cells

After 24 hours of co-incubation, cells were harvested, separately treated with BD Pharm Lyse lysing buffer (BD) for RBC rupture, and washed twice with auto-MACS Rinsing Solution (Miltenyi Biotec). NK cells were enriched from PBMCs by negative selection with the NK Cell isolation Kit (Miltenyi Biotec) according to the manufacturer’s instructions. NK cells were counted and tested for viability with trypan blue, and purity was determined by flow cytometry. A purity of ≥95% CD56^+CD3^- cells was considered acceptable (Figure S1).

RNA extraction and Microarrays

Total cellular RNA was isolated from the enriched NK cells with RNeasy Mini Kit (Qiagen, Hilden, Germany). The quality of each specimen was checked using an Agilent BioAnalyzer 2100 (Agilent, Germany). RNA was processed for Affymetrix Gene Chips using the Affymetrix Whole Transcript Sense Target Labeling Kit (Affymetrix, Santa Clara, USA). Fragmented and labeled cDNA were hybridized onto human HuGene1.0 ST Gene Chips (Affymetrix). The staining of biotinylated cDNA and scanning of arrays were performed according to the manufacturer’s recommendations. The complete microarray data is deposited at the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI) under the series number GSE24791. Validation of the method was performed by RT-PCR.

Rea- time PCR

cDNA was synthesized from total RNA using the Quantitect Reverse Transcription kit (Qiagen) with the elimination of Genomic DNA according to the manufacturer’s instructions. Amplification of IFIT1, IFIT3 and IFI44L genes was carried out in duplicates using the Rotor Gene Syber Green PCR Kit (Qiagen) with QuantiTec Primer Assay (both from Qiagen). Cycling conditions for fast two-step RT-PCR on Rotor-Gene cycles were applied according to the Primer Assay Handbook (Qiagen). Levels of target mRNA expression were determined using the 2^(-ΔΔCt) method with GAPDH as the endogenous reference gene and the CM samples as calibrators.

NK cells expansion and co-culture with parasites for growth inhibition assay

PBMCs and NK cells from donor E were respectively purified and isolated as described above. NK cells (≥95% CD56^+CD3^-) were cultured in IMDM medium (Sigma) with 5% autologous serum, 200 U/ml IL-2 (Peprotech) and irradiated JY cells at a 1:3–3:1 ratio (NK:JY). Purified NK cells expanded for 2–4 weeks (eNK) were co-incubated at 37°C with ring stage 3D7-iRBCs in parasite growth medium at a 1:3 or 3:1 ratio (NK:3D7) in parasite atmosphere. Additionally, IFN-alpha2b (500 U/10^6 cells) or a mixture of IL-12 and IL-18 (200 ng/10^6 cells each) were added to the system. Parasites were cultured alone as a control and the initial parasitemia was set as 0.05% in 1.5% hematocrit. After 24 h and 48 h of incubation, culture samples were frozen at −20°C, then thawed and inhibition of parasite growth was quantified by a
Histidine-Rich Protein 2 (HRP2) ELISA assay performed as described elsewhere [13].

Bioinformatic analysis

Raw CEL-files were imported into Expression Console 1.0 (Affymetrix, Santa Clara). RMA16 was used for array normalization and signal calculation. Normalized signal values were imported into Genespring 11 (Agilent Technologies). Significance was calculated using a t-test without multiple testing correction, selecting all the transcripts with a minimum change in expression level of 1.5-fold together with a p-value of <0.05. Subsequently, transcripts in common for all donors in each treatment were compiled and gene network analysis and functional categorization was performed with Ingenuity Pathway Analysis (IPA) (www.ingenuity.com). The p-value associated with a biological process or pathway annotation for IPA is a measure of its statistical significance with respect to the Functions/Pathways/Lists Eligible molecules for the dataset and a reference set of molecules (which define the molecules that could possibly have been Functions/Pathways/Lists Eligible). The p-value is calculated with the right-tailed Fisher’s Exact Test. The ratio is calculated by taking the number of genes from the dataset that participate in a Canonical Pathway, and dividing it by the total number of genes in that Canonical Pathway. The ratio indicates the percentage of genes in a pathway that were also found in the uploaded gene list (or the Functions/Pathways/Lists Eligible). The p-value is calculated with the right-tailed Fisher’s Exact Test. The ratio is calculated by taking the number of genes from the dataset that participate in a Canonical Pathway, and dividing it by the total number of genes in that Canonical Pathway. The ratio indicates the percentage of genes in a pathway that were also found in the uploaded gene list (or the Functions/Pathways/Lists Eligible) if a cut off was specified)

