Untersuchung der Effektormechanismen von Natürlichen Killerzellen gegenüber *Plasmodium falciparum* infizierten Erythrozyten

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Nur der Tor ist über den Zweifel erhaben…

gewidmet meinem ursprünglichen Betreuer
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Diese Dissertation basiert auf den nachfolgend angeführten unveröffentlichten sowie bereits publizierten Ergebnissen:

Liste der Veröffentlichungen

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Böttger E, Grangeiro de Carvalho E, Meese S, Kun JFJ IL-6 family receptors in NK92 cells are regulated rather by cytokines than by *Plasmodium falciparum*-infected erythrocytes. Manuskript in Vorbereitung

Böttger E, Multhoff G, Kun JFJ, Esen M (2012) Plasmodium falciparum-Infected Erythrocytes Induce Granzyme B by NK Cells through Expression of Host-Hsp70. *PLoS ONE* 7(3): e33774. doi:10.1371/journal.pone.0033774

Weitere Veröffentlichungen, die nicht in diese Dissertation eingeflossen sind:

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INHALT

ZUSAMMENFASSUNG

Natürliche Killerzellen (NK-Zellen) sind wichtige Effektorzellen der angeborenen Immunantwort, die entscheidend sind für die initiale Reaktion auf *Plasmodium falciparum*-Infektion sowie die Regulation nachfolgender adaptiver Prozesse. In der vorliegenden Arbeit sollte ihr Einfluss auf *P. falciparum* infizierte Erythrozyten (iRBZ) *in vitro* untersucht werden. Insbesondere sollten Mechanismen der direkten Aktivierung von NK-Zellen und deren Einflussnahme auf die Parasitenentwicklung durch Ausübung von Effektorfunktionen analysiert werden. Zunächst wurde ermittelt, inwiefern die NK92-Zelllinie als Modell bei der untersuchten Interaktion geeignet ist. Die Transkriptionsanalyse von NK92-Zellen zeigte, dass die Mehrheit der regulierten Gene nach 24h Kokultur mit iRBZ im Zusammenhang mit Zellzyklusprogression, Antiapoptose und Zellwachstum stand. Weiterhin waren einige Gene aktiviert, die Immunantwort und Aktivierung der NK-Zellen regulieren. Allerdings sollten diese Ergebnisse unter dem Aspekt der offensichtlichen Voraktivierung der verwendeten Zelllinie betrachtet werden, die sich als konstitutive Expression von Aktivierungsmarkern und Interferon-γ-Produktion äußerte. Obwohl die NK92-Zellen einen wachstumshemmenden Einfluss auf *P. falciparum* auszuüben schienen, war dieser nicht spezifisch und die Zelllinie erwies sich unter den beobachteten Bedingungen als ungeeignetes Modellsystem.

Nachfolgende Analyse von Rezeptoren der IL-6 Familie machte deutlich, dass diese durch unterschiedliche Zytokine (IFN-α, IL-6 und IL-2/-12/-18), aber nicht die parasitierten Erythrozyten beeinflusst werden konnten. IL-6 scheint in einem negativen Feedback-Loop die Beendigung der Signalkette durch verminderte Expression der untersuchten Rezeptoren zu bewirken, während die anderen Zytokine in unterschiedlichem Maße eine verstärkte Rezeptorexpression induzierten.

Weitere Untersuchungen von Rezeptoren und Liganden ergaben einerseits keinen Einfluss auf den aktivierenden Rezeptor NKG2C und andererseits auf MICA/B sowie HLA-E. Jedoch könnte der Korezeptor CD94 von sowohl aktivierenden als auch inhibierenden NKG2-Rezeptoren bei der Bindung von Hitzeschockprotein 70 (Hsp70) auf der Erythrozytenmembran eine Rolle spielen, da er immer auf der NK-Zelloberfläche exprimiert war. Aufgrund der durchgeführten Untersuchungen wurde eine Hypothese zur Erkennung und Eliminierung der iRBZ erstellt. Diese beinhaltet, dass NK-Zellen über Hsp70 auf der iRBZ-Membran zur erhöhten Granzym B-Freisetzung stimuliert werden, was in der Eryptose der parasitierten Erythrozyten resultiert. In der vorliegenden Arbeit konnte diese Hypothese bestätigt werden. Des Weiteren konnte der Effekt durch das Hsp70-Epitop TKD-Peptid

verstärkt werden. Es ist daher denkbar, TKD oder Hsp70 selbst als Immunostimulans oder als Adjuvans in der Impfstoffentwicklung zu nutzen, wodurch sich neue Alternativen für die Prävention oder Behandlung von Malaria eröffnen.

ABSTRACT

Natural Killer (NK) cells are important effector cells of the innate immune system and are thought to play a crucial role in early responses to *Plasmodium falciparum*-infection and by regulating adaptive immunity. The aim of this thesis was to investigate the interaction between NK cells and *P. falciparum*-infected erythrocytes *in vitro.* Particular interest was on elucidation of mechanisms occurring during such interaction and to rule out whether the intracellular parasite has a direct impact on activation of NK cell effector functions.

