Immunity to Malaria: Insights on the Mechanisms Behind Effector Functions of Natural Killer Cells

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Immunity to Malaria: Insights on the Mechanisms Behind Effector Functions of Natural Killer Cells

This thesis is based on the following publications:

I. **Grangeiro de Carvalho E**, Böttger E, Hoang VT, Kremsner PG, Kun JF (2011). Limited activity of NK92 cells against *Plasmodium falciparum*-infected erythrocytes. *Malaria Journal* 10:311 doi:10.1186/1475-2875-10-311

II. **Grangeiro de Carvalho E**, Bonin M, Kremsner PG, Kun JF (2011). *Plasmodium falciparum*-Infected Erythrocytes and IL12/IL18 Induce Diverse Transcriptomes in Human NK cells: IFN-α/β Pathway *versus* TREM signaling. *Plos One* 6(9):e24963

Further publications not included in this thesis:

III. Faik I, **de Carvalho EG**, Kun JF (2009). Parasite-host interaction in malaria: genetic clues and copy number variation. Genome Medicine, Sep 2; 1(9):82.

IV. Kun JF & **de Carvalho EG** (2009). Novel therapeutic targets in *Plasmodium falciparum*: aquaglyceroporins. Expert Opinion on Therapeutic Targets, Apr; 13(4):385-94.

ZUSAMMENFASSUNG

Malaria ist eine der wichtigsten lebensbedrohlichen Infektionen weltweit, die Tausende von Todesfällen pro Jahr verursacht. Natürliche Killerzellen spielen eine Rolle bei der angeborenen Immunantwort gegen *Plasmodium falciparum*. Allerdings sind die beteiligten Mechanismen immer noch nicht vollständig aufgeklärt. In dieser Arbeit wurde untersucht, inwieweit die NK92-Zelllinie als Modell zur allgemeinen Analyse der NK-Zell-Aktivierung durch *P. falciparum*-infizierte Erythrozyten geeignet ist, um ein besseres Verständnis der Mechanismen, die das Erkennen und die Reaktion auf Parasiten durch NK-Zellen bewirken. Mit dem Ziel ein tieferes Verständnis über die Auswirkungen von *P. falciparum* auf NK-Zellen auf transkriptioneller Ebene zu erlangen, wurden Genexpressionsprofile von primären NK-Zellen 3 gesunder Spender untersucht, die mit *P. falciparum*-iRBCs kokultiviert wurden, und mit den Expressionsmustern von IL-12/IL-18 stimulierten NK-Zellen verglichen. Die Ergebnisse zeigten, dass NK92-Zellen einen Einfluss auf die Parasitämie haben, aber offensichtlich nicht von iRBCs aktiviert werden, obwohl auf transkriptioneller Ebene Anzeichen von Aktivierung und Proliferation nach der Kokultur mit iRBC sichtbar wurden. Die stimulierten primären NK-Zellen wiesen ein sehr ähnliches Genregulationsmuster bei allen Spendern auf. So konnte gezeigt werden, dass die Parasiten vor allem Gene modulieren, die durch IFN-α/-β reguliert werden sowie weitere wichtige Moleküle, die an antimikrobiellen Reaktionen beteiligt sind. IL-12/IL-18-behandelte NK-Zellen hingegen zeigten eine ganz andere Gensignatur: hier wurden IFN-γ- und TREM-1-gesteuerte Genen überexprimiert. Diese Ergebnisse deuten darauf hin, dass, obwohl NK92 scheinbar das Parasitenwachstum beeinflussen, aufgrund der Abwesenheit von klaren Anzeichen einer Aktivierung, NK92 als Modellsystem zur Untersuchung der NK-Zellreaktion auf

Parasiten ungeeignet sind. Darüber hinaus scheinen *P. falciparum*-iRBC und IL-12/IL-18 unterschiedliche Auswirkungen auf das Transkriptom von primären humanen NK-Zellen zu haben. IFN-α-gesteuerte Gene erwiesen sich als die von Parasiten primär induzierten Moleküle und sollten deswegen in der Zukunft weiter hinsichtlich ihres potenziellen Einsatzes in der Malariakontrolle untersucht werden.

ABSTRACT

Malaria is a one of the major life-threatening infections worldwide causing thousands of deaths every year. Natural killer cells are thought to play a role in innate responses against *Plasmodium falciparum* although the means by which such responses occur are still not fully clear. In this thesis, the suitableness of the NK92 cell line as models for general NK cell activation by *P. falciparum*-infected red blood cells was analyzed to better understand the mechanisms behind NK sensing and responding to parasites. Aiming to cover the current lack of information regarding the impact of *P. falciparum* on NK cells in a transcriptional level, the gene expression profile of 3 healthy donors' primary NK cells that were co-cultured with *P. falciparum*-iRBCs was examined and the expression pattern was compared to the same NK cells following stimulation with IL-12/IL-18. The results demonstrate that NK92 cells have an impact on parasitemia but are not evidently activated by iRBCs although showing transcriptional signs of priming and proliferation upon co-culture. Analysis of the stimulated primary NK cells showed a very similar pattern of gene regulation among all donors. Parasites modulated genes especially involved in the IFN-α/β arm of the "Interferon Signaling" and other essential molecules in antimicrobial response. IL12/IL18-treated NK cells showed a complete different gene signature: here IFN-γ and TREM-1-related genes were over-expressed. These findings show that, although NK92 appear to influence parasite growth, the lack of clear signs of activation and response disqualifies such cells as models for the NK response to parasites. In addition, *P. falciparum* and IL12/IL18 appear to impose diverse imprints on the transcriptome of human primary NK cells. IFN-α-related genes were the prominent molecules induced by parasites and deserve to be further investigated as potential new tools in malaria control.

1 INTRODUCTION

1.1 MALARIA

1.1.1 General aspects

Malaria is a disease caused by an intracellular *Apicomplexa* parasite of the genus *Plasmodium* and is transmitted by a bite of an infected female mosquito of the *Anopheles* species [1]. It is the most important protozoal disease that affects man and it is probably one of the oldest infectious diseases known. It has infected humans for over 50.000 years and may have been a human pathogen for the entire history of our species. Close relatives of the human malaria parasites remain common in chimpanzees [2].

The term malaria is derived from the Italian “mal’aria” which means ‘bad air’, from the early association of the disease with marshy areas [1]. The disease had its origins supposedly in Africa and accompanies human migration to the Mediterranean shores, India and South East Asia. The earliest references to malaria are descriptions of splenomegaly with fever from China in the Nei Ching Canon of Medicine in 1700 BC and from Egypt in the Ebers Papyrus in 1570 BC. The protozoa was identified by Alphonse Laveran in 1880 and, in 1897, Sir Roland Ross was the first to demonstrate that malaria parasites were transmitted by a mosquito [3].

The number of malaria deaths worldwide has been estimated at around 1 million per annum. Until today, transmission has been documented in 103 countries which puts over two billion people, more than 40% of the world’s population, at risk of contracting malaria (WHO 2010). Children under 5 years, pregnant women and non immune people are at major threat. The disease is devastating and the situation is worsened by the emergence of drug resistant parasites, global climate change, the

lack of health services and population migration/displacement.

1.1.2 Geographic distribution, vectors and parasites

Malaria is broadly distributed in both subtropical and tropical areas, with many parts of the tropics endemic for the disease (Figure 1). The countries of sub-Saharan Africa account for the majority of all malaria cases with the rest mostly clustered in India, Brazil, Afghanistan, Sri Lanka, Thailand, Indonesia, Vietnam, Cambodia and China [4,5]. However, in many temperate areas such as Europe and the USA, public health measures and economic development have been successful in achieving near or complete elimination of the disease [6].

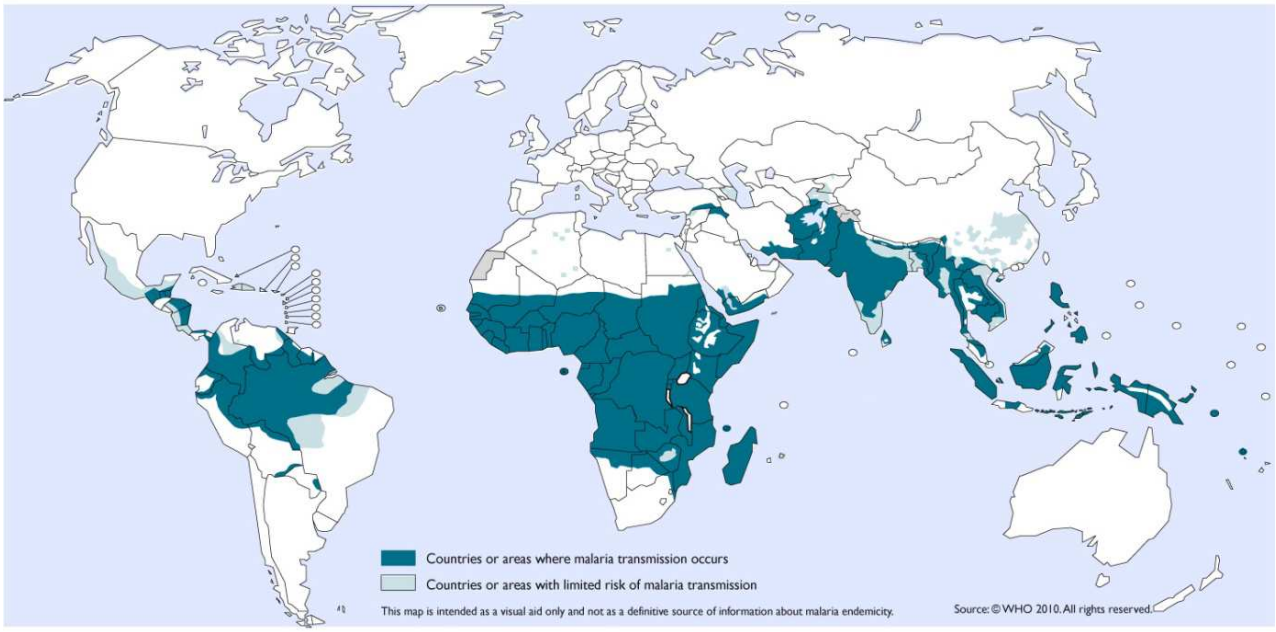

Figure 1. Countries or areas at risk of Malaria transmission

Source: WHO, 2010

Among approximately 400 species of *Anopheles* throughout the world, about sixty are malaria vectors under natural conditions and from these thirty are of major

importance. More than 100 species of *Plasmodium* can infect numerous animal species such as reptiles, birds and various mammals. Only five species can infect humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae and Plasmodium Knowlesi.* The agent of severe, potentially fatal malaria and the principal cause of malaria deaths in young children in Africa is *P. falciparum* [7]. The least common malaria parasite is *P. ovale*, which is restricted to West Africa while infective foci of *P. Knowlesi* have been identified in the past decade in Malaysia. *P. malariae* is found worldwide, but also with relatively low frequency. The most widespread malaria parasite is *P. vivax* although infections with this species are rarely fatal [6].

1.1.3 The life cycle of *Plasmodium spp*

Despite the different species of the parasite, the basic life cycle of each follows the same pathway (Figure 2). Malaria parasites not only have a complex life cycle in two alternating hosts systems, an arthropod vector and a vertebrate host (the female *Anopheles* mosquito and the human host in the case of *P. falciparum*), but are also capable of developing within highly specialised red blood cells. The life cycle involves developmental stages in the liver cells (exo-erythrocytic schizogony), in red blood cells of the human host (asexual schizogony) and in the tissues of the mosquito vector (sexual schizogony).

Infection is initiated with the bite of an infected female Anopheles mosquito and the release of sporozoites from the salivary glands into the bloodstream during feeding (Figure 2A). Within minutes, they are carried along by the circulatory system to the liver where they invade hepatocytes (Figure 2B) and undergo an asexual replication that results in the production of schizonts which, upon maturation, burst

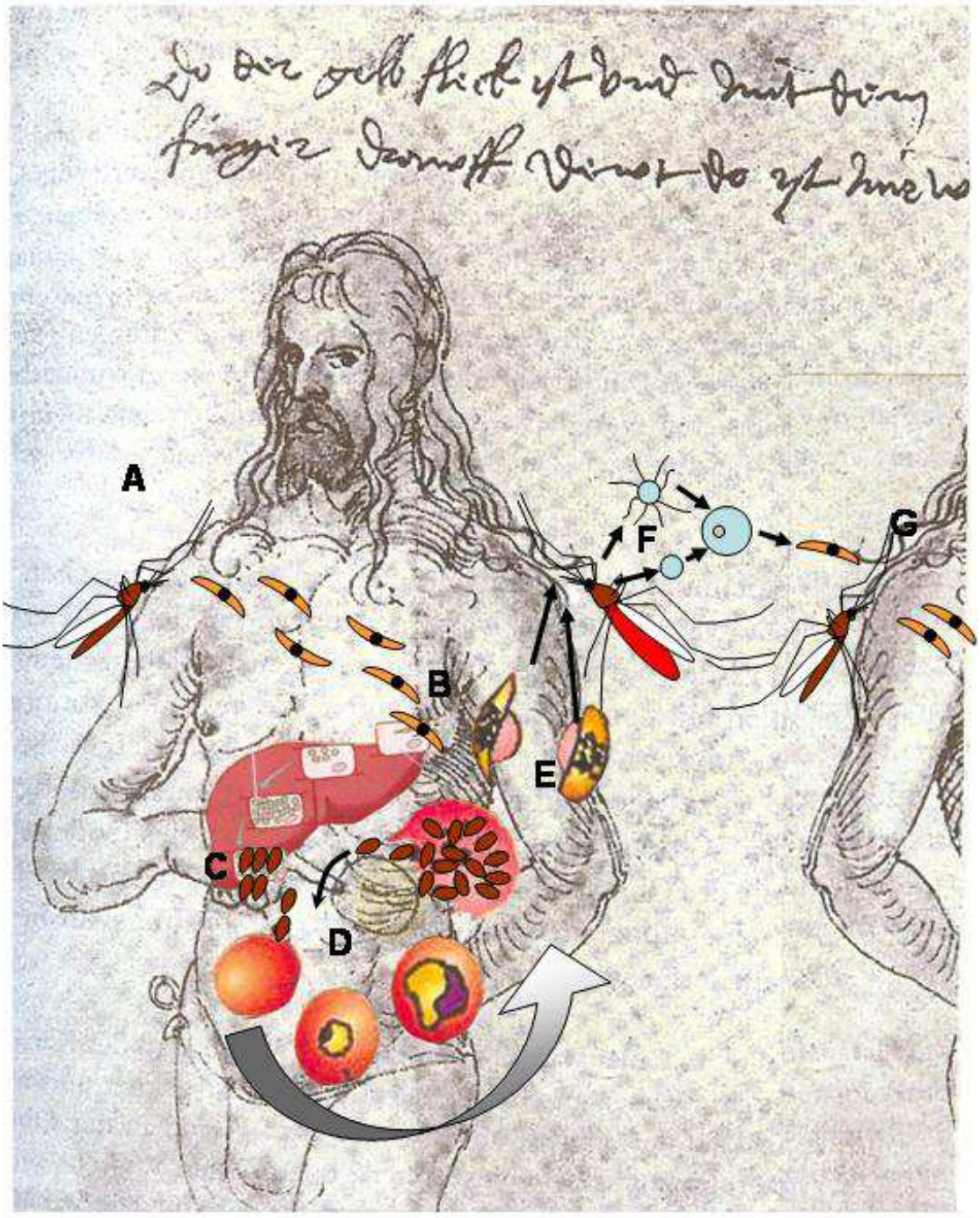

Figure 2. Life cycle of *P. falciparum.* As background a self-portrait of the German Renaissance artist Albert Dürer (1471 – 1528) who contracted malaria and supposedly died of it. In this picture Dürer points at his enlarged spleen in a letter to his doctor describing his symptoms.