Results

P. falciparum-iRBCs induce the up-regulation of type I interferon-related genes in NK cells

Affymetrix microarrays were used to evaluate the gene expression profile of NK cells isolated from PBMCs (purity ≥93%; Figure S1) that were incubated with iRBCs to detect the changes that Plasmodium-iRBCs impose on the gene repertoire of NK cells. The analysis showed that 192 genes were commonly modulated for all donors in response to iRBCs contact. Of those genes, nine were down-regulated and 183 were up-regulated in comparison to untreated cells (Table S1). The expression profile was characterized by the induction/suppression of genes mainly related to immune response and response to virus (IFIT1, IFIT3, OAS3, KLRG1), chemotaxis (CXCL10, CCR1, CCL4L1), signal transduction (CD36, IFITM1, EAS), regulation of transcription (STAT2, IRF7, STAT3), intracellular signaling pathway (JAK, RASGRP3, RASGRP2), and NK cytoxicity (SLAMF7), among others. A summary of the most highly up- and down-regulated genes is depicted in Table 1. A portion of the most highly up-regulated genes (fold change ≥10) encode proteins mostly related to interferon signaling (IFIT1, IFIT3, IFI44L, IFIT2, IFI6, and IFI44), especially via IFN-α. The most highly down-regulated genes (fold change ≤−1.5) are mainly involved in chromatin assembly, receptor activity in the immune response and signal transduction. Three representative genes were chosen for microarray validation by RT-PCR (Figure 1).

Top Networks and Pathways related to iRBCs-induced genes

Ingenuity Systems generated top networks, with a score higher than 40, based on the analysis of the iRBCs-regulated NK genes. Antimicrobial/Inflammatory Responses and Infection Mechanism are the main functions associated with the top-scoring networks (Table 2 and Table S2). Moreover, Interferon signaling (p = 2,17E-14) and Canonical Pathway. The ratio indicates the percentage of genes in a pathway that were also found in the uploaded gene list (or the Functions/Pathways/Lists Eligible) if a cut off was specified)

Table 1. Top up/down-regulated genes on NK cells due to co-culture with P. falciparum-iRBCs.

| Symbol   | Aff. ID  | FC   | Location | Type   |
|----------|----------|------|----------|--------|
| IFIT1    | 7929065  | 34.50| Cytoplasm| other  |
| R5AD2    | 8040080  | 21.55| unknown  | enzyme |
| IFIT3    | 7929052  | 19.71| Cytoplasm| other  |
| OAS3     | 7958895  | 15.48| Cytoplasm| enzyme |
| MX1      | 8068713  | 15.42| Nuclear   | enzyme |
| IFI44L   | 7902541  | 15.33| unknown  | other  |
| IFIT2    | 7929047  | 12.47| unknown  | other  |
| IFI6     | 7914127  | 12.10| Cytoplasm| other  |
| OAS1     | 7958884  | 11.60| Cytoplasm| enzyme |
| IFI44    | 7902553  | 11.32| Cytoplasm| other  |
| MX2      | 8068697  | 11.00| Nuclear   | enzyme |
| CXCL7    | 8076185  | −1.79| Nuclear   | other  |
| KLRG1    | 7953835  | −1.83| Plasma MB| other  |
| RASGRP2  | 7949104  | −1.91| Cytoplasm| other  |
| SYNE1    | 8130211  | −1.92| Nuclear   | other  |
| HERC1    | 7989516  | −1.96| Cytoplasm| other  |
| CMKL1    | 7966089  | −2.03| Plasma MB| GPCR   |
| AHNAK    | 7948667  | −2.27| Nuclear   | other  |
| FGR      | 7914112  | −2.49| Nuclear   | kinase |
| PTGDR    | 7974363  | −2.58| Plasma MB| GPCR   |

Transcriptome of NK Cells

Figure 1. Validation of the microarray results by RT-PCR. Three representative iRBCs-induced NK genes are depicted. Values represent the mean of the relative fold change obtained for each replicate per donor. Levels of target mRNA expression were determined using the 2−ΔΔCT method with GAPDH as the endogenous reference gene and the untreated samples (CM) as calibrators.