First, assessment of suitability of the NK92 cell line as a model to study NK cell-iRBC-interaction was addressed. Transcription analysis of NK92 cells showed that the majority of regulated genes after 24h co-culture were linked to cell cycle progression, anti-apoptosis and cell growth. Additionally, genes important for regulation of immune responses and NK cell activation were altered. However, those results have to be interpreted in the context of obvious pre-activation of the used cell line. Although NK92 seemed to exert a growth-limiting influence on *P. falciparum*, this effect was not specific. Given these circumstances, NK92 cells cannot be considered as a good model to study NK cell-parasite-interactions in malaria *in vitro*.

Subsequent analysis of IL-6-family receptors revealed a differential regulation by several cytokines (IFN-α, IL-6 und IL-2/-12/-18-mix), but not the parasitized erythrocytes themselves. IL-6 seemed to stop signal transduction via a negative feed-back loop, while the other cytokines resulted in increased receptor expression.

Further investigation of receptors and ligands demonstrated no involvement of the activating receptor NKG2C or ligands like MICA/B and HLA-E. However, CD94 could serve as a co-receptor of erythrocyte membrane-associated heat shock protein (Hsp) 70, since it was constitutively expressed on the NK cell surface. Due to Hsp70 on iRBC, NK cells were stimulated to elevated granzyme B release leading to subsequent eryptosis of the parasitized erythrocytes. This proposed mechanism is promising since the recognised Hsp70 epitope TKD-peptide could serve as an immunostimulants or Hsp70 itself as an adjuvants in vaccine development which could open the way for new alternatives of malaria prevention or treatment.

1 EINLEITUNG

Malaria ist trotz verstärkter Anstrengungen immer noch die häufigste parasitäre Infektion weltweit. Jährlich gibt es etwa 216 Millionen Erkrankte, von denen ca. 655000 der Krankheit zum Opfer fallen (1), wobei andere Berechnungen sogar von weit höheren Mortalitätsraten ausgehen (2). Die Infektion beim Menschen wird durch fünf humanpathogene Spezies des Protozoen *Plasmodium* hervorgerufen. Während Infektionen mit *P. vivax, P. ovale* und *P. malariae* zumeist mild verlaufen, kommt es bei der Malaria tropica ausgelöst durch *P. falciparum* auch zu schweren Komplikationen bis hin zum Tod. *P. knowlesi* ist ein erst kürzlich in Südostasien von Primaten auf den Menschen übergesprungener Erreger (3).

1.1 Generelle Aspekte zu Malaria

Menschen, die in malariaendemischen Gebieten leben und damit beständig dem Erreger ausgesetzt sind, können nach mehreren Infektionen eine sogenannte Semiimmunität entwickeln, die sich dahingehend äußert, dass die Krankheit nur noch asymptomatisch oder mit einem unkomplizierten Verlauf auftritt (4). Zusätzlich zur zellulären und humoralen Immunität beeinflussen auch genetische Faktoren den Verlauf der Krankheit (5). Besonders gefährdet an schwerer Malaria zu erkranken sind Kleinkinder bis zum Alter von etwa fünf Jahren, die noch keine ausreichende Immunität aufgebaut haben (6) und auch Schwangere, selbst wenn sie vor der Schwangerschaft bereits semi-immun waren (6;7). Abgesehen von natürlichen Resistenzmechanismen gegen den Erreger entwickelt der Parasit selbst immer neue Resistenzen gegenüber bisher verfügbaren Medikamenten (8;9). Um die Morbidität und Mortalität in den betroffenen Regionen signifikant zu senken, arbeiten Forscher weltweit an der Entwicklung einer effektiven Vakzine. Mehrere Ansätze sind dabei bereits getestet worden, indem man bestrahlte (10) oder attenuierte (11) Sporozoiten als auch Antigene, gerichtet gegen das Leberstadium (12) oder das erythrozytäre Stadium (13;14) als Impfstoffkandidaten verwendete. Jedoch ist es bisher noch nicht gelungen, einen effektiven Impfstoff auf den Markt zu bringen. Dies wird einerseits erschwert durch die fehlende protektive, natürlich entwickelte Immunität gegen das Leberstadium sowie andererseits durch antigene Variation und Polymorphismen im erythrozytären Stadium (15). Der bisher vielversprechendste Kandidat ist RTS,S/AS01E, der sich in Phase III befindet und 2015 als erste Malariavakzine für Kinder in malariaendemischen Gebiet auf den Markt kommen soll. Allerdings wird auch hier nur eine circa 50%-ige Protektion erreicht (16). Um ein tieferes

generelles Verständnis involvierter Abwehmechanismen des Immunsystems bei der natürlichen Infektion zu erlangen und dringend notwendige neue Alternativen für die Impfstoff- und Medikamentenentwicklung zu entdecken, ist es notwendig, weitere Anstrengungen in der Grundlagenforschung zu unternehmen.

1.2 Überblick über das Immunsystem

Das menschliche Immunsystem setzt sich aus diversen Komponenten wie beispielsweise mechanischen Barrieren, humoralen Faktoren wie chemischen Botenstoffen und Antikörpern als auch zellulären Faktoren, den Leukozyten (weiße Blutzellen), zusammen. Es werden hinsichtlich zeitlicher und zellulärer Komponenten das angeborene und das erworbene, adaptive Immunsystem unterschieden, welche in stetiger Wechselwirkung miteinander stehen und sich dadurch gegenseitig beeinflussen. Zu den Leukozyten des angeborenen Immunsystems zählen Granulozyten, Dendritische Zellen (DC), Monozyten, Makrophagen und Natürliche Killerzellen (NK-Zellen). DCs und Monozyten/Makrophagen stellen dabei als antigenpräsentierende Zellen eine Verbindung zwischen angeborener und erworbener Immunität dar. Die Zellen der adaptiven Immunantwort umfassen B- und T-Zellen.
NK-Zellen bilden mit 5-15% die drittgrößte Population an im Blut zirkulierenden Lymphozyten und werden auch verstärkt in Leber, Lunge, Milz (17) sowie unter bestimmten physiologischen Bedingungen auch in Lymphknoten, Plazenta (18) und Bauchhöhle (19;20) gefunden.