Source: Kun & de Carvalho, 2009 [8].

(Figure 2C) and release merozoites into blood stream (Figure 2D). The released merozoites can then invade red blood cells (RBCs) and initiate the asexual phase.

Merozoites undergo a trophic period in which the parasite develops from the ring through trophozoite stage which by multiple rounds of nuclear division, results in the formation of a schizont. Invasion of the new erythrocytes by merozoites re-initiates another round of the blood–stage replicative cycle. The length of this erythrocytic stage of the cycle depends on the parasite species and varies between 24 hours to 72 hours.

Alternatively, some parasites differentiate endoerythrocytically into sexual forms, the male and female gametocytes. These gametocytes are taken up by a female anopheles mosquito (Figure 2E). Within the mosquito midgut, the male gametocyte undergoes a rapid nuclear division, producing flagellated microgametes which can fertilise the female macrogamete (Figure 2F). The resulting ookinete traverses the mosquito gut wall and encysts on its exterior as an oocyst. Soon the oocyst ruptures releasing hundreds of sporozoites into the mosquito body cavity where they eventually migrate to the mosquito salivary gland awaiting injection into the human host [9,10].

1.1.4 Diagnosis, clinical features and pathophysiology

Malaria is diagnosed using a combination of clinical observations, case history and a diagnostic test which is mainly the microscopic examination of blood [11]. Ideally, blood should be collected when the patient's temperature is rising, as that is when the greatest number of parasites is likely to be found. Thick blood films are used in routine diagnosis and as few as one parasite per 200ul of blood can be detected [6]. To support the clinical management of malaria, WHO recommends rapid

diagnostic tests (RDTs) which are based on the detection of parasite antigens and other products to be used together with microscopic tests (WHO 2010). However, polymerase chain reaction (PCR) is the current most accurate diagnostic test. It is not used as a routine test but can be performed in specialized reference laboratories.

The clinical spectrum of malaria is wide, spanning from asymptomatic infections to fulminant disease and death. Malaria symptoms can develop as soon as 6-8 days or as late as several months after the bite of an infected mosquito. The classical symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever/sweating lasting for 4-6 hours. It occurs every second day in *P. falciparum, P. vivax* and *P. ovale* infections, while every third day *for P. malariae.* This variation in the body temperature is due to the rupture of the red cells by merozoites. This event releases certain factors and toxins which could directly induce the release of cytokines resulting in chills and high grade fever. Severe malaria is usually complex and several key pathogenic processes such as jaundice, kidney failure, severe anemia and cerebral malaria can combine to cause serious and often fatal disease [12,13].

1.1.5 Treatment and Vaccines

Malaria is a curable disease if treated adequately and promptly. Current treatment relies on an “intercultural collaboration” consisting in the use of mosquito nets, DDT and multi-drug cocktails. Combination therapy was introduced to overcome the problem of rapidly development of resistance to common antimalarial drugs such as chloroquine. Due to chloroquine resistant malaria, sulfadoxine – pyrimethamine (SP), a combination of antifolate class of drugs was gradually introduced. However, resistance to SP was rapidly observed. A novel drug derived

from the plant *Artemisia annua*, artemisinin, is an extremely effective antimalarial recommended by WHO to be used as first line treatment. This drug, or its derivatives such as artesunate or artemether, is being used in mainly combinations with several other drugs such lumefantrine, amodiaquine, fansidar and mefloquine [14].

Currently available anti-malarial drugs include aryl amino alcohols (quinine, quinidine, mefloquine and halofantrine), 4-aminoquinolines (chloroquine and amodiaquine), folate synthesis inhibitors (proguanil and pyrimethamine), 8-aminoquinolines (primaquine), antimicrobials (tetracycline, clindamycin, azithromycin, fluoroquinolones), peroxides (artemisinin derivatives and analogues), naphthoquinones (atovaquone) and iron chelating agents (desferrioxamine) [15]. In *P falciparum*, unfortunatelly, resistance has been observed to all current anti-malarial drugs and more recently also to artemisinin derivatives [16].

Around the year 2000, the knowledge of the genetic map of malaria parasite placed trust that new action programs as well as new therapeutic targets and vaccines could be developed. An effective vaccine has been the subject of research for over 70 years with various antigens being identified as potential targets.

Currently, there are 38 *P. falciparum* and 2 *P. vivax* malaria vaccines candidates either in advanced preclinical or clinical development. However, the only one showing protective efficacy in field trials so far is RTS,S/AS01, a pre-erythrocytic stage vaccine consisting of the *P. falciparum* circumsporozoite protein combined with HBsAg. The candidate was developed by GlaxoSmithKline in partnership with PATH Malaria Vaccine Initiative and was recently shown to succeed in providing protection against both clinical and severe malaria in African children and is currently in Phase III trial [17].

1.1.6 Immune response in malaria

Immunity resulting from natural exposure to the *Plasmodium* parasites takes years to fully develop, fails to maintain sustained protection in absence of re-exposure and is rarely able to completely clear the parasites from the host. Nevertheless, infection gives rise to host responses which are regulated by both innate and adaptive immune systems as well as by environmental factors.

Unless restrained by immune mechanisms or by anti-malarial drugs, blood parasitemia increases exponentially to the point at which almost all of the available erythrocytes are infected [18]. Most of the elimination of the parasite occurs in the spleen under normal circumstances, although the liver has been shown to function as an alternative clearing site. Innate immune mechanisms are triggered when parasites density crosses a predefined threshold at which partial clearance of infected cells occurs.

Several elements of the immune system such as antibodies, CD4 and CD8 subsets of αβ T cells, γδ T cells, natural killer (NK) T cells, dendritic cells (DCs), macrophages and NK cells are involved in the reaction against *P. falciparum* [18,19]. The optimal immune response is characterized by early, intense, pro-inflammatory cytokine-mediated effectors mechanisms that kill or clear parasite infection [20].

Studies in both mice and humans have repeatedly shown that pro-inflammatory cytokines, specifically interferon gamma (IFN-γ), IL-12, and TNF-α, are essential mediators of protective immunity to erythrocytic malaria [21,22]. These cytokines can derive from either the innate or adaptive arm of the immune system. IFN-γ production is correlated with resistance to re-infection with *P. falciparum* [23,24] and protection from clinical attacks of malaria [25]. Plasma TNF-α and nitrogen oxide levels are associated with resolution of fever and parasite clearance

[26,27] and plasma TNF-α and IFN-γ mediate loss of infectivity of circulating gametocytes [28]. Clearance of remaining parasites and prevention of recrudescence or reinfection appears to be primarily antibody-mediated [29].

Although Innate or adaptive immune effector mechanisms can limit the peak of parasitemia, prevent severe pathology and reduce the load of circulating infected cells, the typical failure in complete elimination of infection leads to many months or years of persistent low-grade parasitemia, frequently undetectable by microscopy [30]. Chronic and/or severe infection might therefore result from an imbalance of the timing of innate and adaptive responses to the parasite [18].

1.2 NK CELLS

1.2.1 General aspects

NK cells were originally characterized as large granular lymphocytes of the innate immune system on basis of their morphology, their expression of many lymphoid markers and their origin (majority) from common lymphoid progenitors in the bone marrow. They compose around 7% of the total lymphocytes and circulate through the blood and lymphatics residing virtually in all organs where they can promptly recognise and kill stressed, virally infected or transformed cells without prior sensitization [31].

The most specific phenotypic NK cell marker across mammalian species appears to be the natural cytotoxicity receptor NKp46, although small subsets of T cells also express it [32]. Traditionally, NK cells are characterized by the lack of CD3 and, based on their surface density of CD56 (neural cell adhesion molecule, NMCAM) and CD16 (FcγRIIIA receptor), can be divided into two broad

subpopulations. The $CD56^{dim}/CD16^{+}$ subset (85-90%) has little intrinsic secretory capability but potent cytolytic activity (perforin expressing cells) whereas the $CD56^{bright}/CD16^{-}$ subset (10-15%) lack perforin and is a potent cytokine secretor upon activation [31,33].

NK cell functions are controlled by a unique array of germ-line encoded receptors (NKRs) that can inhibit or enhance NK cell killing. Because they are powerful effector cells, their activation must be tightly regulated. To avoid autoreactivity therefore, NK cells are trained or educated during their maturation in the bone marrow whereby they become able to distinguish healthy from abnormal tissues.

Although normally classified as part of the innate immunity, it has recently been accepted that NK cells share with T and B cells properties normally associated with the adaptive immunity [34,35]. Like typical innate cells they can respond at peripheral sites of infections, trafficking rapidly to mediate cytotoxicity and cytokine release without previous priming. As adaptive responders, NK cells posses the features of clonal expansion, longevity and the ability to respond more robustly during subsequent encounters with the same pathogen. Such newly found overlapping characteristics break old concepts about defined borders between the innate and adaptive arms of the immune system.

1.2.2 NK cell receptors and education

Despite significant contribution of cytokines to NK cells activation, these cells can directly discriminate between normal and abnormal (potentially dangerous cells) cells without prior sensitization and immediately respond to this stimulus [36,37]. NK cells lack the capacity to somatically rearrange antigen-specific receptors but have

the ability to recognize major histocompatibility complex (MHC) class I or class I-like molecules on target cells through a unique class of germline encoded receptors that can either stimulate NK cell reactivity (activating receptors) or suppress it (inhibitory receptors). Such complex repertoire allows NK cells to recognize pathogen-encoded molecules (non-self-recognition), self-proteins whose expression is up-regulated in transformed or infected cells (stress-induced self-recognition), or self-proteins that are expressed by normal cells but down-regulated by infected or transformed cells (missing-self-recognition) [38-40].

Three major superfamilies of NKR have been described: the killer immunoglobulin (Ig)-like receptors (KIR) superfamily which recognizes classical MHC class I molecules (HLA A, B and C), the C-type lectin superfamily recognizing non-classical MHC class I or class I-like molecules (includes CD94 and NKG2 receptors recognizing HLA-E), and the natural cytotoxicity receptors (NCR) whose ligands remains poorly defined. Other NKR that function primarily as co-receptors have been described but their ligands and significance remain unknown in some cases [41].

During NK cell development, expression and signalling via NKG2 and KIR receptors play a pivotal role in the education of NK cells. They become self-tolerant via licensing, a process of MHC-dependent education [42-45]. Engagement of inhibitory receptors with self-MHC before acquiring functional competence ensures that NK cells lacking such receptors are not fully functional and thereby detrimental to the host. In addition, heterogeneity comes with maturation. It has been demonstrated that when $CD56^{bright}$ cells turn $CD56^{dim}$, they lose expression of NKG2A, of natural cytotoxic receptors, of CD27 and CD62L while acquiring CD16, LIR-1, Siglec-9, KIRs and, in the final stage of activation, they acquire of CD57 [46-48].

1.2.3 Effector functions

NK cells use a wide range of different mechanisms to kill their targets (figure 3). They are able to recognize a variety of stressed cells in the absence or presence of antibodies (figure 3; blue arrows). They can mediate antibody-dependent cellular cytotoxicity (ADCC) through their Fc-receptor complex (CD16) which allows them to participate in the elimination of antibody-targeted cells recognized as nonself [49]. Apoptosis through granule-released perforin / granzyme is the principal pathway used by natural killers to eliminate infected or transformed cells [50]. Cell-mediated-bound and secreted cytokines of the TNF family are also able to mediate direct cytotoxicity. NK cells can express at least three TNF-family ligands – FAS ligand (FASL), TNF and TNF-related apoptosis inducing ligand (TRAIL) all shown to induce tumour-cell apoptosis [51,52].

Additionally, they are able to produce chemokines and cytokines (figure 3; red/green arrows) depending on the cytokine microenvironment and on interactions with other cells such as T cells, dendritic cells (DCs) and macrophages [53]. Type I IFN, IL-12, IL-18 and IL-15 are potent activators of their cells efector functions [54]. It is well known that IL-12 promotes NK cells proliferation, cytotoxicity and that alone or in combination with IL-18, IL-15 and IL-2 it induces cytokine secretion, including IFN-γ, TNF-α and MP1-α [55].

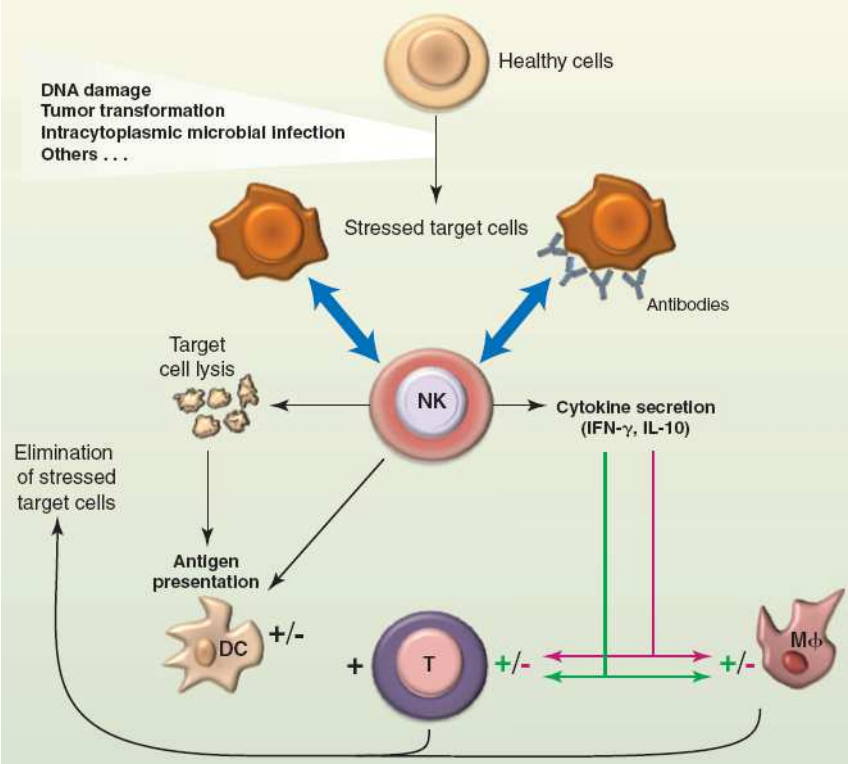

Figure 3. The biological functions of NK cells

Source: Vivier et al., 2011 [35].

Moreover, NK cells are also source of macrophage-activating factor, lymphotoxin-α (LT-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP), IL-15, IL-10, IL-13 and IL-3 [55,56]. Although not yet completely characterized regarding its origin, a novel population of IL-22 producing NK cells (NK22) has been identified in mouse and human mucosal tissues [57,58].