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Type II interferon-induced NK genes are up-regulated by IL-12 and IL-18

To compare patterns of NK cell activation, PBMCs were also treated with IL-12 and IL-18, well-described NK cell stimulators. Treatment with the cytokine mix resulted in the regulation of 576 NK genes in both donors E and K. Down-regulated genes totalled 160, whereas 416 genes were up-regulated (Table S4). Modulated genes included those related to the immune response (IFI-γ, CD25), signal transduction (P2RX5, MT2A, KLRF1), complement activation (CD55), antigen presentation (CD83), chemotaxis (CCR4, CXCL10, CX3CR1), DNA replication/repair (CHEK1, TMS), transcription (IRF8, MYC), and cytokines (IL26, IL6), among others. The most up and down-regulated genes share similar molecular as well as biological functions such as receptor activity and signal transduction/immune response, respectively (Table 3; fold change ≥12 and fold change ≤−5.5).

Top Networks and Pathways related to IL-12 and IL-18-induced genes

The highest score upon analysis of the IL-12 and IL-18 modulated genes was given to the network that listed Gene Expression, Infection Mechanism, RNA Post-Transcriptional Modification as associated functions (score: 50; Table 2 and Table S5). The most significant canonical pathways obtained from Ingenuity analysis were TREM1 Signaling (p = 2.69E-07) and the Protein Ubiquitation Pathway (p = 3.31E-07; Table S6). The former canonical pathway is composed of 69 molecules and 13 out of those were found to be modulated in NK cells, resulting in a high strength of association (ratio: 0.188; Figure 2B). The top canonical pathway and its modulated genes are depicted in Figure S2.

Gene expression similarities between iRBCs- and IL mix-treated NK cells

There were about 400 additional transcripts regulated by IL-12 and IL-18 in comparison to iRBCs-induced genes. In total, only 40 were modulated by both treatments. Among those are transmembrane receptors (IL2RA, CCR1, IL12RB2), cytokines (CXCL10, CCL3), transcription regulators (STAT3, LBA1) and enzymes (PTPN2, HSPA8).

For a general overview, members of the Interferon signaling pathway, TREM-1 signaling pathway and other genes that play a role in immune response were arranged in a heatmap (Figure 4). The image depicts the comparison of the gene fold change between the three different donors in response to the treatment type. It is clear that, although the same pattern of gene regulation is generally maintained among the donors within the different treatments, parasites and IL-12/IL-18 affect the transcription of NK genes in a different manner.

Influence of uninfected erythrocytes on NK cells

Since the RBCs and the NK cells used in this study are from different donors, PBMCs from donor E and K were incubated with uRBCs in order to control the allogeneic responses that might affect gene expression in NK cells. This analysis showed that in total only nine genes were modulated due to uRBCs treatment. NK cells from donor E up-regulated six genes, whereas donor K cells showed up-regulation of three different genes. The biological processes or molecular functions of some of these genes have not been described (NRUSE, SNORD47), and others are known to play a role in RNA splicing (SNRP5) and translation (EEF1A1).

Patterns of NK activation and inhibition of parasite growth by expanded NK cells

The activation characteristics of NK cells from the three donors studied were next examined. NK cell up-regulation of the CD69 membrane surface protein and production of IFN-γ were examined in response to incubation with iRBCs, iRBCs plus IFN-α, IL-12 and IL-18, and culture medium alone. All the donors’ NK cells up-regulated CD69 due to iRBCs incubation, although the strength of the responses differed among donors (Figure 5A). In response to parasite stimulation, only 10.7% of the NK cells from donor K up-regulated CD69, while 16.9% and 39.5%, respectively, of the NK cells from donors V and E responded. The addition of IFN-α to the system contributed to NK activation by increasing the percentage of cells that up-regulated CD69 for all donors. IL-12/IL-18 treatment induced around 80% of the donors’ cells to express CD69. None of the coculture conditions induced IFN-γ release by NK cells to a large extent, except for the IL-12/IL-18 treatment (Figure 5B).