1.3 NK-Zellrezeptoren und ihre Liganden

Ursprünglich wurden NK-Zellen als zytotoxische Lymphozyten beschrieben, die ihre Zielzellen ohne vorherige Sensibilisierung abtöten können (21;22). In den letzten Jahren wurde allerdings gezeigt, dass sie von einer Vielzahl keimbahnkodierter aktivierender und inhibierender Rezeptoren reguliert werden (23;24). Die Rezeptoren dienen zur Unterscheidung von „Selbst“ und „Nicht-Selbst“ bzw. auch zum Erkennen infizierter oder transformierter Zellen (25). Beim Menschen werden die NK-Zellen durch im Haupthistokombatibilitätskomplex auf Chromosom 6 kodierte humane Leukozyten-Antigen-Klasse-I-Moleküle beeinflusst (26;27). Entsprechend ihres Polymorphismus unterscheidet man die hochpolymorphen klassischen HLA-Klasse-Ia-Moleküle, zu welchen HLA-A, -B und -C zählen und die wenig polymorphen, nicht-klassischen HLA-Klasse-Ib-Moleküle HLA-E,-F

und –G. Die erkennenden Rezeptoren werden in 4 Familien unterteilt: immunoglobulin-ähnliche Killerrezeptoren (KIR) (28;29), natürliche Zytotoxizitätrezeptoren (NCR) (30), lektinähnliche C-Typ-Rezeptoren (NKG2 beim Menschen und Ly49 bei der Maus) (31;32) sowie immunoglobulinähnliche Leukozytenrezeptoren (LIR/ILT) (31). Spezifische MHC-Klasse-I-Molekülerkennung durch KIR als auch LIR/ILT und Ly49 dient wesentlich der Selbst/Nicht-Selbstunterscheidung. Nicht-klassische MHC-Klasse-Ib-Moleküle werden von Heterodimeren aus NKG2 mit CD94 erkannt (33). NKG2-A, -B, -C und –E erkennen alle HLA-E, wobei die ersten beiden inhibierende und letztere aktivierende Rezeptoren darstellen. CD94 wird von allen NK-Zellen exprimiert, wobei CD94/NKG2A (inhibierend) ungefähr zehnmal häufiger als CD94/NKG2C (aktivierend) vorkommt. Der inhibierende Komplex dient dazu, Autoreaktivität von NK-Zellen ohne KIR-Expression über HLA-E zu verhindern (34). NKG2D-Homodimere sind aktivierende Rezeptoren, die MICA/B oder ULBP auf Zielzellen binden und entscheidend für Tumorimmunität und antivirale Abwehr sind (35).

1.4 Effektormechanismen der NK-Zellen

NK-Zellen nehmen entweder direkt durch zytotoxische Mechanismen ähnlich denen von zytotoxischen T-Zellen oder indirekt über die Ausschüttung von Chemokinen und Zytokinen Einfluss auf ihre Zielzellen. Ihre Effektorfunktionen werden durch Typ-I-Interferon (IFN), Interleukin (IL)-12, IL-15 und IL-18 stimuliert (36). NK-Zellen stellen keine einheitliche Zellart dar, sondern bestehen aus verschiedenen Subpopulationen, von denen hauptsächlich Zellen mit geringer Oberflächenexpression von CD56 ($CD56^{dim}$) und solche mit starker Expression von CD56 ($CD56^{bright}$) unterschieden werden. Während $CD56^{dim}$ nur zu etwa 10% im peripheren Blut vorkommen, stellen die weiterentwickelten $CD56^{dim}$ den restlichen Anteil an peripheren NK-Zellen. Beide unterscheiden sich nicht nur in der Quantität des Oberflächenmarkers CD56, sondern auch in ihren Effektormechanismen und der Expression des FcγIII-Rezeptors CD16 (37). $CD56^{bright}$ weisen wenig oder kein CD16 auf und können vermehrt Zytokine wie IFN-γ produzieren (37). $CD56^{dim}$ sind eher zytotoxisch, schwache IFN-γ-Produzenten und exprimieren CD16, worüber sie auch antikörpervermittelte zelluläre Zytotoxizität ausüben können. Weitere generelle Effektormechanismen, die letztendlich alle in der Apoptose der Zielzellen münden, sind sekretorische Lyse mittels Perforin und Granzymen sowie nonsekretorische Lyse, die Todesrezeptoren unter Einbeziehung der entsprechenden Liganden wie FasL (Fas-Ligand) oder TRAIL (TNF-α bezogener, Apoptose induzierender Ligand) involvieren (38). Degranulation der zytotoxischen Granula führt zur

Ausschüttung von Perforin, Granzymen und Granulysin (39) in die immunologische Synapse, die sich zwischen Zielzelle und NK-Zelle bildet (40). Wie genau die Effektormoleküle in die Zielzelle aufgenommen werden, ist Gegenstand momentaner Forschung. Einerseits wurde angenommen, dass Perforinmultimere Ca^{2+} abhängig Poren in die Zellmembran inserieren, durch welche Granzyme ins Zytosol gelangen könnten (41). Durch neuere Erkenntnisse geht man jedoch davon aus, dass beide per Endozytose aufgenommen werden und Perforin dann zur Freisetzung von Granzym (Gzm) aus dem Endosom benötigt wird (42). Weiterhin wurde auch Perforin unabhängige, GzmB vermittelte Apoptose beobachtet, die im Zusammenhang mit membranassoziierten Hitzeschockprotein (Hsp) 70 stand (43).