Traditionally, NK cell function can be regulated by transforming growth factor (TGF)-β (29) and by regulatory T cells through a TGF-β-dependent mechanisms in human and mice [59,60]. NK-cell production of T helper (Th) 1 and Th2 cytokines occurs at distinct stages of NK-cell differentiation. Mature NK cells lose the ability to produce the type 2 cytokines and acquire the ability to produce IFN-γ [52].

IFN-γ is central to host resistance to many infections, regulating several genes associated with immune system functions and recruiting other effector cells to the site

of infection [61]. It activates macrophages and neutrophils, stimulates the differentiation of Th1 $CD4^+$ lymphocytes, enhances antigen-presentation by the up-regulation of MHC and MHC-associated molecules, and induces IgG subclasses switching in B cells, thereby bridging the innate and adaptive arms of the immune system [61]. IFN-γ secretion by NK cells has been demonstrated to play an important role in triggering potent immune responses to various pathogens [62].

1.2.4 Memory

It has recently been observed that NK cells exhibit many features normally associated with adaptive immunity. Notions originally restricted to T and B cells as the ability to expand due to pathogen encounter, to mount a form of antigen-specific immunological “memory” and to generate an enhanced secondary recall response to rechallenge, now apply to NK cells [63-65].

The current “NK memory” knowledge is still primary but a few probable characteristics have been already described. Apparently, changes in NK behaviour based on prior activation seem to happen in response to both specific and non-specific activation. In addition, memory NK cells were shown to have a $KLRG1^{hi}CD43^{hi}CD27^-$ phenotype and, when compared to naive mature NK cells, they produce more IFN-γ and have a higher cytolytic capacity before and after stimulation. Such characteristics are apparently heritable and passed on to daughter cells that were never previously activated. NK cells can persist far longer than previously estimated. The identification of such long-lived cells in mice raises hopes for the existence of similar populations in humans. The confirmation of this hypothesis would consolidate NK cells as potential subjects for vaccine development.

1.2.5 NK cells and immunity to malaria

The key element in the cell-mediated effector responses to malaria is the rapid induction of IFN-γ. It was shown that human natural killer cells can be an important early source of IFN-γ after exposure of leukocytes to *P. falciparum* infected red blood cells (iRBCs), although in an extremely heterogeneous manner [66,67]. NK cells are activated during the first 12-15 h of exposure to iRBCs whereas γδT cells and NK T cells respond after 24 to 48 h. However, the responses given by each cell type are highly correlated indicating that NK cells might initiate a cascade of immune responses to parasites [68].

Both $CD56^{bright}$ and $CD56^{dim}$ NK subsets were observed to produce abundant IFN-γ when stimulated by malaria-infected erythrocytes. In addition, RBCs infected by several different strains of *P. falciparum* induced up-regulation of the activation markers CD69 and CD25 on NK cells [67]. Purified NK cells and the NKL cell line were found to produce IL-18 in response to iRBCs in addition to up-regulation of CD69. This proves the existence of detectable functional consequences even in the absence of accessory cells [66,67,69].

Another human NK cell line, NK92, was also shown to interact with iRBCs [70]. They were observed to be able to form rosettes with erythrocytes infected by certain *P. falciparum* strains whereas no rosettes were formed with uninfected RBCs (figure 4).

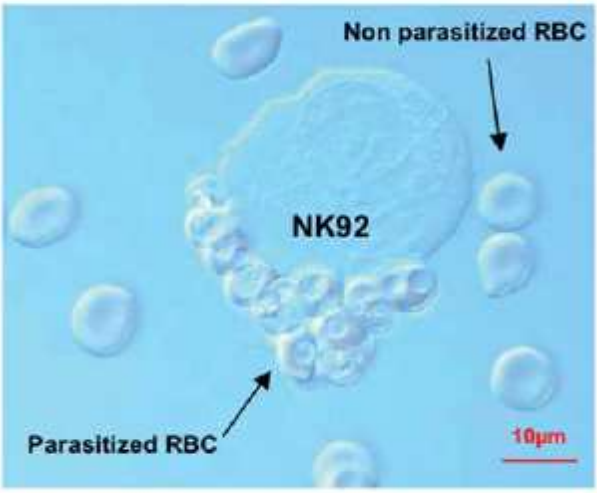

Figure 4. NK92 cell line and iRBCs: rosettes

Source: Baratin et al., 2007 [70].

Along this line, iRBCs and freshly isolated NK cells have been reported to form conjugates [66,67]. Such a specific interaction suggests that one or more receptors, although not defined yet, might be involved in the direct recognition of iRBCs [1]. In humans, KIR molecules might play a role since associations between KIR genotype and susceptibility to malaria were already described [66].

Signals from accessory cells such as macrophages, monocytes and dendritic cells (DCs) were demonstrated to be absolutely necessary for optimal NK cell reaction to many infectious agents [71-73]. The same is true for the responses to *Plasmodium*. NK cell activation appears to depend on direct contact with iRBCs and is regulated by co-stimulatory molecules [74] (e.g. CD40) and by several accessory cell-derived cytokines (e.g. IL-2, IL-12, IL-18 and type I IFNs) [66,69,74]. Such findings postulated a new model of NK response against non-viral pathogens: activation of accessory cells precedes rather than follows NK activation (figure 5) [75].

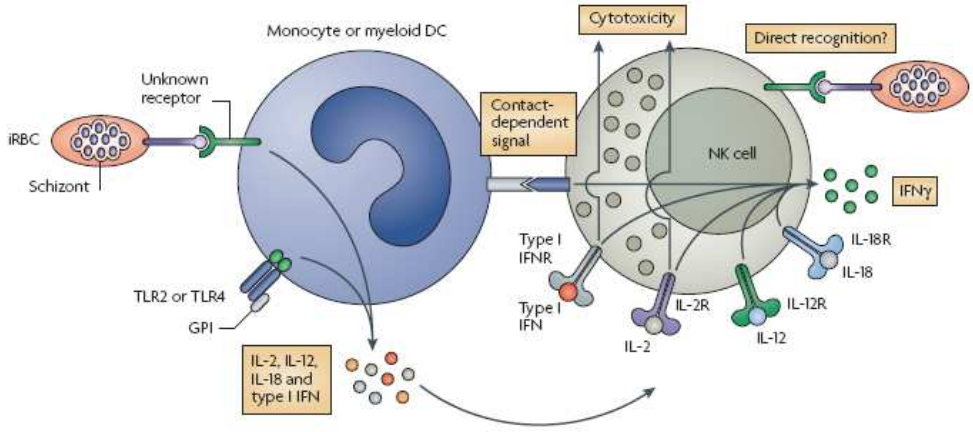

Figure 5. NK cells response to *P. falciparum*-iRBCs

Source: Newman & Riley, 2007 [75]

Intracellular pathogens convert host cells into targets for NK cells by diverse mechanisms. Soluble Fas ligand (sFasL) interactions with Fas expressed on iRBCs surface and release of granzyme B are two of the possible mechanisms shown to probably be related to the direct lysis of iRBCs by NK cells derived from naive and infected people [76,77]. The DBL-alpha domain, part of the *P. falciparum*-infected erythrocyte membrane protein 1 (PFEMP1), was pointed to be the molecule through which NK cells recognize iRBCs [78]. MYD88-associated IL18R in NK cells was described to be the major indirect sensor for *P. falciparum* infection [69].

Activation of NK cells *in vivo* is also inferred from evidence that PBMCs from children with acute *P. falciparum* infection enhance lytic activity against the NK-sensitive cell line K562 [79] and that serum levels of soluble granzymes and IFN-γ increase concomitantly two days before the onset of clinical symptoms in experimental malaria infections [80]. In this line, elevated IFN-γ producing NK cells in the placenta and CD45RO memory-like and CD4T cells in peripheral blood were suggested to possibly be involved in protection against malaria in pregnancy [81].

In mice, no clear role for either NK cells or IFN-γ in controlling primary *P. yoelli*

infection was suggested [82]. However, the same authors observe that depletion of monocytes/macrophages exacerbated parasite growth and anemia during lethal and nonlethal acute *P. yoelli* infections, indicating that there is an IFNy-, NK cell- and T cell –independent pathway for induction of effector macrophages during acute malaria infection. More recently however, early response to mice *P. chabaudi* blood infection was shown to be marked by a primary wave of interferon with a subsequent response by NK cells [83]. In contrary, suppression of acute *P. chabaudi* infection by cell mediated immunity was demonstrated to be independent of NK-derived but dependent of γδT cell-derived IFN-y [84].

In humans nevertheless, malaria specific heterogeneity in early IFN-γ responsiveness was observed among children. High-level of early IFN-γ were associated with protection from high-density and clinical *P. falciparum* infections. Parasite-induced IFN-γ was predominantly derived from γδT cells (68% of which expressed the natural killer marker CD56) and αβT cells, whereas natural killer and other cells made only minor contributions [85]. Conversely, a study with a large cohort of malaria-naive donors shows that the majority of IFN-γ^+ T cells are αβ and not γδ T cells. They also describe that NK cells dominate the early (around 18h) IFN-γ response, that NK and T cells contribute equally to the response at 24h and that T cells dominate afterwards [86].

The current knowledge points to the fact that NK cells make a significant contribution to total IFN-γ production in response to iRBCs as consequence of their dependency on memory T cell-derived IL-2 in an MHC class II-dependent manner. This indicates that the innate response to infections actually relies upon complex interactions between NK cells, T cells and accessory cells [87,88]. In general, both indirect signalling via accessory cells, T cell-derived cytokines and direct signalling

presumably through NK receptors are needed for optimal stimulation of NK cell activity upon *P. falciparum* encounter.

2 AIMS

The major objective of the two studies presented in this thesis was to provide the scientific community with a better knowledge of the mechanisms behind the NK cell sensing and responding to *Plasmodium falciparum* parasites. For such, it was performed as follows:

With the notion that once an appropriate model is obtained it can be useful for understanding the behavior of a system:

- the suitability of the NK92 human cell line as models for the NK mechanisms involved in the immune response against malaria was investigated.

To overcome the current lack of information regarding the impact of *P. falciparum* on NK cells in a transcriptional level :

- the gene expression profile of three healthy donors' primary NK cells that were co-cultured with *P. falciparum*-iRBCs was analysed and subsequently compared to the transcriptome induced by IL12/IL18 in the same freshly isolated NK cells.

3 RESULTS

3.1 PAPER I

Limited activity of NK92 cells against *Plasmodium falciparum*-infected erythrocytes

To investigated suitability of NK92 cells as models for the NK mechanisms involved in the immune response against malaria, experiments to observe NK sensing of parasites as IFN-γ release, detection of activation and formation of rosettes were performed. Parasitemia was followed upon NK92 and iRBCs co-culture. By performing microarrays, the impact of parasites on NK92 cells in a transcriptional level could be observed.

Although not evidently activated by iRBCs (Figure 1, Table 1 - Paper I) and minor engagement with iRBCs to form real rosettes (Figure 2 - Paper I), NK92 cells showed transcriptional signs of priming and proliferation upon contact with *Plasmodium (*Figure 3, Tables 2, Table 3 - Paper I). Decreased parasite growth was observed due to co-incubation with NK92 cells (Figure 5 - Paper I). However, it is probable that such effect is not NK-specific since irrelevant cells also affected parasitemia *in vitro* (Figure 6 - Paper I).

3.2 PAPER II

***Plasmodium falciparum*-infected erythrocytes and IL12/IL18 induce diverse transcriptomes in human NK cells: IFN-α/β pathway versus TREM signaling**

To better understand the mechanisms involved in NK sensing and responding to *P. falciparum,* a affymetrix oligonucleotide microarray analysis was conducted on NK cells from three 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 (Table 1, Table 2, Figure 2A, Figure 3 - Paper II).

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 (Table 2, Table 3, Figure 2B, supplementary Figure S2 - Paper II). For a general overview, significantly regulated genes were arranged in a heatmap. This clearly shows 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 (Figure 4 - Paper II).

After co-culture with parasites, cells from all donors were shown to up-regulate the activation marker CD69 and this activation was improved upon addition of IFN-α to the system (Figure 5 - Paper II). However, parasite growth was not affected upon co-culture with expanded NK cells associated with IFN-α and IL12/18 (Figure 6 - Paper II).

4 GENERAL DISCUSSION

The knowledge of the immune mechanisms involved in malaria is still partial. Studies regarding the in vivo functions of NK cells will lead to a more comprehensive understanding of the immunological basis for protective immunity. This thesis therefore is focused on the cellular and transcriptional effects that *Plasmodium* parasites impose on freshly isolated human NK cells and on a NK cell line.

Cell lines are potential resources frequently adopted in studies aiming to investigate pathological mechanisms, particularly in diseases where primary material is of difficult access. The well-characterized NK92 cell line was already shown to directly interact with *P. falciparum*-iRBCs [66,67]. The choice therefore for immortalized cells stands for the purpose of establishing a “model” for NK cell innate response to *Plasmodium*. The need of accessory cells in the system for proper NK activation is acknowledge. However, a reductionist approach (without accessory cells) was chosen in order to observe the very specific behaviour of NK92 cells.

The results obtained were surprising in many aspects. NK92 cells neither responded to iRBCs stimulus through IFN-γ release nor via up-regulation of the CD69 and CD25 activation markers, events already shown to occur when fresh NK cells meet parasites. Besides the lack of accessory cells in the system, another potential explanation for the weak NK response to stimulus might be the influence of the genetic background of the NK92 cells` donor. It is known that the response to iRBCs is not homogeneous among people and that genes may be variably expressed among NK clones, which might therefore influence NK activation. Such lousy signs of activation, however, are contradictory to what was observed with the arrays. Genes related to cell proliferation and T cell priming were modulated, what appears to be the result of parasite sensing. Moreover, the analysis of the NK92

microarrays showed that parasites induce similar mechanisms in human NK cells compared to mouse models. Corroborating the transcriptional signs of the possible NK response to parasites, the co-cultures showed an impact of NK92 cells (and also T cells) on parasite growth. Cells were kept at usual and viable concentrations for this type of assays, as commonly performed in experiments where fresh cells are used. However, C32 and HeLa cells, irrelevant to immune response in malaria, also suppressed parasite development which suggested that the growth inhibition detected was an effect of competition for limited resources in the presence of growing/dividing cells. Still, a “parasite-strain-biased” growth inhibition could be observed, but due to the lack of an NK cell-specific response it is difficult to ensure that parasites die as a reflection of NK92 cytotoxicity, raising the importance of further investigations.

With the fresh NK cells, the main aim was to observe transcriptional changes that malaria parasites impose on NK cells within PBMCs. For such, in vitro experiments were performed and the NK cells’ gene expression profile and activation characteristics were measured at one time point (24h) due to prior observations that the peak of NK IFN-γ release occurs between 15h-24h after parasite stimulus [68]. However, up-regulation of IFN-γ related genes was not observed after 24 hours. Instead, type-I interferon related genes were modulated. Such findings were in accordance to those from studies where IFN-α was added to the system as a stimulus. In such studies, the up-regulation of IFIT1, IFIT3, IFI44, IFIT2, OASs and other interferon-α related molecules was observed in whole PBMCs and in NK cells isolated from healthy donors [89,90]. Over-expression of such genes was also reported in diseases such as Lupus Erythemathosus and Tuberculosis providing primary evidences for a role of type-I-interferons in human disease pathogenesis [91-

93]. It is possible, however, that such IFN-α signature induced by parasites is the first step of a cascade of events that will lead to the release of IFN-γ. This co-induction concept was already demonstrated by studies with healthy people and parasitic diseases [94-96]. *Plasmodium*-mediated IFN-α responses have been reported by few studies in the past [97-99]. 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) [100]. 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 used in our study appear to respond in concert with their PBMCs from malaria-infected individuals.