To further examine the cytotoxic characteristics of the cells used in the study, we co-cultured expanded NK cells (eNK) from donor E with 3D7-iRBCs. Neither the 24 h/48 h co-culture time nor the different 3D7:eNK ratios (1:5; 3:1) appeared to have a significant

Table 2. Top networks and functions associated with 3D7- or IL-12/IL-18-induced transcripts on NK cells.

| NW ID | Network functions related to 3D7-induced transcripts | Score |
|-------|-----------------------------------------------------|-------|
| 1     | Antimicrobial Response, Inflammatory Response, Infection Mechanism | 61    |
| 2     | Antimicrobial Response, Inflammatory Response, Infection Mechanism | 54    |
| 3     | Infection Mechanism, Organismal Injury and Abnormalities, RNA Damage and Repair | 44    |
| 4     | Infection Mechanism, Antimicrobial Response, Inflammatory Response | 40    |
| 5     | Post-Translational Modification, Protein Folding, Cell Morphology | 31    |

| NW ID | Network functions related to IL-12/IL-18-induced transcripts | Score |
|-------|-------------------------------------------------------------|-------|
| 1     | Gene Expression, Infection Mechanism, RNA Post-Transcriptional Modification | 50    |
| 2     | Cell Death, Post-Translational Modification, Protein Folding | 43    |
| 3     | DNA Replication, Recombination, and Repair, Cellular Growth and Proliferation | 40    |
| 4     | Cell Death, Genetic Disorder, Immunological Disease | 37    |
| 5     | Cell Morphology, Hematological System Development and Function, Cancer | 36    |

NW ID—network identification; IL—interleukin.

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effect on parasitemia. Additionally, parasite growth was not affected by the addition of IFN-α and IL-12/IL-18 to the system (Figure 6).

Discussion

The objective of this study was to observe the transcriptional changes that malaria parasites impose on NK cells in order to gain a deeper knowledge of the mechanisms behind such interaction. An in vitro approach was used to investigate the specific immune response to malaria. Such an approach is widely used in research especially in diseases where primary material is difficult to access. The gene expression profile and activation characteristics of NK cells incubated with iRBCs at a 1:3 ratio (PBMCs:iRBCs) were examined at one time point (24 h) after co-culture. These conditions were chosen based on prior observations showing that optimal NK cell IFN-γ production occurs at either 1×10^6 or 1×10^7 iRBCs per 10^6 PBMCs and that the peak of IFN-γ release occurs between 15 and 24 hours after stimulation [3].

First, these results demonstrate that all donors' NK cells have a very similar pattern of gene regulation for each different treatment. An interferon signaling gene expression signature is
induced by iRBCs on NK cells, in which genes involved in a pro-inflammatory response, mainly mediated by type I interferon were modulated. A recent microarray study has described the up-regulation of IFIT1, IFIT3, and CXCL10 (after 1 h of activation) and IFI44, IFIT2, and ISG20 (after 18 h of activation) in IFN-α-treated whole PBMCs from healthy donors. Arrays performed with isolated cell subsets (NK cells, monocytes and T cells) showed the up-regulation of OAS2, OASL, ISG20 and IFI44 [14]. Another group has reported the up-regulation of TNFSF10 (TRAIL), IFIT and OAS genes in NK cells isolated from IFN-α-2b-stimulated PBMCs from five healthy donors [15]. The expression profile of IFN-α-treated cells in these studies was very similar to the profile detected in iRBCs-activated NK cells in the current study, which did not utilize IFN-α. Therefore, such similarities provide support of a clear iRBCs-induced type I interferon-related response in NK cells. Moreover, the regulated molecules were linked to top canonical pathways, all belonging to the “cellular immune response” category. Some of the regulated genes were not yet assigned to a clear role in this category, but most genes were linked to well-known biological functions, mainly in infection control and inflammation. As in systemic lupus erythematosus (LE) [16,17], such inflammatory components were recently detected as increased IFN-α/β-inducible genes in the blood of patients with Tuberculosis (TB), especially in their purified neutrophils [18]. Its correlation with disease severity provided primary data supporting a role for type I IFN in the pathogenesis of human disease. Here, we have observed that mostly the same transcripts found in the mentioned studies were overexpressed in our NK cells due to parasite co-incubation. It is difficult to extrapolate the LE/TB findings to malaria without confirmation with longitudinal studies; however, it is possible that the role of type I interferon signaling in diseases caused by intracellular pathogens will be a marker of disease progression and immune response development.