1.5 IL-6-Familierezeptoren und NK-Zellen

Interleukin-6 (IL-6) ist ein pleiotropes, NK-Zellen stimulierendes Zytokin und in Prozessen wie Hämatopoese, Immunregulation und Akute-Phase-Reaktionen involviert. Sein Rezeptor IL-6Rα wird auf vielen unterschiedlichen Geweben und Zelltypen wie Knochenmarkszellen, Hepatozyten und peripheren mononukleären Blutzellen (PBMZs) exprimiert (44). IL-6 gehört der helikalen neuropoetischen IL-6-Familie an, welche sich aus IL-6, IL-11, Leukämie inhibierender Faktor (LIF), Onkostatin M (OSM), ziliarer neurotropher Faktor (CNTF), Cardiotrophin-1 (CT-1) und Cardiotrophin ähnliches Zytokin (CLC) zusammensetzt. Mitglieder der Familie teilen alle den gemeinsamen signaltransduzierenden β-Rezeptor CD130, der auch als Glykoprotein gp130 bezeichnet wird (45). Während membrangebundenes gp130 ubiquitär vorkommt, ist die Expression der α-Rezeptoren streng reguliert (46). Zytokinen kommt eine bedeutende Rolle bei der Immunantwort auf *P. falciparum* zu. Bei schwerer Malaria korrellieren Level von proinflammatorischen Zytokinen wie IFN-γ, IL-12, IL-6 und TNF-α mit dem Schweregrad der Krankheit (47-50). Klinische Manifestation von periodischen Fieberschüben, Rigor und Schüttelfrost korrellieren mit dem Peak von proinflammatorischen Mediatoren, welche vom angeborenen Immunsystem als Antwort auf freigesetzte Parasitenantigene während der Erythrozytenruptur produziert werden (48). Im Plasma von Patienten mit zerebraler Malaria oder Nierenversagen wurden erhöhte Konzentrationen an IL-6 and IL-6R gefunden (51). Aufgrund der ubiquitären Expression von gp130 und löslichen Rezeptoren von IL-6 und CNTF können praktisch alle Zellen auf IL-6 reagieren, u.a. auch NK-Zellen. Jedoch ist bisher nichts über die Regulation der IL-6-Familierezeptoren in NK-Zellen bekannt. Ein möglicher Einfluss ergibt sich aber, weil *Plasmodium ssp.*-Infektion zur Aktivierung der NK-Zellen durch proinflammatorische

Zytokine führt und ein Zusammenhang zwischen IL-6 und IFN-α-Signaltransduktion über gp130 besteht (52).

1.6 Hitzeschockprotein 70 und NK-Zellen

Erste Hinweise, dass normalerweise im Zytosol lokalisiertes Hitzeschockprotein (Hsp) 70 in die Membran inseriert werden kann, kamen durch Hitzeschockexperimente mit *P. knowlesi*-iRBZ in Rhesusaffen (53) und wurden später auch für *P. falciparum*-iRBZ belegt (54). Multhoff *et al.* zeigten, dass rekombinantes Hsp70, aber nicht homologe Hsps wie Hsc70 oder DnaK, spezifisch die NK-Proliferation anregen und IFN-γ-Produktion stimulieren können. Durch Peptidanalyse wurde das Hsp70-Epitop TKD gefunden, welches ein Oligomer aus vierzehn Aminosäuren des C-Terminus darstellt (55). Stimulation von Zelllinien mit Hsp70 bzw. TKD und niedrig dosiertem IL-2 induzierten Zytotoxizität in NK-Zellen, die zur Apoptose der Zielzellen führte. Später wurde belegt, dass Hsp70 sowie der C-Terminus von Hsp70, gekoppelt an GST spezifisch an NK YT-Zellen binden und internalisiert werden können und bei der Erkennung CD94 involviert ist (56). Gross *et al.* deckten mittels Affinitätschromatographie mit NK YT-Zellen auf, dass GzmB als Bindungspartner von Hsp70 fungiert (43). Autologes Hsp70 erhöhte signifikant die IFN-γ-Produktion durch periphere mononukleäre Blutzellen (PBMZ) nach 48h Stimulation in CML-Patienten. Dies wurde hervorgerufen durch MICA/B auf DCs, welches einen Liganden für NKG2D auf NK-Zellen darstellt (57). Die Bedeutung von MICA/B und NKG2D wurde auch in einer weiteren Studie gezeigt (58). In gesunden Probanden und Patienten mit akuter myelogener Leukämie wurde herausgefunden, dass Stimulation mit TKD+IL-2/-15 die Produktion von GzmB durch NK- aber nicht T-Zellen anregt, wobei Hsp70 auf der Oberfläche der Zellen ein stimulierendes Signal darstellte und HLA-E inhibitorisch wirkte (59). Zusammengefasst konnte also eine NK-Zellen stimulierende Wirkung von Hsp70 nachgewiesen werden, die über CD94 vermittelt wird und über NKG2D-MICA/B-Wechselwirkungen aktivierende Signale transduziert als auch über HLA-E blockiert werden kann. Aktivierung der NK-Zellen mittels Hsp70 führt daraufhin über GzmB zur Apoptose von Zielzellen.