More recently, studies with human and mice *Plasmodium* infections have claimed a role for IFN-α in the course of the disease [83,101]. However, the importance of type-I-interferon in malaria is still contradictory. The cytokine was shown to be dispensable in *P. chabaudi* infections [102] but to have a protective effect on disease caused by *P. berghei* and *P. yoelli* [103,104].

The results obtained by FACS analysis showed that IFN-α boosted up-regulation of CD69 (and not IFN-γ) but did not interfere with parasite growth. This can be due to donor-related characteristics (low IFN-γ responder) or simply to cell type features since there has still been considerable debate regarding the importance of NK cells and T cells in immunity to malaria. Some authors show that suppression of infections is dependent on γδT cells and not on NK cells [84] while NK cells and αβT cells (and not γδT) were demonstrated to be the main contributors for the IFN-γ

response by others [86].

There were no similarities between the transcripts induced by IL mix and iRBCs in our study. The interleukins induced genes related to signaling triggered by TREM-1, an immune regulatory molecule that is expressed in monocytes, dendritic cells, NK cells and neutrophils and is important for innate and adaptive immune responses [105-107]. The Protein Ubiquitination pathway was also induced by the cytokines. Such pathway is responsible, among others, for immune surveillance where IFN-γ is a prominent molecule. Such cytokine was the top up-regulated molecule induced by IL12+IL18 in our study. It probably induces immune cells to express immunoproteasomes that impose changes on the normal cascade of actions of the ubiquitin-proteasome system, leading to host defense stimulation [108].

Overall, the studies here compiled show that NK92 cells do not suit as models for NK immune responses to *Plasmodium falciparum.* These parasites provoke transcriptional changes in human NK cells that differ from that incited by the well known NK stimulators, IL12 and IL18. The IFN-α signature induced by *Plasmodium* brings new insights into the mechanisms behind host-parasite interactions in malaria. Such information could be used as new tools for malaria vaccine development, a past and present high-priority goal.

5 PERSONAL CONTRIBUTIONS

Hereby I declare that I am the sole author of this thesis, which was carried out under the supervision of Dr. Jürgen Kun in the Department of Parasitology, Institute for Tropical Medicine, University of Tübingen, Germany.

My contributions to the papers include:

Paper I:

- Design of the study
- Parasite culture
- NK92 cells culture
- Co-culture of NK92 cells with *Plasmodium*
- Rosettes experiments
- ELISA IFN-γ
- Flow cytometry experiments
- Data analysis
- Writing of the manuscript

Paper II:

- Design of the study
- Parasite culture
- NK92 cells, JY cells and primary cells culture
- PBMC isolation
- Co-culture of PBMCs/NK cells with *Plasmodium*
- NK cell isolation
- NK cell RNA extraction
- Flow cytometry experiments
- Reverse transcription
- Real time PCR
- JY cell line irradiation
- Primary NK cell expansion
- ELISA HRP
- Knock-out experiments
- Data analysis
- Writing of the manuscript

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Limited response of NK92 cells to *Plasmodium falciparum*-infected erythrocytes

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Abstract

Background: Mechanisms by which anti-malarial immune responses occur are still not fully clear. Natural killer (NK) cells are thought to play a pivotal role in innate responses against *Plasmodium falciparum*. In this study, the suitability of NK92 cells as models for the NK mechanisms involved in the immune response against malaria was investigated.

Methods: NK92 cells were assessed for several signs of activation and cytotoxicity due to contact to parasites and were as well examined by oligonucleotide microarrays for an insight on the impact *P. falciparum*-infected erythrocytes have on their transcriptome. To address the parasite side of such interaction, growth inhibition assays were performed including non-NK cells as controls.

Results: By performing microarrays with NK92 cells, the impact of parasites on a transcriptional level was observed. The findings show that, although not evidently activated by iRBCs, NK92 cells show transcriptional signs of priming and proliferation. In addition, decreased parasitaemia was observed due to co-incubation with NK92 cells. However, such effect might not be NK-specific since irrelevant cells also affected parasite growth *in vitro*.

Conclusions: Although NK92 cells are here shown to behave as poor models for the NK immune response against parasites, the results obtained in this study may be of use for future investigations regarding host-parasites interactions in malaria.

Background

More than any other disease restricted to tropical areas, malaria has a widespread impact and is considered one of the main public health problems in the world. The disease causes thousands of deaths annually and its burden continues to grow especially in areas of poverty.

The human immune system fails to completely eliminate malarial infections and the reason for this is still not known. Nevertheless, it is clear that immunity to malaria involves the innate and adaptive arms of the immune system, engaging macrophages, dendritic cells, γδT cells, Natural Killer T (NKT) and NK cells to participate in the response developed by the host against parasites [1,2]. Natural killer lymphocytes are thought to play an important role in combating infections. Without requiring clonal expansion ("naturally") and balanced by a repertoire of activating and inhibitory receptors, these cells are promptly triggered to develop their biological functions: cytotoxicity, cytokine and chemokine secretion and, therefore, co-stimulation of other cells of the immune system [3].

Experimental evidence suggested that NK cells are one of the first cells to sense a malarial infection and produce type 2 interferon [4-6]. Interferon-γ is described to be important for limiting parasitaemia in early infections. It presumably inhibits parasite development in hepatocytes and activates macrophages to promote phagocytose of intra-erythrocytic parasites and merozoites. Indeed, the need of accessory cells for complete NK activation via cross talk with dendritic cells and monocytes was already reported [7-9]. Moreover, killer cells derived from patients with malaria as well as from donors with no prior exposure to the disease were described to be cytotoxic to and lyse *Plasmodium*-infected erythrocytes (iRBCs) [10,11].

The immune response in malaria has been extensively investigated over the years. However, further studies are still required for a clear knowledge of the many unresolved

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issues regarding the *in vivo* functions of NK cells in malaria. NK cell lines are potential resources frequently adopted in studies aiming to investigate pathological mechanisms, particularly in diseases where primary material is of difficult access. A valuable use of these cells includes attempts to mimic the processes by which fresh NK cells recognize non-self, stress induced-self and missing-self molecules that trigger their activation and further response to infections.

The well-characterized NK92 cell line was already shown to directly interact with red blood cells infected with *P. falciparum* [4,5]. With the notion that once a model is appropriate it can be useful for understanding the behaviour of a system, the NK cell and the *Plasmodium* side of such host-parasite interaction was investigated to examine whether NK92 cells can be used as models for the mechanisms involved in the NK fight against malaria.

Methods

Cells

The NK92 cell line was purchased from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany) and kept in culture at 0.2-0.6 × 10^6 cells/ml in alpha-MEM (Sigma-Aldrich) supplemented with FBS (12,5%; Sigma-Aldrich), horse serum (12,5%; Sigma-Aldrich), L-glutamine (2 mM; Sigma-Aldrich), penicillin-streptomycin (10 ml/L; Invitrogen) and recombinant human interleukin-2 (rIL-2; 10 ng/ml; Invitrogen).

Jurkat cells were obtained from the German Resource Centre for Biological Material (DSMZ; Braunschweig, Germany). Cells were kept in culture at 0.2-0.6 × 10^6 cells/ml in RPMI 1640 (Sigma-Aldrich) supplemented with FBS (10%; Sigma-Aldrich), L-glutamine (2 mM; Sigma-Aldrich) and penicillin-streptomycin (10 ml/L; Invitrogen).

HeLa cells were purchased from the German Resource Centre for Biological Material (DSMZ; Braunschweig, Germany). Cells were grown to maximum 70%/80% confluency in DMEM (Sigma-Aldrich) supplemented with FBS (10%; Sigma-Aldrich), L-glutamine (2 mM; Sigma-Aldrich) and penicillin-streptomycin (10 ml/L; Invitrogen).

C32 cells were obtained from American Type Culture Collection (ATCC; Rockville, MA, USA). Cells were grown to maximum 70%/80% confluency in DMEM (Sigma-Aldrich) supplemented with FBS (10%; Sigma-Aldrich), L-Glutamine (2 mM; Sigma-Aldrich), Gentamycin (50 μg/ml; Invitrogen) and MEM non-essential amino acid solution (1%; Sigma-Aldrich).

Mycoplasma free cells of maximum 12^{th} passage were utilized for all the experiments described in this study.

Plasmodium falciparum culture

The laboratory strains *P. falciparum* 3D7, FCR3-CSA, FCR3-CD36 and Dd2 were maintained in continuous culture as described elsewhere [12] and frequently tested for Mycoplasma contamination by PCR. Prior to each experiment, FCR3-CSA parasites were selected for CSA adhesion as previously described [13]. Ring stages of all strains were obtained by constant culture synchronization with 5% sorbitol and mature schizont-infected erythrocytes were purified by magnetic cell sorting LD columns (Miltenyi Biotec, Berg. Gladbach, Germany).

Cytokine EASIA assay

In a 96 flat-bottomed well plate, NK92 cells (10^5) previously cultured with recombinant human interleukin (rIL-)2 and without rIL-2 (24 hours starvation conditions) were co-incubated with 3D7 schizont-iRBCs (10 × 10^5) and uninfected erythrocytes (10 × 10^5) in RPMI-1640 medium (200 ul/well) at 37°C in 5% CO_2. After 24 hours of incubation, IFN-γ was measured in the supernatants by a solid phase enzyme amplified sensitivity immunoassay kit (EASIA; Biosource). As controls, pure RPMI-1640 and supernatants of NK92 cells cultured in their normal growth medium (+ rIL-2) and under a 24 hours period of "starvation" (cell medium without rIL-2) as well as supernatants of iRBCs and uRBCs incubated without cells in RPMI-1640 were analysed for presence of IFN-γ. All samples were tested in duplicate according to the manufacturer's recommendations.

NK92/iRBCs co-culture and flow cytometry

NK92 cells were kept in two different environments for 24 hours prior to the co-culture: in normal cell medium (+rIL2; NK92 nm) and in cell medium without rIL-2 (starvation medium; NK92s). Cells from both environments were co-cultured with 3D7 schizont-infected erythrocytes and uRBCs (NK92-RBCs ratio: 1:3) in their respective growth medium. As a positive control, cells were also incubated with a mixture of IL-12 and IL-18 (Peprotech and MBL, respectively; 100 ng/10^6 cells each). After the indicated time of incubation at 37°C and 5% CO_2, NK cells from the co-culture as well as cells incubated without RBCs were stained for 30 min at 4°C with fluorochrome-conjugated antibodies for surface CD56 (APC), CD3 (PE), CD16 (FITC), CD69 (FITC), CD25 (PE) in parallel with the appropriate isotype controls. Cells were also internally stained with the IFN-γ (PE) antibody (all BD Biosciences). Dead cells were excluded from the analysis based on scatter signals and 7AAD fluorescence. Acquisition of samples was carried out in a FACS canto flow cytometer (BD Biosciences). Data were analysed with BD FACS Diva 6.0 software. Gates were set on the events compatible to lymphocytes regarding "size of the cells" × "internal complexity" (FSC × SSC). A total of 10.000 events were collected for each sample.

Cytoadhesion assay

NK92 cells were incubated with 3D7 and FCR3-CSA schizont and ring-infected erythrocytes (NK - iRBCs ratio: 1:1, 1:3, 1:10) in RPMI 1640 medium, in a 6-well plate at 37°C and 5% CO_2 for 1 h under continuous shaking. As a control for unspecific binding, the FCR3-CD36 strain was submitted to the same conditions. After incubation, the co-culture was stained with acridine orange and the adhesion of iRBCs to NK cells (rosettes) was observed by fluorescence microscopy.

RNA isolation and microarray analysis of NK92 after co-culture

After 0, 6, 12 and 24 hours of co-culture of NK92 with 3D7-schizont-iRBCs or uninfected erythrocytes (uRBCs; 1:3) at 37°C in 5% CO_2, RBCs were lysed (Lysis Buffer, BD) and NK cell RNA was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany). Quality of RNA specimen was validated on a Agilent BioAnalyzer 2100 (Agilent, Germany) and processed for Affymetrix Gene Chips using Affymetrix Whole Transcript Sense Target Labeling Kit (Affymetrix, Santa Clara, USA). Fragmented and labeled cDNA was hybridized onto human HuGene1.0 ST Gene Chips (Affymetrix, Santa Clara, USA). Staining of biotinylated cDNA and scanning of arrays were performed according to the manufacturer's recommendations. Analysis was done with 3 biological replicates. The MIAMI-compliant complete microarray data is deposited at the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO) under the entry name GSE 26876.

Microarray data analysis

Raw data were imported into Expression Console 1.0 (Affymetrix, Santa Clara). Robust multichip average algorithm (RMA16, Bolstad 2003) was applied for array normalization and signal calculation [14]. Normalized signal values were imported into Genespring 11 (Agilent Technologies) and intensity values for the biological replicates were averaged for each time point and treatment. Significance was calculated using a Student *t* test without multiple testing correction, considering all transcripts with a minimum fold change in expression level of 1.5-fold together with a p-value < 0,05 compared to time point 0. Principal component analysis was performed based on the covariance matrix of normalized gene expression values to reduce the complexity of high-dimensional data structures and compare inter-variability of the array. Differentially expressed genes were annotated with Affymetrix database and their corresponding protein was ascribed. Lists of regulated genes were further analysed with Ingenuity Pathway Analysis (IPA). Expression profiles were visualized as heat maps using Genesis. Functional annotation of genes was performed according to the three gene ontologies (GO) describing gene products in terms of their "biological processes," "molecular functions" and "cellular compartments".

Real-time PCR

Quantitative Real-time PCR was performed as described elsewhere on a Corbett Rotor-Gene Cycler (Corbett Research, New South Wales, Australia). The used primers were pre-designed Quantitect Primer Assays from Qiagen for the following genes of interest: CECR1, Fyb, KLRC2/C3, Lax, PTGDR and TNFSF4 (Ox40L). Experiments were ruled out in duplicate with samples from all three biological replicates. The specificity of primers was verified by melting curve analyses and all had similar amplification efficiencies. mRNA levels were normalized to glycerinaldehyde-3-phosphate dehydrogenase (GAPDH), and expressed relative to control samples at time point 0 using the $2^{-\Delta\Delta CT}$ method.

Growth inhibition assay

3D7 or Dd2 sorbitol-synchronized ring-stage-iRBCs were co-incubated with NK92 (pre-cultured overnight without IL2) in different ratios in parasite growth medium, 0,125% starting parasitaemia and 1% haematocrit. As controls, co-culture was also performed with HeLa cells, Jurkat cells and C32 cells under the same conditions. After 24 to 48 hours of incubation at 37°C in parasite atmosphere, culture samples were frozen at -20°C, then thawed and parasite growth inhibition was quantified by a Histidine-Rich Protein 2 (HRP2) ELISA assay performed as described elsewhere [15].

Statistical analysis

Analysis was performed using StatView for Windows 5.0.1 (SAS Institute Inc., Cary, North Carolina) running on Windows XP (Microsoft Corp., Redmond, Washington). Results were analysed using either ANOVA test ($p < 0,05$) or Bonferroni test ($p < 0,005$).