In this study, the IFIT family was found to be among the most highly up-regulated NK genes induced by parasites. To date, little is known about their function. Most of the evidence characterizes these proteins as inhibitors of cellular and viral processes such as protein translation [19]. Recent findings indicate that IFIT proteins are substantially induced during infection possibly reflecting a functional role. A complex formed by IFIT1, 2 and 3 was observed to exert antiviral activity by physically engaging microbial triphosphorylated-RNA suggesting that these proteins possibly have the ability to bind to various types of nucleic acids of other diverse microbes [20]. Plasmodium DNA, therefore, could be a target. In fact, DNA sensing and its relation to type I interferons have recently been revealed to be important in innate immunity to malaria [21]. Plasmodium genomic DNA, rich in AT motifs, was shown to generate type I interferon through two suggested innate pathways (a TLR9- and a STING-driven) which converge on the IRFs to regulate IFN gene transcription. Interferon type I, in turn, could possibly influence the outcome of the disease.

Instead of IFN-α/β-related genes though, we expected to observe a direct up-regulation of the IFN-γ gene in all donors treated with iRBCs (as was detected with the IL-12/IL-18 treated NK cells). To our surprise, NK cells from only one donor (donor
Table 3. Top up/down-regulated genes on NK cells due to treatment with IL-12 and IL-18.

| Symbol | Aff. ID | FC  | Location | Type(s)       |
|--------|---------|-----|----------|---------------|
| IL18   | 8121727 | 4.05| Extr. Space| cytokine      |
| IFNγ   | 7964787 | 92.46| Extr. Space| cytokine      |
| IL28A  | 7931914 | 37.33| Plasma MB | Tmb R         |
| IFIT1  | 8178601 | 34.30| unknown   | other         |
| CDC57  | 7983650 | 20.71| Cytoplasm | transporter   |
| DPP4   | 8056222 | 17.57| Plasma MB | peptidase     |
| CD97   | 8154233 | 16.19| Plasma MB | other         |
| CDC6   | 8007071 | 14.25| Nucleus   | other         |
| MYD188 | 8047127 | 13.25| Cytoplasm | other         |
| P2RX5  | 8011415 | 13.10| Plasma MB | ion channel   |
| TNFSF4 | 7922343 | 13.05| Extr. Space| cytokine      |
| PTGDR  | 7974363 | 5.54 | Plasma MB | GPCR          |
| YPE1   | 8074780 | 5.56 | Nucleus   | enzyme        |
| FGFBP2 | 8099471 | 5.63 | Extr. Space| other         |
| PIK3IP1| 8075483 | 5.78 | unknown   | other         |
| KLHL24 | 8084219 | 5.79 | unknown   | other         |
| AIN1   | 7948667 | 5.96 | Nucleus   | other         |
| CX3CR1 | 8086344 | 6.68 | Plasma MB | GPCR          |
| FAIM3  | 7923917 | 7.37 | unknown   | other         |
| SH2D1B | 7921900 | 7.56 | unknown   | other         |
| KLF6   | 7953892 | 10.20| Plasma MB | Tmb R         |
| IL7R   | 8104901 | 11.12| Plasma MB | Tmb R         |

IL-interleukin; Aff. ID- Affymetrix identification; FC-fold change; MB-membrane; Extr.-extracellular; Tmb R-transmembrane receptor; GPCR-G protein coupled receptor.