1.7 Immunologisches Gedächtnis bei NK-Zellen

Klassischerweise wird zwischen einer schnellen angeborenen und einer späteren adaptiven Immunantwort unterschieden. Dabei können angeborene Abwehrmechanismen bereits

innerhalb weniger Minuten nach Pathogenkontakt gestartet werden, während die Entwicklung der spezifischen humoralen und zellulären Antwort des adaptiven Immunsystems mehrere Tage benötigt. Es gibt wachsende Indizidien, dass abgesehen vom spezifischen immunologischen Gedächtnis der B- und T-Zellen auch eine verstärkte angeborene Immunantwort nach Reinfektion möglich ist. Frühe Experimente belegten eine Art Kreuzprotektion gegenüber unverwandten Bakterien (60), welche im Zusammenhang mit IFN-γ-Freisetzung durch Lymphozyten, ausgelöst durch das erste Pathogen, standen. Diese temporär verstärkte angeborene Immunität ließ schnell nach, wenn das Primärpathogen entfernt wurde (61). Dass auch Zellen des angeborenen Immunsystems ein Gedächtnis nach Primärinfektion entwickeln können, ist in Pflanzen und Invertebraten schon länger bekannt. In Vertebraten gibt es diesbezüglich vorwiegend Studien in Mäusen gegen Candidose (62-64) sowie auch erste Hinweise auf NK-Zellen vermittelte Protektion gegenüber CMV (65). Es wurde weiterhin gezeigt, dass BCG-Impfung bei Kindern in Westafrika die allgemeine Morbidität durch andere Infektionen außer Tuberkulose, und damit die Mortalität, senkt (66). Sun *et al.* zeigten, dass bei CMV-Reinfektion spezifische NK-Zellsubpopulationen durch eine sekundäre Expansion, mit schneller Degranulation und Zytokinfreisetzung eine protektive Immunantwort vermitteln können (50). Diese Gedächtnis-NK-Zellen haben eine Proliferationskapazität von bis zu 1000-fach, sind selbsterneuernd und können noch mehrere Monate später aktiviert werden (67). Abgesehen von erhöhter Resistenz gegen Neuinfektion wurde auch eine Bedeutung der NK-Zellen bei Hypersensitivität gezeigt (68). Netea et al. schlugen in diesem Zusammenhang den Begriff der trainierten Immunität vor (69). Charakteristika einer trainierten, angeborenen Immunität sind Induktion nach Primärinfektion oder Impfung, die eine T-/B-zellunabhängige Protektion gegenüber Sekundärinfektion vermittelt sowie verstärkte Resistenz, die weniger spezifisch als die adaptive Immunität ist und eventuell eine Kreuzprotektion gegenüber anderen Pathogenen bewirkt. Außerdem sollte solch eine erhöhte Aktivierung verschiedene Zellen wie Makrophagen aber auch NK-Zellen umfassen, die zu einer verbesserten Pathogenerkennung über spezifische Rezeptoren befähigt sind und eine protektive inflammatorische Antwort auslösen (69).

1.8 Bedeutung der Natürlichen Killerzellen bei Malaria

Es ist weitgehend akzeptiert, dass NK-Zellen eine bedeutende Rolle in der frühen angeborenen Immunantwort auf *P. falciparum*-Infektion spielen. NK-Zellen beeinflussen den Verlauf der Malaria hauptsächlich indirekt durch Ausschüttung proinflammatorischer

Zytokine, wodurch sie Einfluss auf die Aktivierung und das Ausbalanzieren späterer adaptiver Reaktionen nehmen (70-72). NK-Zellen stellen dominierende IFN-γ-Produzenten 12-18h nach Kontakt mit iRBZ dar. Ihre Effektorfunktionen werden durch Zytokine und Kontakt mit myeloischen Nachbarzellen wie Monozyten/Makrophagen und Dendritischen Zellen gesteuert, die IL-12, IL-15 und teilweise IL-18 bereitstellen sowie IL-2, welches von T-Zellen zur vollständigen Aktivierung freigesetzt wird (70;73;74). Die Menge an freigesetztem IFN-γ ist stark heterogen zwischen unterschiedlichen Personen. Dies steht einerseits im Zusammenhang mit der Stärke der Kontakt und Zytokin vemittelten Nachbarzellsignale sowie andererseits dem KIR-Genotyp (75-77). Ob zusätzlich zu den indirekten Mechanismen eine direkte Erkennung der iRBZ möglich ist, konnte bisher nicht eindeutig geklärt werden. Die Beobachtung von Rosettenbildung der NK92-Zelllinie (78) als auch von frisch isolierten NK-Zellen (75;76) mit iRBZ lässt vermuten, dass auch eine direkte Interaktion zwischen Killerzellen und iRBZ stattfinden könnte. Welche Rezeptoren und Liganden hierbei eine Rolle spielen, ist jedoch unbekannt.