Results

NK92 cells constitutively release IFN-γ

To assess whether NK92 cells release IFN-γ upon contact with iRBCs, *in vitro* 24 hours co-cultures of NK92 with 3D7-iRBCs were performed and supernatants were analysed for cytokine presence (Figure 1). NK92 cells grown alone in normal cell medium released 7 IU/ml of IFN-γ compared to 2,4 IU/ml and 2,3 IU/ml when iRBCs and uninfected erythrocytes (uRBCs), respectively were added to the assay. Only a very low level (0,2 IU/ml) of IFN-γ was detected in the supernatant of NK92 cells that were maintained under starvation conditions (NK92s). The addition of iRBCs and uRBCs to NK92s decreased the amount of IFN-γ released. As controls, supernatants of iRBCs, uRBCs and RPMI 1640 were also tested and did

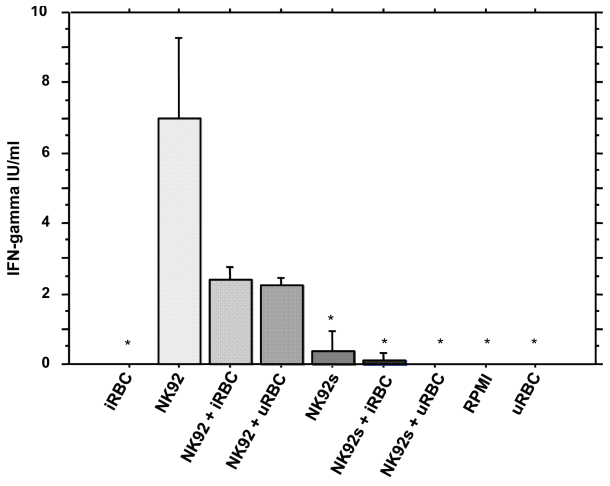

Figure 1 IFN-γ release of NK92 cells after co-culture with iRBCs. NK92 cells previously cultured with rIL-2 and without rIL-2 (24 hours starvation; NK92s) were co-incubated with 3D7 schizont-iRBCs and uninfected erythrocytes in RPMI-1640 medium (24 hours - 37°C - 5% CO_2) and supernatants were subsequently tested for IFN-γ release. iRBCs/uRBCs: supernatants from iRBCs and uRBCs incubated without cells in RPMI-1640; NK92/NK92s: supernatant from cells incubated in normal growth medium and starvation medium, respectively. Values represent mean ± S.E.M.; *p = 0.0001 for comparison with NK92 (p <0,05 - ANOVA).

not present traces of IFN-γ. Significant differences were seen when the levels of IFN-γ released by the cells cultured in normal medium without stimulus was compared to that released by iRBCs alone, NK92s, NK92s + iRBCs, NK92s + uRBCs and uRBCs (p < 0,0001). To sum up, NK92 cells that were submitted to starvation before the experiment did not produce IFN-γ due to co-culture with iRBCs. In addition, cells kept in normal culture conditions (+rIL2) prior to the experiment released greater levels of IFN-γ regardless presence of iRBCs.

3D7 parasites do not induce up-regulation of activation markers in NK92 cells

To further investigate NK92 expression of activation markers due to iRBCs contact, *in vitro* co-cultures were carried out for 24 hours and cells were analysed by flow cytometry. Independently of the medium that NK92 cells were incubated (normal cell medium or starvation medium) the NK phenotypic marker CD56 was always positive, CD3 and CD16 surface antigens were always negative, proving that the cells presented the NK phenotype (Table 1). The activation markers CD25 and CD69 were already positive without addition of stimulus, indicating certain "base level" activation. However, the addition of IL12+IL18 (positive control) induced up-regulation of both activation markers as well as IFN-γ above the "base level" activation, showing that these cells are able to respond to an external stimulus.

As expected, co-culture of NK92 with uRBCs did not change the picture described above. No up-regulation of CD25, CD69 and IFN-γ was observed in comparison to the cells cultured without RBCs. Surprisingly, the addition of 3D7-infected erythrocytes to the system also did not have any significant impact on NK cells. No up-regulation of CD25 and CD69 was detected due to the addition of iRBCs although these membrane proteins, especially CD69, should be the first markers expressed in immune activated cells. In addition, no significant up-regulation of IFN-γ was observed (Table 1).

NK92 form rosettes with FCR3-CSA-iRBCs but not with 3D7

Baratin *et al* described rosettes as conjugates of NK cells with more than two RBCs and that this direct contact with iRBCs could contribute to complete activation of NK cells [16]. In this study, formation of rosettes between 3D7 and selected FCR3-CSA with NK92 in different ratios (1:1, 1:3 and 1:10; NK:RBC) was tested. For the 3D7 strain, no rosettes could be observed in none of the tested ratios. Best results were obtained with FCR3-CSA in 1:10 ratio. Ten percent of NK92 formed conjugates with iRBCs (Figure 2A and 2B). Nevertheless, these conjugates were mostly not real rosettes since mainly one RBC per NK cell was bound. Only around 1% of the cells were able to form rosettes with more than two attached iRBCs. There was no binding of NK92 with uRBCs and no rosettes were formed with FCR3-CD36 (Figure 2C and 2D).

Plasmodium falciparum-iRBCs influence NK92 gene expression only after 24 hours, but not at 6 or 12 hours of co-culture

To avoid missing the right time point of transcriptional changes induced by *P. falciparum*-iRBCs, we performed a time kinetics experiment investigating transcription in NK92 cells after 6, 12 and 24 hours of co-culture with parasites. Expression data show almost no change on transcription level after 6 h (10 genes) and 12 h (12 genes) of co-cultivation compared to control (time point 0). After 24 h of co-culture a total of 167 genes were differentially expressed in the NK92 cells (Figure 3). However, there is a big overlap of 103 genes with NK92 cells co-cultured with uRBCs. Since we cannot use 100% iRBCs for experiments, this overlap can be explained by the influence of the uRBCs present in the iRBCs co-cultures. Only 64 genes were regulated due to iRBCs. Out of these 64 genes, 53 were up-regulated (24 mitochondrial genes) and 11 down-regulated. Analysis of these genes with Ingenuity revealed "cancer/respiratory disease", "cell-to-cell signalling" and "interaction/cell-mediated immune response" as well as "cell cycle/infection mechanism and inflammatory response" as top networks

Table 1 FACS analysis of the NK92 cell line after co-culture with 3D7

Starvation	NS (%/MFI/SD)	uRBCs (%/MFI/SD)	iRBCs (%/MFI/SD)	IL (%/MFI/SD)
CD56	100/51177/26893	100/47072/22400	100/48515/23025	100/62661/30093
CD3	0.1/142/105	0.1/133/103	0.1/135/97	0.7/182/125
CD16	0.5/599/347	0.5/601/389	0.5/605/373	10/724/405
CD25	94.2/3834/2269	95.6/3570/1983	96.5/3809/2040	98.3/14259/12340
CD69	3.4/824/635	1.7/747/473	2.7/800/568	58.1/3883/3127
IFN-γ	0.1/202/102	0.1/189/98	0.1/192/98	40.4/2270/4658
Normal Medium	**NS (%/MFI/SD)**	**uRBCs (%/MFI/SD)**	**iRBCs (%/MFI/SD)**	**IL (%/MFI/SD)**
CD56	99.9/79722/37294	100/72957/35805	100/78647/65793	100/68544/34844
CD3	1.4/209/135	1.7/235/146	1.3/215/137	7.5/302/232
CD16	2.3/859/473	3.2/963/548	2.8/929/544	8.5/1195/792
CD25	6.2/313/722	5.2/318/762	5.6/309/584	95.9/188865/19512
CD69	39.7/2218/1804	44.5/2319/1655	48.5/2485/1904	84.1/5950/4900
IFN-γ	1.6/297/152	1.1/270/130	1.2/291/201	75.3/3504/4701

NS: no stimulus; uRBCs: uninfected erythrocytes; iRBCs: infected erythrocytes; IL: positive control; %: percentage of positive cells; MFI: mean fluorescence intensity; SD: standard deviation

with a score ≥ 25 (Table 2). The majority of up-regulated genes are linked to the biological process of cell-cycle progression and possible entry into G2-phase, an event characterized by cell growth as well as protein and RNA biosynthesis (Table 3). Many guide-RNAs required for splicing were up-regulated (SCARNA7 and 9, SNORA40/JOSD3, SNORD47/GAS5, SNORD50B and SNORD75). Furthermore, genes involved in anti-apoptosis and cell growth (GIMAP5, FAIM3, ZNF780A, Tubulin-γ, MT1E and CECR1) were over-expressed. Another set of interesting genes is linked to immune response and activation of NK cells, especially granule secretion (CECR1, TNFSF4, KLRC2/C3, Centaurin delta 1, Fyb, PTGDR, Tubulin-β). Microarray results were validated by RT-PCR for some representative genes (CECR1, Fyb, KLRC2/C3, LAX, PTGDR and TNFSF4). Normally the fold change was always higher for the RT-PCR analysis (Figure 4).

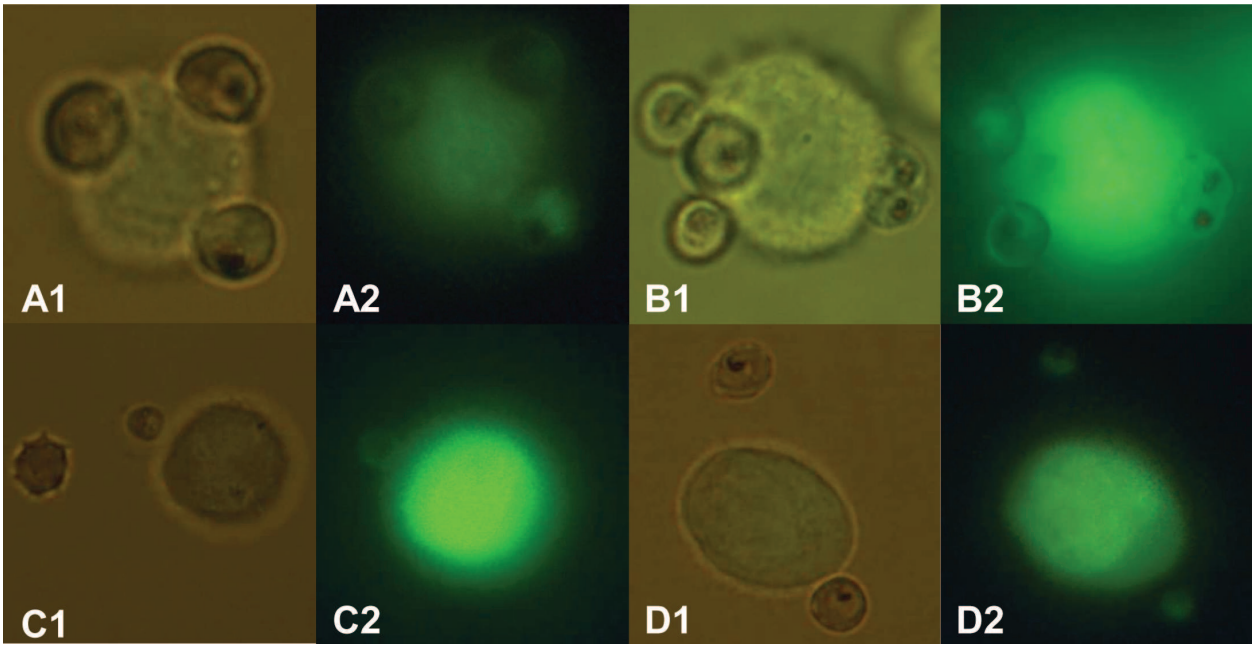

Figure 2 Rosettes of NK92 and FCR3-CSA parasites. NK92 were co-cultured with FCR3-CSA-iRBCs in 1:3 ratio respectively, for 1 h at 37°C/5% CO_2. After incubation, a sample of the co-culture was dyed with acridine orange and analysed in a fluorescence microscope. **A1 and B1**. Rosettes of 1 NK cell with 2-3 iRBCs (normal light). **A2 and B2**. The same rosettes in fluorescent light. **C1, C2, D1, D2**. NK92 and FCR3-CD36 as a control for unspecific binding. The binding is specific for infected erythrocytes. Few uninfected RBCs were found to bind the NK cells.

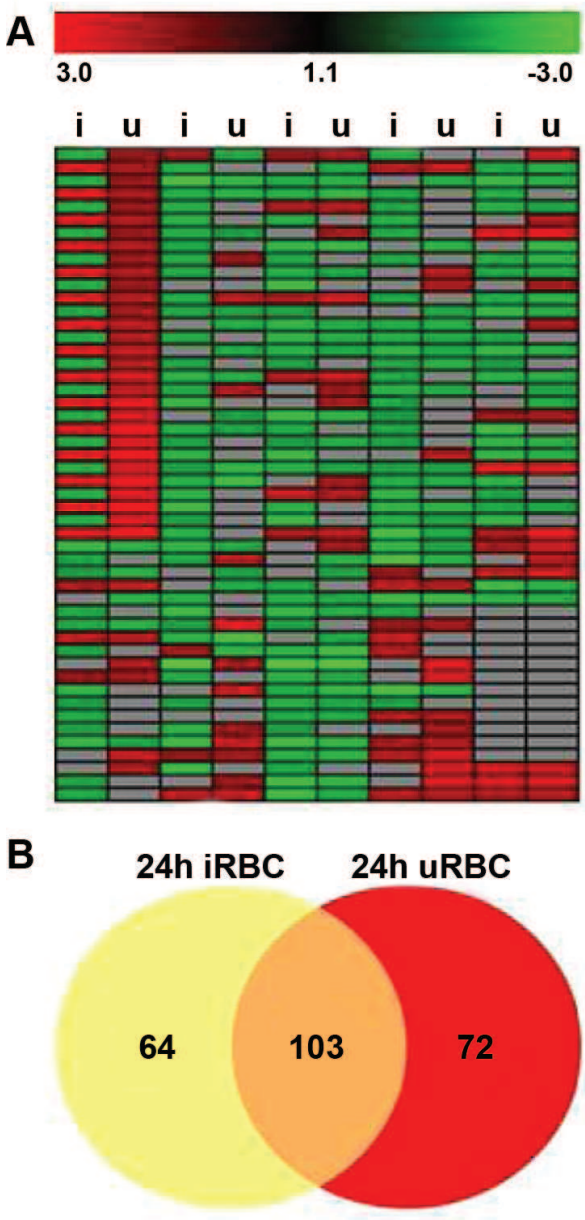

Figure 3 Gene expression patterns in NK92 after 24 h co-culture with *P. falciparum*-iRBCs. Normalised expression patterns were derived from microarrays of NK92 cells after 24 hours co-culture with 3D7-iRBCs or uRBCs (1:3). Data were obtained from 3 biological replicates **A**. Heatmap of altered genes in 24 h co-cultures normalised to untreated NK92 at time point 0 h comparing uRBCs (u) and iRBCs (i)-treated NK92. Up-regulated genes are depicted in red and down-regulated in green. **B**. Venn-diagram of total numbers of the same genes. Only regulated genes with a fold-change ≥ 1.5 ($p \leq 0,05$) are depicted. 167 genes were differentially expressed in the NK92 cells after co-culture with iRBCs, of which 85 genes were up-regulated and 82 down-regulated. However, there is a big overlap of 103 genes with NK92 cells co-cultured with uRBCs.