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K) up-regulated the IFN-γ gene, and the fold change was much lower than that induced by IL-12 and IL-18. Nevertheless, it is very likely that this 3D7-mediated induction of type I-related genes reflects the first steps of a cascade of events leading to IFN-γ release. In the case of a viral infection, there is consensus that the activation of NK cells is critically dependent on type I IFN signaling in vivo and that this activation is achieved by its direct action on NK cells [22]. Others have shown that type I IFNs are an early and critical regulator of NK cell number, activation and antitumor activity and that, in combination with IL-18, type I IFNs play an important role in the immune response [23,24]. In addition to overlapping with type II interferon at multiple levels of the JAK/STAT signaling pathway, type I interferons have unique regulatory mechanisms for both their own signaling as well as IFN-γ signaling [25]. A very recent report showing the responses of human PBMCs to stimulation with type I and II interferons, among other cytokines, is in agreement with this co-induction concept. The authors describe the responses to IFN-γ as being restricted to a subset of type I interferon-inducible genes whereas responses to type I interferon were highly stereotyped and resulted in the up-regulation of genes such as OAS1-3, MX1/2, CXCL10, STAT1/2, IRF2/7 and IFIT1-5. However, after four hours of IFN-α treatment, transcripts of type II interferon itself were induced, which the authors suggested might play a role in the initiation of an IFN-γ-dependent transcriptional programs in type I IFN-treated cells [26]. Along these lines, L. major-induced IFN-α/β was suggested to mediate key events of the innate response to the parasite. NK cell cytotoxicity and IFN-γ secretion early in infection were shown to be decreased in the event of type I interferon blockage in mice [27]. In a recent study with L. mexicana infection, type I interferon was also described to promote the early IFN-γ and IL10 expression [28].

P. falciparum-mediated IFN-α responses have been previously reported by few in vivo and in vitro studies [29–31]. In accordance with our findings, new microarray evidence show that expression profiling of PBMCs derived from patients with P. falciparum malaria show elevated expression of interferon-inducible genes (ISGs) [21]. The study further confirms that PBMCs stimulated with iRBCs induce IFN-α at the protein level and IFN-β mRNA, suggesting a possible role for type I interferons in malaria. Although their gene expression profiling was performed with a mixed group of cells and hence cannot be traced to one specific cell population, the NK cells in the present study appear to respond in concert with PBMCs from malaria-infected individuals in that previous study. Furthermore, human plasmacytoid
In contrast, experimental evidence suggested that IFN-α polymorphisms in the IFN-α blood-stage infections in mice [35,36]. In addition, yoelli evolution of cerebral malaria and inhibits the development of treatment of P. berghei antiviral activity have been reported to be due to IFN-α. IFN-α/β receptor knock-out mice could control P. berghei infection [34]. In contrast, experimental evidence suggested that IFN-α treatment of P. berghei-infected mice has a protective effect on the evolution of cerebral malaria and inhibits the development of P. yoelli blood-stage infections in mice [35,36]. In addition, polymorphisms in the IFN-α receptor 1 were associated with protection against cerebral malaria in humans [37]. High titer of antiviral activity have been reported to be due to IFN-α, and a positive correlation between degree of parasitemia, interferon titers and NK cell activity was observed in acutely ill P. falciparum-infected children [29]. IFN-α, in combination with iRBCs, boosted the up-regulation of CD69 on NK cells but did not up-regulate IFN-γ in the present study. Additionally, when testing the cytotoxicity of expanded NK cells from Donor E against Plasmodium, no significant interference in parasite growth was observed, even with the addition of IFN-α. Such lack of cytotoxicity was likely due to donor-related characteristics (low IFN-γ responder) but it will be important to determine the reason that expanded NK cells treated with IL-12/IL-18 (which lead to IFN-γ release) did not inhibit parasite growth. As mentioned before, others have described that the peak of IFN-γ induction occurs around 15–24 h after co-culture with parasites and that this response is dependent on cross-talk with other cells. Thus, it would be worth observing whether HPR2 is suppressed at earlier time points than 24 h and 48 h after co-culture and whether the addition of accessory cells to the system would interfere with parasite growth. To further investigate the importance of IFN-α on the NK response against parasites, 3D7-iRBCs were co-cultured with NK92 (NK cell line) in which IRF9, STATT1 or STAT2 were knocked down by siRNA and RT-PCR was used to verify the suppression of EBA-175 and BAEBL/EBA-140, which are vital parasite genes involved in invasion (our unpublished observations). However, no differences were observed in cytotoxicity of the siRNA -transfected cells against 3D7. One potential explanation for this is the fact that, due to the difficulties in obtaining large amounts of fresh NK cells, these experiments were performed with an NK cell line, which might not reflect physiological conditions. Another reason could be the choice to evaluate cytotoxicity by the NK population, although there is still considerable debate regarding the importance of NK and T cells in immunity to malaria. A very recent study with P. chabaudi-infected mice shows that the suppression of infection is dependent on γδ T cells and independent of NK cells [38]. Conversely, a study with a large cohort of malaria-naive donors shows that the majority of IFN-γ+ T cells are γδ and not γδ T cells. Moreover, the authors of that study observed that NK cells dominate the early IFN-γ response (around 18 h), that NK and T cells contribute equally to the response at 24 h, and that T cells dominate from there-after [39].