2 ZIEL DER ARBEIT

2.1 Arbeitshypothesen

Nachdem eine indirekte Wirkung der NK-Zellen durch Ausschüttung von IFN-γ weitgehend akzeptiert ist, stellt sich die Frage, inwieweit die Killerzellen den intrazellulären Parasiten direkt erkennen und gegebenenfalls eliminieren können. Geschieht dies ausschließlich durch Mithilfe anderer Komponenten des Immunsystems, d.h. zellvermittelten Kontakt oder bereitgestellte Zytokine oder können NK-Zellen direkt veränderte Strukturen auf den befallenen Erythrozyten erkennen? Der Parasit moduliert nach der Invasion durch Oberflächenexpression vielzähliger Moleküle die Erythrozytenmembran, wodurch die Membranrigidität und Permeabilität verändert wird (79;80). Andererseits ist es notwendig, dass durch diese Veränderungen seine Proliferation durch Aktivierung von Immun-mechanismen nicht komplett verhindert wird. Ziel dieser Arbeit war es, ein tieferes Verständnis über die Interaktion zwischen NK-Zellen und iRBZ zu erlangen.

Die Hypothese umfasst, dass NK-Zellen auch ohne Einfluss von akzessorischen Nachbarzellen unter geeigneten Bedingungen einen direkten Einfluss auf infizierte Erythrozyten nehmen können, der sich in bekannten Aktivierungsmerkmalen wie IFN-γ-Sekretion und Oberflächenmarkern wie CD25 und CD69 oder Zytotoxizität äußern sollte.

Ausgehend davon sollte die Etablierung eines *in vitro*-Modellsystems mit der Zelllinie NK92 und *P. falciparum* Laborstämmen möglich sein, mit Hilfe dessen transkriptionelle und translationelle Veränderungen sowohl von Killerzellen als auch iRBZ untersucht werden können. Des Weiteren wurde postuliert, dass beobachtbare Auswirkungen von konkreten Rezeptor-Liganden-Interaktionen hervorgerufen werden, welche eine direkte Stimulation der NK-Zellen durch iRBZ zur Konsequenz haben. Infolgedessen werden NK-Zellen zur Ausübung von Zytotoxizität mittels Perforin/Granzym oder CD16 stimuliert und damit zur direkten oder indirekten Abtötung des Parasiten per Eryptose angeregt, ein Mechanimus, welcher stimuliert und zur verbesserten Elimination von *Plasmodium* genutzt werden kann.

2.2 Vorgehensweise

Es wurde zunächst ein *in vitro*-Modellsystem mit der Zelllinie NK92 und *P. falciparum* Laborstämmen etabliert. Wichtig dabei war es, das geeignete Verhältnis von Parasit zu Lymphozyt sowie zusätzliche, förderliche Faktoren (Rosettenbildung, externe Stimuli wie Zytokine) für das Kultursystem zu ermitteln. Des Weiteren wurden Aktivierungsmarker auf NK-Zellen als auch Effektormoleküle wie beispielsweise CD16, Perforin, GzmA und GzmB untersucht, die Zytotoxizität vermitteln, als auch ein direkter Einfluss von NK-Zellen auf die Parasitämie *in vitro* analysiert. Mit Hilfe dieses Systems sollte der Einfluss der Parasiten auf NK-Zellen auf transkriptioneller Ebene mittels Microarrays untersucht werden. Wichtige Fragestellungen hierbei waren, welche Gene aufgrund des Kontaktes mit infizierten Erythrozyen in NK-Zellen reguliert werden, welche Rezeptoren und Liganden bei der Interaktion eine Rolle spielen und inwiefern ein Mechanismus existiert, der für die Impfstoff-/ Medikamentenentwicklung genutzt werden könnte.
Konkret wurde im ersten Teil eine Transkriptionsanalyse von NK92-Zellen nach Kokultur mit iRBZ durchgeführt. Aus der Primäranalyse ergab sich als neue Hypothese eine mögliche Bedeutung der IL-6-Familierezeptoren IL-6R, CNTFRα und gp130, deren Regulation durch Zytokine (IL-6, IFN-α, IL-2/-12/-18) und den Parasiten selbst daraufhin im zweiten Teil untersucht wurden. Im letzten Abschnitt wurde die Oberflächenexpression von Liganden wie MICA/B, HLA-E und Rezeptoren wie CD94, NKG2C/E und D untersucht. Schließlich wurde membranassoziiertes Hitzeschockprotein (Hsp) 70 der Erythrozyten als mögliche Zielstruktur für NK-Zellen auf infizierten Erythrozyten analysiert. Nachdem beobachtet wurde, dass NK-Zellen die Parasitämie einzudämmen vermögen, wurde weiterhin die Bedeutung von

Granzymen und Perforin untersucht und ob eine Art Apoptose, in diesem Falle Eryptose, der infizierten Erythrozyten durch NK-Zellen induziert werden kann.