Dd2 and 3D7 parasitaemia decreases significantly after parasite incubation with NK cells and Jurkat cells

In order to investigate whether NK cells affect parasite growth, invasion and/or development, *in vitro* co-cultures were carried out with different NK - iRBCs ratios. Parasite growth was significantly inhibited due to co-culture with NK cells after 48 h especially at the 10:1 ratio (3D7/Dd2:NK92). The Dd2 growth was suppressed in a greater extent (p <0,0001) than the 3D7 growth at such ratio (p = 0,0018) in comparison to the controls. Development of both strains was slightly but not significantly inhibited at 3:1 ratio while at 1:1 ratio no differences were observed in comparison to the controls (Figure 5A).

To verify whether the observed growth inhibition was due to a specific response of NK cells, we have performed the same co-culture experiment with diverse cell lines. C32 cells have equally suppressed growth of 3D7 and Dd2 (p <0,0001) after 48 h co-culture in comparison to controls (Figure 5B). In contrast, HeLa cells restrained Dd2 development to a larger extent at 10:1 ratio (Dd2:HeLa; p <0,0001) although no significant effect of such cells was observed on 3D7 growth (Figure 5C). In the presence of Jurkat cells, Dd2 parasitaemia was drastically decreased (p <0,0001) at the 10:1 ratio (Dd2:Jurkat) in comparison to the control. In addition, the growth curve of the 3D7 parasites was considerably inhibited in comparison to the control at 10:1 (3D7:Jurkat; p <0,0001) and 3:1 (p <0,0002) co-culture ratios (Figure 5D).

Comparing the effects that all four cells lines imposed on parasitaemia after 48 hours, Dd2 was observed to be more sensitive to the co-cultures than 3D7 (Figure 6). NK92 and Jurkat cells showed a stronger impact than C32 and HeLa cells on Dd2 growth. Moreover, NK92 and C32 cells have similarly affected 3D7 development in a stronger manner than HeLa and Jurkat cells. Taken together, these data shows that all cell lines influenced parasite growth at 10:1 (parasite:cell) ratio except for the HeLa:3D7 co-culture; that Dd2 is affected by the cells in a greater extent than 3D7 and that the impact observed on *in vitro* parasite growth is therefore not NK cell-specific.

Discussion

PBMCs are frequently adopted tools for studies of immune responses in malaria. NK cell cross talk with accessory cells triggers their activation and effector functions. In this study, however, a reductionist approach (without accessory cells) was chosen, aiming to investigate the very specific effect of NK92 cells towards *Plasmodium* parasites.

NK92 cells were already producing IFN-γ when incubated alone in normal growth medium. This is probably

Table 2 Top networks related to *P.falciparum*-induced NK92 genes

Associated Network Functions	*Score*
Cancer, Genetic Disorder, Respiratory Disease	49
Cell-Cell Signaling/Interaction, Cell-Mediated Immune Response, Cellular Development	28
Cell Cycle, Cellular Compromise, Infection Mechanism	25
Cell Cycle, Nervous System Development and Functions, Inflammatory Response	25
Cell Cycle, Cellular Assembly/Organization, DNA Replication, Recombination and Repair	24

due to the presence of IL-2, a cytokine already reported to induce IFN-γ secretion in mice macrophages [17]. In addition, external and intrinsic factors might also play a role in the release of IFN-γ. The handling of the cultures and the fact that these are tumour/continuous cells (and for this reason already went through a process of activation) has to be taken into account. Co-incubation with iRBCs did not induce IFN-γ release by NK92 cells as a response to *P. falciparum* antigens. It was already shown that signals from accessory cells such as macrophages, monocytes and dendritic cells (DCs) are required for full NK cell commitment. Moreover, it is known that PBMCs from malaria-unexposed donors can produce heterogeneous responses, including IFN-γ release, when stimulated by iRBCs [4-6]. A possible explanation to these results is, of course, the lack of accessory cells but it might be possible that the donor of such NK cells is a low IFN-γ responder [5]. If this is the case, accessory cells in the system would not change the picture. In a study using NK92 cell lines as models for IL-18-mediated signal transduction it was shown by RT-PCR and ELISA that the activation of the cell lines with IL-18 alone failed to stimulate IFN-γ protein production despite inducing expression of IFN-γ mRNA [18]. By the NK92 microarrays however, no induction of the IFN-γ gene up to 24 hours of co-culture with parasites was observed. It would be interesting, therefore, to test whether mRNA expression can be detected at a later time point.

CD69 is a C-type lectin-like glycoprotein known to be a sensitive early marker of leukocyte activation and cytotoxic activity of NK cells [19]. However, only a slight up-regulation of CD25, another activation marker, was observed when NK92 grown under starvation conditions were incubated with iRBCs. The same was detected when uRBCs were added to the system suggesting that the weak activation detected is not iRBCs-specific. Genetic differences between people appear to influence NK cell response to iRBCs. It is claimed that relevant gene(s) may be variably expressed among different NK clones [5] which might, therefore, influence NK cell activation. In addition, no binding of NK92 with 3D7-iRBCs was detected although physical interactions between NK cells and iRBCs have already been described with freshly isolated NK cells and NK cell lines [4,5,16,20]. However, real rosettes were observed with FCR3-CSA-iRBCs what might explain a probable engagement of *var2csa* on the surface of iRBCs. It was suggested that CSA, claimed to be involved in pregnancy-associated malaria [21] is the element through which PfEMP1 of FCR3-CSA strain will form the rosettes. If this is the only factor involved in that event, it explains the reason that adherence with 3D7 was not detected.

The microarray results suggest that NK92 cells proliferate in response to *P. falciparum*-iRBCs after 24 hours of co-culture. Another study on mice experimental malaria already reports a similar observation [22]. After an early interferon type-I response a second wave of differential expression was apparent at 24-32 hours post infection with *Plasmodium chabaudi*. Such expression was linked to NK cell proliferation in the peripheral blood although signs of activation were absent. Interestingly, the same

Table 3 Top bio-functions related to *P.falciparum* -induced NK92 genes

Molecular and Cellular Functions	*p-Value*	*N° Molecules*
Cell Cycle	3,41E-09 - 3,51E-02	34
DNA Replication, Recombination and Repair	2,31E-06 - 3,00E-02	36
Cellular Assembly and Organization	5,27E-06 - 3,18E-02	21
Gene Expression	5,69E-05 - 3,00E-02	13
Cell Death	6,58E-05 - 3,00E-02	32
Physiological System Development and Function	***p-Value***	***N° Molecules***
Embryonic Development	2,30E-05 - 3,19E-02	7
Connective Tissue Development and Function	2,30E-05 - 3,19E-02	11
Cell-mediated Immune Response	1,16E-03 - 1,83E-02	10
Hematological System Development and Function	1,16E-03 - 3,00E-02	18
Hematopoiesis	1,16E-03 - 2,80E-02	12

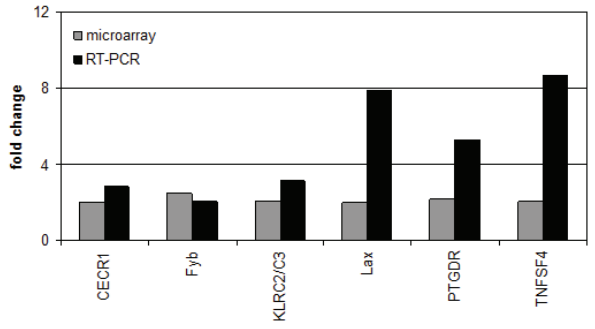

Figure 4 RT-PCR validation of microarray results. Fold change in gene expression of NK92 cells after 24 hours of 3D7-iRBCs co-culture comparing microarray and qRT-PCR results for 6 genes of interest (CECR1, Fyn-binding protein, KLRC2/C3, LAX, PTGDR, and TNFSF4 (Ox40L)) linked to priming of adaptive immune responses, NK granule secretion and cell activation.

pattern was observed in the present study at 24 hours, since an activated status of NK92 cells after iRBCs co-culture could be detected neither by microarray analysis nor by flow cytometry. Of course, not the same set of genes was found at earlier time points, because in this study no accessory cells or any additional cytokines were applied. However like in another report, genes important for DNA replication, cell proliferation, spindle formation and microtubule cytoskeleton formation were altered. In the study of Kim *et al*. the main KEGG pathways were cell cycle and small cell lung cancer comparable to the pathways of cancer/respiratory disease and cell cycle (infection mechanism/inflammation) presented in this study [22].

In addition to the proliferative signs, few other altered genes gave a hint for possible NK cell triggering. After 24 hours KLRC2 and KLC3 were up-regulated, both activating NK cell receptors. KLRC2 together with CD94 is involved in NK cell-mediated cytotoxicity and ligand binding leads to granule release as well as TNF-α and IFN-γ secretion. Up-regulation of CECR1 in NK92 cells might be a sign for T cell priming since it was shown that inhibition will lead to less signal transduction via CD3 and TCR [23]. Another important gene in this context is TNFSF4 (OX40L): it serves as a ligand for OX40 and results in higher $CD4^+$ T cell proliferation and cytokine production, especially IFN-γ. It is selectively induced in IL-2/-12 or -15 treated NK cells after stimulation via NKG2D, CD16 or KIR2DS2 [24]. Additionally, Centaurin delta 1 that signals via phosphatidylinositol-3-kinase pathway to induce cytoskeleton remodelling and thus influencing granule secretion was altered [25]. Also interesting in this context is FYB, a Fyn-binding protein, which can phosphorylate IKKa/b and ubiquitinylate IKKγ resulting in NF-kB activation in T cells [26] and degranulation. IKK was one of the few up-regulated genes after 6 hours. Among the few down-modulated genes is tubulin-β. Tarazona *et al* have reported an important role in killing of target cells, cell polarisation, cellular movement and granule secretion [27]. Furthermore, PTGDR expression was up-regulated. This receptor is known to increase intracellular cAMP concentration and subsequently inhibit NK cell function through blocking Th1-cytokine production and cytotoxicity/promotion of Th2-type responses [28]. Although not many conclusions can be drawn from these results, it is possible that the adaptive immunity is primed and NK cells become activated by iRBCs to release their granule contents. However, complete pathways linked to activation were not found to be switched on in the present study. Still, the microarrays showed the existence of similar mechanisms altered in human NK cells after *P. falciparum*-iRBCs encounter comparable to those reported from mouse model experiments.

To validate the picture observed with the arrays, the impact of NK92 cells on parasite growth, invasion and development upon co-culture was investigated. The experiments show that NK92 cells interfere with the parasite life cycle, especially with that from the Dd2 strain. In addition, Jurkat cells, a T cell line, strongly diminished Dd2 parasitaemia. These results would be in concert with the general knowledge that immune responses against malaria parasites relies upon NK and T cells [1,2]. Surprisingly, however, other cell types irrelevant to immune response in malaria (C32 and HeLa) also suppressed parasite development. These results might suggest that the inhibition of parasite growth caused by NK92 cells is an effect of competition for limited resources in the presence of growing and dividing cells. Upon co-culture however, cells were kept at usual and viable concentrations for this type of assays, as commonly performed in experiments were fresh cells are used. It could be observed that the decrease of parasitaemia caused by irrelevant cells was somehow related to the parasite strain used. HeLa cells for example had a very strong impact on Dd2 parasitaemia but did not influence growth of 3D7 parasites. The same is true for the C32 cells. NK92 and Jurkat cells had an even stronger impact on the growth of Dd2. However, due to the lack of a cell-specific response, these results cannot be claimed to reflect a cytotoxic effect of NK92 cells against parasites. However, if the effect is there, it appears to be subtle and is not comparable to that imposed by primary NK cells.

If characteristics from primary NK cells could be extrapolated to NK cell lines, one could assume that NK92 cells belong to the CD56bright sub-population, which is known to be CD16dim/neg. These cells cannot elicit ADCC but are potent IFN-γ producers what is then in accordance to the results presented in this study (cells release IFN-γ even

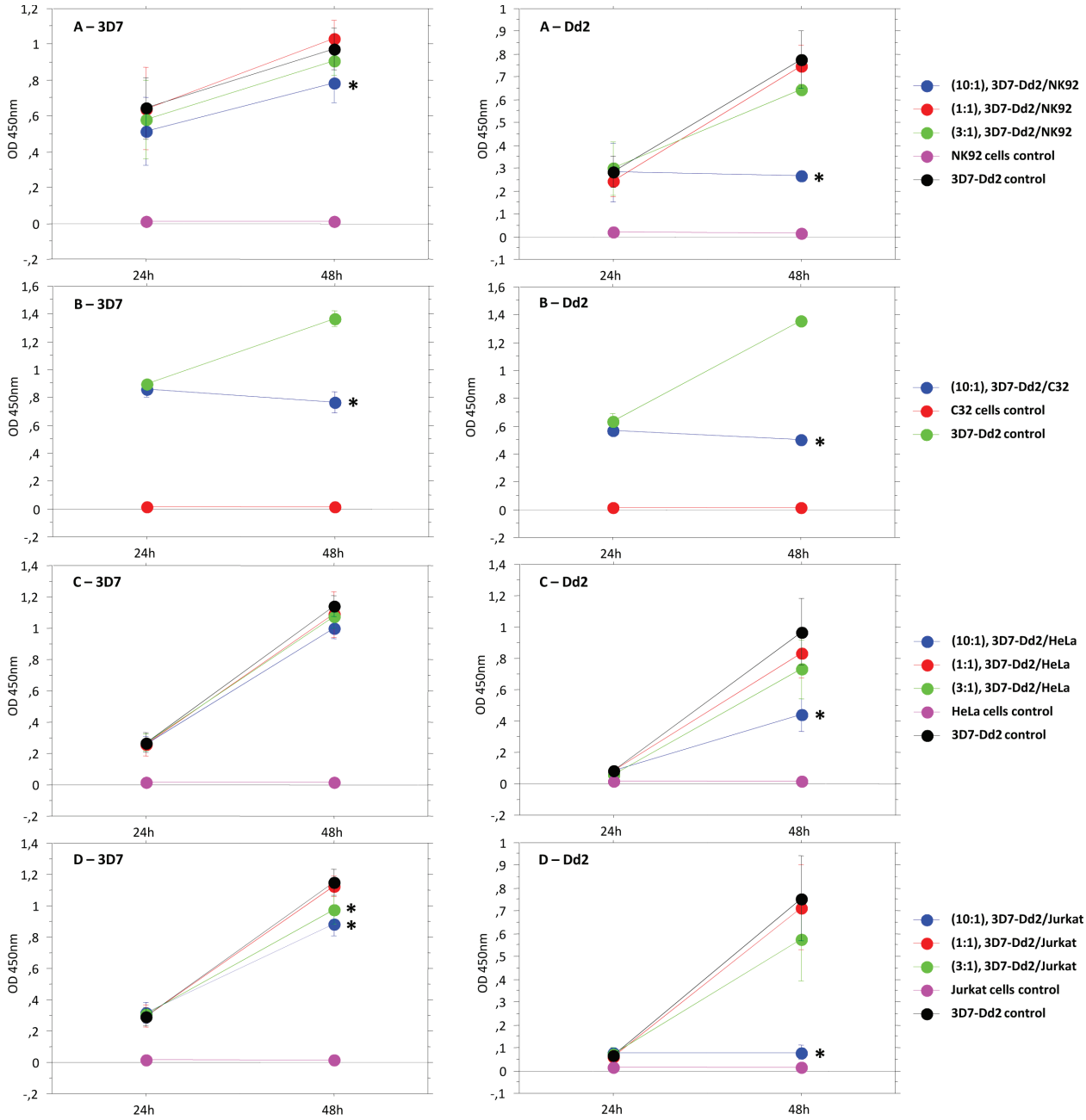

Figure 5 Parasite growth inhibition assay. 3D7 (left column) or Dd2 (right column) sorbitol-synchronized ring-stage-iRBCs were co-incubated with NK92 (A) in different ratios (10:1, 1:1, 3:1 - parasites:cells) in parasite growth medium. Starting parasitaemia was 0,125% in 1% haematocrit. As controls, co-culture was also performed with C32 cells (B), HeLa cells (C), and Jurkat cells (D) in the same conditions. After 24 hours to 48 hours of incubation at 37°C in parasite atmosphere, parasite growth inhibition was quantified by measuring HRP2 release by ELISA. *$p < 0.002$ for paired comparisons ($p < 0.005$ - Bonferroni).

without stimulus). The lack of parasite-induced activation could be solely linked to the fact that no accessory cells were adopted. In addition, a more specific NK cell effect against parasites might have been detected if NK92 cells belonged to the CD56dim population, which is known to be important for their cytotoxicity against *Plasmodium*.