The combination of IL-12 and IL-18 augments NK cell activity and stimulates NK production of IFN-γ, a cytokine suggested to control P. falciparum infection [3–5]. PBMCs stimulation with high doses of IL-12 and IL-18 (as performed in this study) was previously shown to up-regulate NK cell expression of CD69 and CD25, and to stimulate the release of IFN-γ [5]. We were interested in determining whether there were similarities between the transcripts induced by IL-12/IL-18 and iRBCs. However, this was not the case. IL-12/IL-18 treatment induced genes strongly correlated to the signaling pathway triggered by TREM 1, an immune regulatory molecule that plays a role in innate and adaptive immune response [40]. The molecule is expressed on monocytes/macrophages, dendritic cells, NK cells, and neutrophils [41,42] and its activation triggers molecules involved in cell-to-cell signaling/interactions and inflammatory responses (including CD83, IL-6 and TNF among others). NK cells induced by iRBCs in our study also modulated some genes related to this pathway although not as strongly as the IL-12/IL-18 treatment. The second strongest gene association was found with the protein ubiquitination pathway, which consists of a concerted action of enzymes indispensable for the rapid removal of proteins, the regulation of gene transcription, translational quality control and immune surveillance, to mention some of the functions. A prominent molecule in immune surveillance is IFN-γ, which was found to be the top molecule (FC = 92,460) up-regulated by IL-12/IL-18 treatment in this study. The ubiquitin-proteasome system is essential for antigen presentation on MHC class I
molecules and this process is enhanced by IFN-γ. This cytokine induces immune cells to express immunometaproteasomes that impose changes on the normal cascade of actions of the pathway, consequently leading to the stimulation of host defence [43]. Overall, this study provides evidence that P. falciparum parasites induce IFN-α-associated transcripts in human NK cells within the first 24 hours of interaction. This study also demonstrated that the NK transcriptional changes induced by IL-12 and IL-18 are diverse from those induced by 3D7. Whether both patterns of NK transcriptional changes induced by IL-12 and IL-18 are first 24 hours of interaction. This study also demonstrated that the role of IFN-α in malaria is still controversial and understudied. This study suggests inherent regulatory molecules of the NK response to parasites that might be potential targets to be considered in malaria vaccine development.

Supporting Information
Figure S1 Purity of the isolated NK cells measured by FACS. Values represent the percentage of pure NK cells (CD56+CD3-) before and after isolation within the four different co-culture conditions: CM: culture medium only; iRBCs: +infected erythrocytes; uRBCs: +uninfected erythrocytes; IL-12+I-18: IL-12 and IL-18. E1, E2 and E3 represent the three replicates for donor E; K1, K2 and K3 represent the three replicates for donor K and V1, V2 and V3 represent the three replicates for donor V. (TIF)

Figure S2 IL-12/IL-18 treatment of NK cells induces transcripts related to the TREM-1 signaling pathway. The “Triggering receptor expressed in myeloid cell 1” (TREM-1) signaling pathway was identified by the Ingenuity Pathways knowledge base as highly associated with the IL-12/IL-18-regulated genes on NK cells. Up-regulated genes are highlighted to IL-12/IL-18-induced genes. (TIF)

Table S1 Complete list of P. falciparum-iRBCs induced genes. (XLS)
Table S2 Complete list of Networks related to P. falciparum-iRBCs induced genes. (XLS)
Table S3 Complete list of Canonical Pathways related to P. falciparum-iRBCs induced genes. (XLS)
Table S4 Complete list of IL-12/IL-18-induced genes. (XLS)
Table S5 Complete list of Networks related to IL-12/IL-18-induced genes. (XLS)
Table S6 Complete list of Canonical Pathways related to IL-12/IL-18-induced genes. (XLS)

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Author Contributions
Conceived and designed the experiments: EGdC JFJK. Performed the experiments: EGdC. Analyzed the data: EGdC MB PGK. Contributed reagents/materials/analysis tools: MB PGK. Wrote the paper: EGdC JFJK.

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