3 ERGEBNISSE

3.1 Limitierte Antwort von NK92-Zellen auf *P. falciparum* infizierte Erythrozyten

LIMITED RESPONSE OF NK92 CELLS TO *PLASMODIUM FALCIPARUM*-INFECTED ERYTHROCYTES

Elisandra Grangeiro de Carvalho*, Evelyn Böttger*, Van Tong Hoang, Peter G. Kremsner und Jürgen F. J. Kun

Präaktivierung in NK92-Zellen

Untersuchung der IFN-γ-Produktion und Oberflächenexposition der Aktivierungsmarker CD25 und CD69 zeigten eine Voraktivierung der NK92-Zellen. Die Zelllinie exprimierte konstitutiv IFN-γ und zu ca. 10% CD25, aber kein CD69. Nach Kokultur mit iRBZ verringerte sich die Menge freigesetzten Interferons. Des Weiteren konnte keine vermehrte Expression von CD25 und CD69 als Reaktion auf iRBZ detektiert werden.

Transkriptionsanalyse nach Kokultur

Sowohl nach 6 und 12 Stunden Kokultur mit iRBZ konnten keine nennenswerten Veränderungen des Genexpressionsprofils von NK92-Zellen beobachtet werden. Die wenigen regulierten Gene deuteten darauf hin, dass möglicherweise die NF-κB gesteuerte Genexpression aktiviert wurde. Nach 24h waren 167 Gene verschieden exprimiert, wovon allerdings nur 64 spezifisch für den Kontakt mit iRBZ im Vergleich zu uRBZ waren. Die Mehrheit der hochregulierten Gene stand im Zusammenhang mit Zellzyklusprogression, Antiapoptose und Zellwachstum. Weiterhin deuteten einige Gene auf Regulation der Immunantwort und Aktivierung der NK-Zellen hin.

Reduktion der Parasitämie nach Kokultur

Nach 24h Kokultur wurde mittels indirektem ELISA für das Parasitenprotein Histidinreiches Protein (HRP) 2 eine reduzierte Parasitämie detektiert, die allerdings auch bei der Kokultur von iRBZ mit anderen Zellarten (HeLa, C32 und Jurkat) festgestellt wurde.

3.2 Regulation von IL-6-Familierezeptoren durch *Pf*iRBZ und Zytokine in NK-Zellen

IL-6 FAMILY RECEPTORS IN NK92 CELLS ARE REGULATED RATHER BY CYTOKINES THAN BY *PLASMODIUM FALCIPARUM* INFECTED ERYTHROCYTES

Evelyn Böttger, Elisandra Grangeiro de Carvalho, Stefanie Meese, Jürgen F.J. Kun

Regulation der α-Rezeptoren der IL-6 Familie

NK92-Zellen wurden für 24h entweder mit IL-2/-12/-18, IL-6, IFN-α oder infizierten bzw. uninfizierten Erythrozyten stimuliert und die Veränderung auf transkriptioneller Ebene sowie Beeinflussung der Oberflächenexpression von CNTFR-α und IL6R-α wurde anschließend untersucht. Es wurde ein ähnlicher Einfluss auf die Regulation der α-Rezeptoren CNTFR-α und IL6R-α nach 24h Stimulation durch die verschiedenen getesteten Zytokine beobachtet. IL-6 hatte generell einen geringen Effekt im Vergleich zu IFN-α und IL-2/-12/-18 auf die Oberflächenexpression der α-Rezeptoren. Der Interleukinmix erhöhte die Oberflächenexpression der Rezeptoren, während die zytosolische Menge verringert wurde. Es zeigte sich eine ausgeprägte Verminderung auf transkriptioneller Ebene nach Stimulation mit Interleukinen. Durch IFN-α war dieser Effekt bei IL6R-α nicht so stark und führte sogar zu einer 2,2-fachen Erhöhung der CNTFR-α-Transkription.

Regulation des Korezeptors gp130

Erhöhte gp130-Oberflächenexpression zeigte sich nach Stimulation mit IL-6 nach 1h, welche allerdings nach 24h Stimulation wieder auf das ursprüngliche Niveau zurückgegangen war. IFN-α-Behandlung führte nach einstündiger Stimulation zu einem Trend in Richtung verstärkter Expression. Nach 24h war die Oberflächenexpression signifikant erhöht. Alle anderen Stimuli (IL-2/-12/-18 oder i/uRBZ) zeigten keinen nennenswerten Einfluss. Auf transkriptioneller Ebene jedoch bewirkten auch diese Stimulationen einen leichten Anstieg (1,5 – 2,4-fach) der Expression von gp130. IL-6 und IFN-α erhöhten ebenfalls die gp130-Transkription nach 24h.

Einfluss von P.falciparum-iRBZ auf NK-Zellen

Die Kokultur von NK-Zellen mit iRBZ hatte keinen Einfluss auf die Expression der untersuchten IL-6-Familierezeptoren. Teilweise wurde eine erhöhte Expression von gp130 detektiert, die aber in gleicher Weise von uRBZ ausgelöst wurde. Die α-Rezeptoren wurden nach iRBC-Kontakt eher vermindert exprimiert.