Conclusions

On the one hand, this study shows that there is a lack of primary signs of NK92 activation in response to *Plasmodium* stimulus although NK92 transcription of proliferation- and priming-related genes was clearly changed in response to such interaction. On the other hand, a drastic

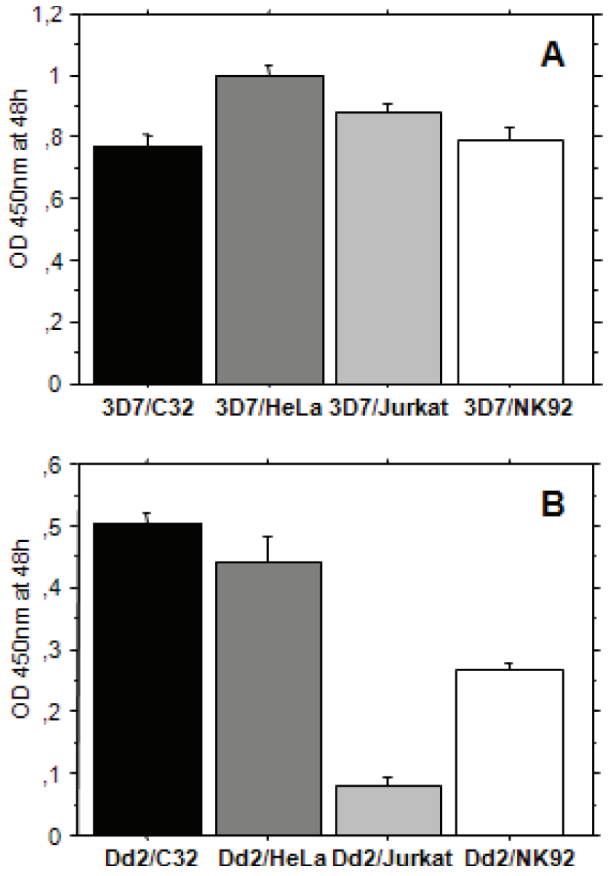

Figure 6 3D7 and Dd2 growth inhibition induced by different cell lines. 3D7 or Dd2 sorbitol-synchronized ring-stage-iRBCs were co-incubated with NK92, C32 cells, HeLa cells and Jurkat cells in the same conditions. After 24 to 48 hours of incubation, parasite growth inhibition was quantified by measuring HRP2 release by ELISA. Comparison of the inhibition effect of each cell line on 3D7 **(A)** and Dd2 **(B)** is depicted.

impact on *P. falciparum* parasitaemia linked to NK cell contact was observed. Whether it reflects only consequences of competition with the co-cultured cells or whether there is the addition of the cytotoxic effects of NK92 needs to be further investigated. Although NK92 alone were observed to disqualify as good models for the NK immune response to *Plasmodium*, interesting information regarding the mechanisms behind NK effector responses to parasites were acquired and will be of use for future basic research in malaria.

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Authors' contributions
EGC and EB designed the study, carried out the laboratory work, analysed and interpreted the data, wrote the manuscript; VTH carried out the laboratory work and analysed the data; PGK provided scientific leadership and corrected the manuscript; JFJK provided scientific leadership, designed the study, analysed the data, wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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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.

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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-naive 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 IL18R 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 Tübingen, 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 co-cultivation 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 (16I.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 (Invitrogen) 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 (Myltenyi 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% CO_2, 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-PE, 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 ≥93% $CD56^+CD3^-$ 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 Quantitec 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 $2^{-\Delta\Delta CT}$ 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 (≥93% $CD56^+CD3^-$) 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 5:1 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 genes if a cut off was specified) and is therefore useful for determining which pathways overlap with most of the genes in the dataset.

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 $\geq$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 (*CD38*, *IFITM1*, *FAS*), regulation of transcription (*STAT2*, *IRF7 STAT3*), intracellular signaling pathway (*JAK*, *RASGRP3*, *RASGRP2*), and NK cytotoxicity (*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 $\geq$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

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
RSAD2	8040080	21,55	unknown	enzyme
IFIT3	7929052	19,71	Cytoplasm	other
OAS3	7958895	15,48	Cytoplasm	enzyme
MX1	8068713	15,42	Nucleus	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	Nucleus	enzyme
CBX7	8076185	−1,79	Nucleus	other
KLRG1	7953835	−1,83	Plasma MB	other
RASGRP2	7949104	−1,91	Cytoplasm	other
SYNE1	8130211	−1,92	Nucleus	other
HERC1	7989516	−1,96	Cytoplasm	other
CMKLR1	7966089	−2,03	Plasma MB	GPCR
AHNAK	7948667	−2,27	Nucleus	other
FGR	7914112	−2,49	Nucleus	kinase
PTGDR	7974363	−2,58	Plasma MB	GPCR

iRBCs-infected erythrocytes; Aff. ID-affymetrix identification; FC-fold change; MB-membrane; GPCR-G protein coupled receptor.
doi:10.1371/journal.pone.0024963.t001

Activation of IRF by cytosolic pattern recognition receptors (p = 2,9E-09) were identified as the top canonical pathways linked to the modulated genes (Figure 2A and Table S3). The highest strength of association was found with the *Interferon Signaling* canonical pathway. Eleven of the 30 molecules that compose the pathway were regulated on NK cells by co-culture with parasites (ratio: 0.367). The pathway and the modulated genes are depicted in Figure 3.

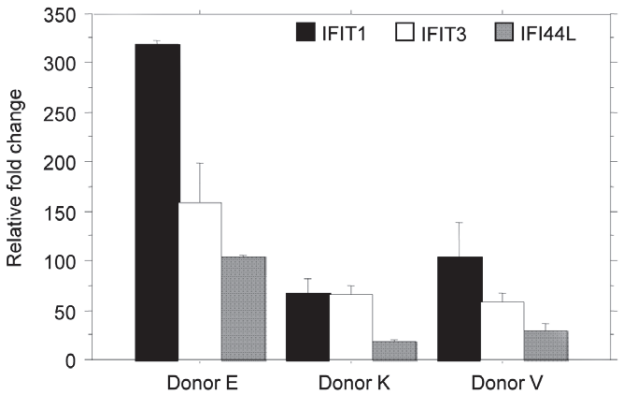

Figure 1. Validation of the microarray results by RT-PCR. Three representative iRBCs-induced NK genes are depicted. Values represent the mean of the relative fold change obtained for each replicate per donor. Levels of target mRNA expression were determined using the $2^{-\Delta\Delta CT}$ method with GAPDH as the endogenous reference gene and the untreated samples (CM) as calibrators.
doi:10.1371/journal.pone.0024963.g001

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.
doi:10.1371/journal.pone.0024963.t002

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 (*IFN-γ*, *CD25*), signal transduction (*P2RX5*, *MT2A*, *KLRF1*), complement activation (*CD55*), antigen presentation (*CD83*), chemotaxis (*CCR4*, *CXCL10*, *CX3CR1*), DNA replication/repair (*CHEK1*, *TYMS*), 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 $\geq$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 Ubiquitination Pathway* (p = 3,38E-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 (*RNU5E*, *SNORD47*), and others are known to play a role in RNA splicing (*SNRPN*) 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 co-culture 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

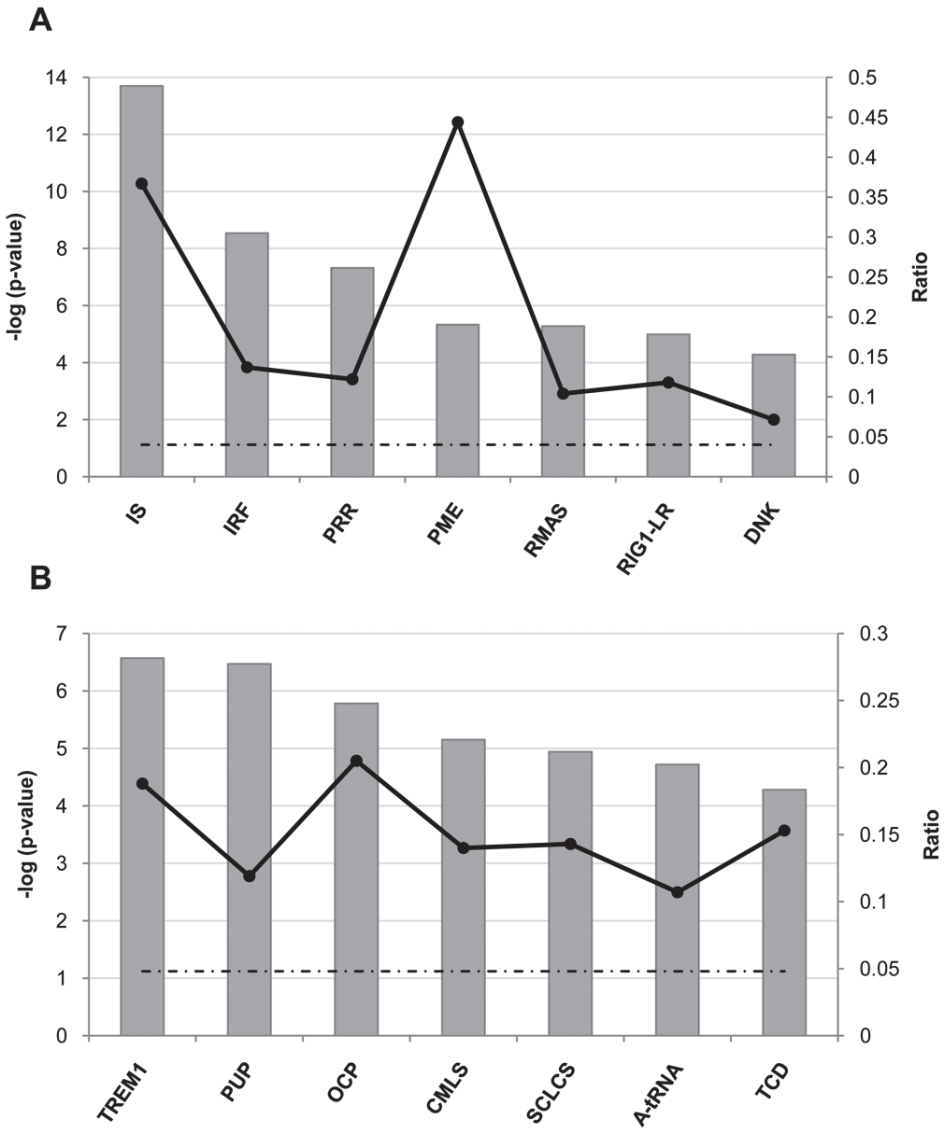

Figure 2. Top canonical pathways associated with 3D7- and IL-12/IL-18-induced NK cell genes. Canonical pathways were obtained using the Ingenuity System upon analysis of the genes differentially modified in NK cells. Top canonical pathways are indicated by the grey bars on the x-axis. The association of the data set with the given pathway is depicted in the y-axes and was determined based on the p-value and on the ratio (strength of the association - black line). The threshold (dashed black line) is shown at $p<0.05$. **A. 3D7-induced**: IS: Interferon Signaling; IRF: Activation of Interferon Regulatory Factor family of transcription factors by Cytosolic Pattern Recognition Receptors; PRR: Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses; PMS: Pathogenesis of Multiple Sclerosis; RMAS: Retinoic acid Mediated Apoptosis Signaling; RIG1-LR: Role of Retinoic Acid Inducible gene 1-like receptors in Antiviral Innate Immunity; DNK: Crosstalk between Dendritic Cells and Natural Killer Cells. **B. IL-12+IL-18-induced**: TREM1: Triggering Receptor Expressed in Myeloid Cell 1 Signaling; PUP: Protein Ubiquitination Pathway; OCP: One carbon Pool by Folate; CMLS: Chronic Myeloid Leukemia Signaling; SCLCS: Small Cell Lung Cancer Signaling; A-tRNA: Aminoacyl-tRNA Biosynthesis; TCD: T Helper Cell Differentiation.
doi:10.1371/journal.pone.0024963.g002

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

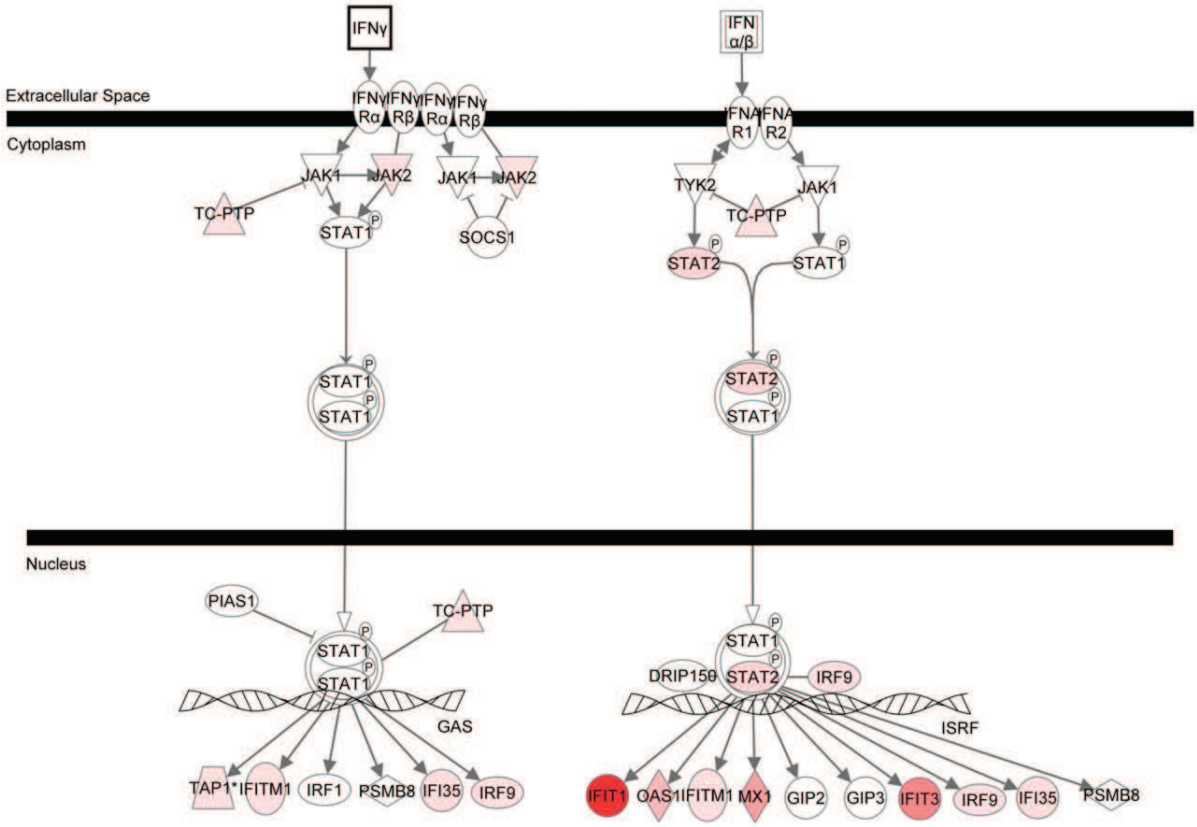

Figure 3. Type I interferon transcripts are induced by parasites on NK cells. Ingenuity Pathways Analysis identified the canonical pathway "Interferon Signaling" as highly associated with the 3D7-regulated genes on NK cells. Mainly IFN-αβ transcripts are induced. The differentially-regulated genes are marked in red (up-regulation) or green (down-regulation).
doi:10.1371/journal.pone.0024963.g003

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 one 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)
IFNG	7964787	92,46	Extr. Space	cytokine
IL2RA	7931914	37,33	Plasma MB	Tmb R
MIR155HG	8068022	34,30	unknown	other
SLC27A2	7983650	20,71	Cytoplasm	transporter
DPP4	8056222	17,57	Plasma MB	peptidase
CD274	8154233	16,19	Plasma MB	other
CDC6	8007071	14,25	Nucleus	other
MYO1B	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
YPEL1	8074780	−5,56	Nucleus	enzyme
FGFBP2	8099471	−5,63	Extr. Space	other
PIK3IP1	8075483	−5,78	unknown	other
KLHL24	8084219	−5,79	unknown	other
AHNAK	7948667	−5,96	Nucleus	other
CX3CR1	8086344	−6,68	Plasma MB	GPCR
FAIM3	7923917	−7,37	unknown	other
SH2D1B	7921900	−7,56	unknown	other
KLRF1	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.
doi:10.1371/journal.pone.0024963.t003

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 IFN plays an important role in inducing IFN-γ production by NK cells [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

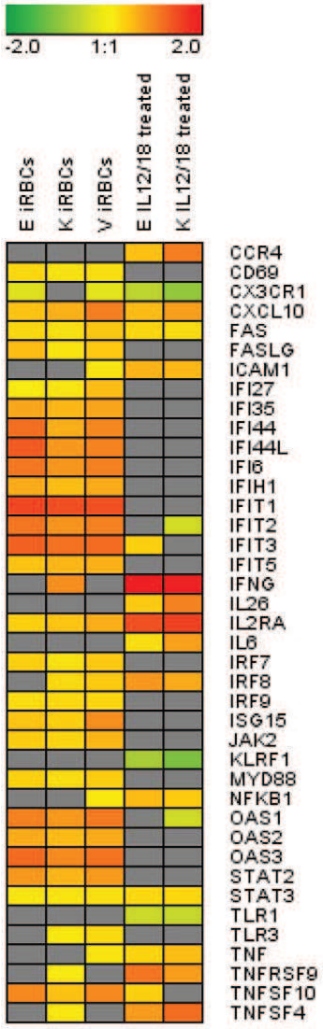

Figure 4. Comparison of the gene expression patterns between donors and treatments. Members of the *Interferon signaling pathway*, the *TREM-1 signaling pathway*, and other genes that play a role in the immune response were selected. The image depicts the log of the fold change of NK genes regulated due to co-culture with 3D7-infected erythrocytes (iRBCs) or with the cytokines IL-12 andIL-18. The expression profile is shown for the three different donors (E, K, and V).
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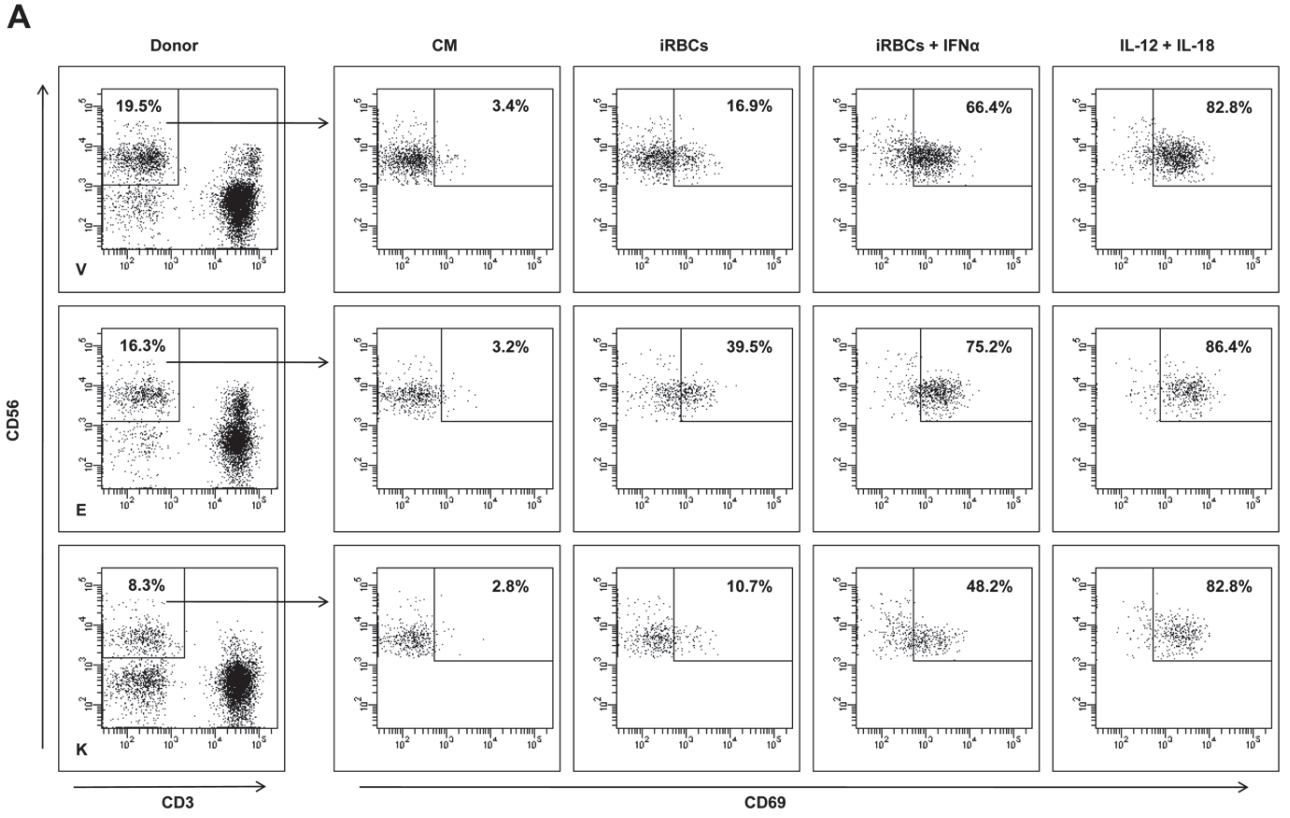
and IFN-γ secretion early in infection were shown to be decreased in the event of type I interferon blockage in mice [27]. In a recent study with *L. mexicana* infection, type I interferon was also described to promote the early IFN-γ and IL10 expression [28].

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 plasmatocytoid


A
Donor
CM
iRBCs
iRBCs + IFNα
IL-12 + IL-18
19.5%
3.4%
16.9%
66.4%
82.8%
V
16.3%
3.2%
39.5%
75.2%
86.4%
E
8.3%
2.8%
10.7%
48.2%
82.8%
K
CD56
CD3
CD69

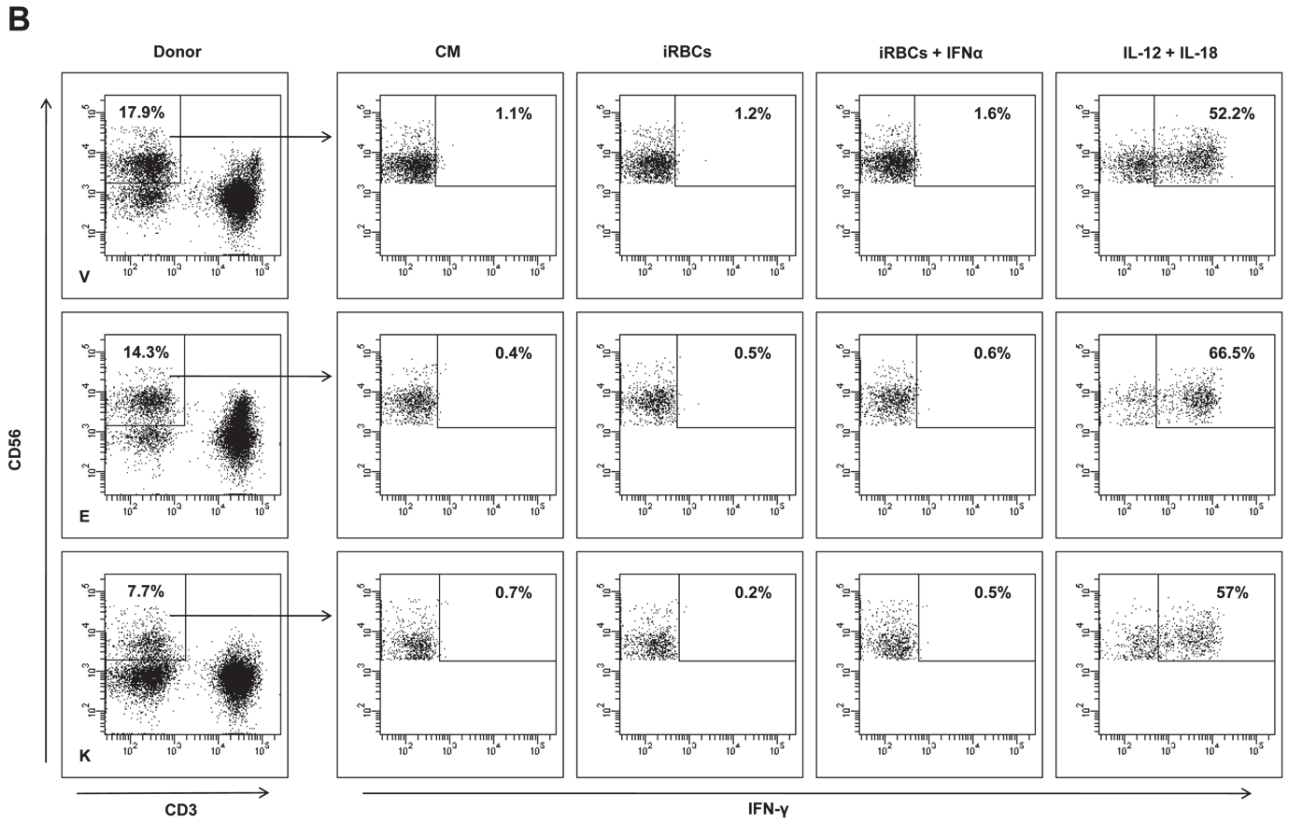

B
Donor
CM
iRBCs
iRBCs + IFNα
IL-12 + IL-18
17.9%
1.1%
1.2%
1.6%
52.2%
V
14.3%
0.4%
0.5%
0.6%
66.5%
E
7.7%
0.7%
0.2%
0.5%
57%
K
CD56
CD3
IFN-γ

Figure 5. Regulation of CD69 and IFN-γ on NK cells. PBMCs from the three donors were incubated with 3D7 schizont-infected erythrocytes (iRBCs), with iRBCs plus human IFN-α or with a mixture of IL-12 and IL-18, or were leftuntreated in culture medium (CM) for 24 hours and analyzed by flow cytometry. The gating strategy for NK cells ($CD56^+CD3^-$ lymphocytes) and the percentages of $CD69^+$ (**A**) and IFN-γ^+ (**B**) cells for each treatment are depicted. Upper rows: donor V; middle rows: donor E; lower rows: donor K.
doi:10.1371/journal.pone.0024963.g005

dendritic cells (pDCs) were suggested to produce IFN-α in response to *P. falciparum*-schizonts, which in turn promote γδ T cell proliferation and IFN-γ production [32]. Microarray analysis of whole-blood cells from *P. chabaudi*-infected mice [33] demonstrated that the predominant responses at about 16 h to 24 h post-infection were dominated by interferon-induced genes and that after 32 hours there was a dramatic change in the regulated transcripts. However, there are contradictory studies regarding the importance of IFN-α in the immune response against malaria. A recent study has described that neither pDCs nor IFN-α/β were essential for parasite clearance as mice depleted of pDCs or IFN-α/β receptor knock-out mice could control *P. chabaudi* 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 titers 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

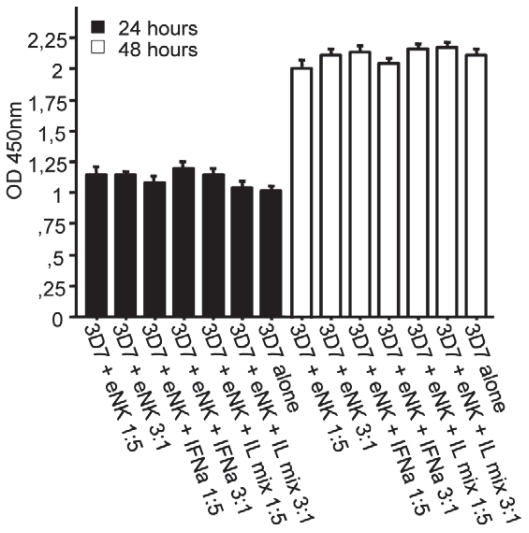


Figure 6. The influence of expanded NK cells and IFN-α on parasite growth. Expanded NK cells (eNK) from donor E were co-cultured with 3D7 ring-infected erythrocytes (iRBCs), with iRBCs plus human IFN-α, or with a mixture of IL-12 and IL-18. Parasites were incubated alone as a control. After 24 h and 48 h, culture samples were frozen at −20°C, thawed and inhibition of parasite growth was tested by HRP2 ELISA assay. Values represent the mean of three different experiments. Ratios are depicted as 3D7:eNK (1:3 and 5:1).
doi:10.1371/journal.pone.0024963.g006

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*, *STAT1* 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. chaubadi*-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 αβ 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 immunoproteasomes 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 expression converge at one stage and whether IFN-α-related transcripts result in IFN-γ signaling, should be further investigated by microarrays and functional studies at different time points. 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+IL-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 in red and the down-regulated genes are highlighted in green.
(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 JFJK. Contributed reagents/materials/analysis tools: MB PGK. Wrote the paper: EGdC JFJK.

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