3.3 *P. falciparum* infizierte Erythrozyten induzieren Granzym B in NK-Zellen durch Expression von Wirts-Hsp70

Plasmodium falciparum-infected erythrocytes induce Granzyme B by NK cells through expression of host-Hsp 70

Evelyn Böttger, Gabriele Multhoff, Jürgen F.J. Kun, Meral Esen

Verlangsamtes Wachstum von Plasmodium falciparum durch NK-Zellen

Kokultur von NK-Zellen mit iRBZ (stimuliert oder nicht stimuliert mit TKD-Peptid) führte zu einer Inhibition der normalen Parasitenentwicklung sowie zu einer Reduktion der Parasitämie. Hemmung der Parasitenentwicklung zeigte sich durch Induktion von kompakten Formen von *P. falciparum*, die üblicherweise vor dem Absterben der Parasiten *in vitro* beobachtet werden. Unstimulierte NK-Zellen induzierten nach 24h Kokultur in 85.7 ± 4.9% der Parasiten und nach 5-tägiger Voraktivierung mit TKD in nahezu allen Parasiten (94.0 ± 4.3%) diesen kritischen Zustand. Dieser Effekt war vernachlässigbar mit einer äquivalenten Menge an PBMZ. Wurden PBMZ mit dem NK-Zellen aktivierenden Hsp70-Peptid TKD stimuliert, konnten auch morphologisch veränderte Parasitenformen (27.8 ± 8.9%) induziert werden.

Wirts-Hsp70 an der Oberfläche von infizierten Erythrozyten

Mittels Westernblot und Durchflusszytometrie wurde die Präsenz von Wirts-Hsp70 an der Oberfläche von iRBZ sowie seneszenten uRBZ gezeigt. HLA-E und MICA/B hingegen wurden nicht oberflächenexprimiert. NKG2C fand sich nach 24h nicht an der NK-Zelloberfläche, dafür aber CD94, welches als potentieller Hsp70-Rezeptor daher nicht auszuschließen ist.

Erhöhte Granzym B-Translation und Ausschüttung nach iRBZ-Kontakt

Auf transkriptioneller Ebene wurde keine signifikante Beeinflussung der Expression durch iRBZ von GzmA, GzmB oder Perforin detekiert. Hingegen zeigte sich nach 24h Kokultur mit iRBZ eine verstärkte zytosolische Proteinexpression und Freisetzung von GzmB. Durch Blockade der Hsp70-Oberflächenexpression konnte die GzmB-Ausschüttung zu basalen Levels reduziert werden. Auch Stimulation mit TKD erhöhte die GzmB-Freisetzung signifikant.

NK-Zellen induzieren Eryptose der iRBZ

Die Freisetzung von GzmB durch NK-Zellen nach 24h Kokultur induzierte Eryptose der infizierten Erythrozyten. Die Eryptose äußerte sich durch verringerte Größe und Exposition von Phosphatidylserin an der Zelloberfläche der Erythrozyten. Wurden die iRBZ hingegen mit PBMZ kultiviert, war der Grad an Eryptose weniger stark ausgeprägt, jedoch auch signifikant erhöht im Vergleich zu iRBZ, die ohne Lymphozyten in Zellkulturmedium kultiviert wurden. Vorherige Stimulation der Lymphozyten mit TKD erhöhte die Eryptoserate zusätzlich.

4 DISKUSSION

Es ist bekannt, dass NK-Zellen eine essentielle Rolle bei der angeborenen Immunantwort gegen den Malariaerreger *P. falciparum* spielen. Jedoch fehlt ein grundlegendes Verständnis, welche Rezeptoren und Liganden eingebunden werden und inwieweit abgesehen von IFN-γ-Freisetzung auch Zytotoxizität ausgelöst wird.

4.1 NK92 als ungeeignetes Modell zur Untersuchung der NK-iRBZ-Interaktion

Die Zelllinie NK92 sollte als Modellsystem etabliert werden, weil sie bereits in anderen Studien zur Untersuchung von iRBC-NK-Wechselwirkungen eingesetzt wurde (64). Zelllinien haben den Vorteil, dass die Reproduzierbarkeit der Experimente sowie unlimitierte Quantität an Ausgangsmaterial gewährleistet ist. Werden *ex vivo* isolierte Zellen von verschiedenen Donoren verwendet, muss außer begrenzten Ressourcen zusätzlich starke Heterogenität zwischen Individuen berücksichtigt werden (76). Aus den dargestellten Ergebnissen wurde ersichtlich, dass die Wahl der untersuchten Zellen entscheidend ist. Die anfangs verwendete NK92-Zelllinie erwies sich als bereits voraktiviert, was sich durch konstitutive IFN-γ-Freisetzung und partielle CD25-Expression äußerte. Zudem ließen sich keine weiteren Aktivierungsmerkmale wie erhöhte Expression von CD25 und CD69 nach Kontakt mit iRBZ detektieren. Präsenz derartiger Aktivierungsmarker wurde aber bereits nach Stimulation von PBMZ mit iRBZ beobachtet (72). Aufgrund dieser Ergebnisse wurden im Verlauf der Studien frisch isolierte NK-Zellen bevorzugt. Durch Verwendung frisch isolierter Zellen limitiert sich der störende Einfluss von Voraktivierung und somit kann die Aktivierung der zu messenden Einflüsse besser eruiert werden. Außerdem können mit *ex vivo* isolierten NK-Zellen auch die verschiedenen Subpopulationen evaluiert werden. Vergleich der Microarray-Ergebnisse von NK92-Zellen mit dem Transkriptionsprofil von frisch isolierten Zellen nach iRBZ-Kokultur zeigte ein komplett verschiedenes Bild. So wurden in *ex vivo* isolierten NK-Zellen vornehmlich Typ-I-Interferon gesteuerte Signalwege beeinflusst, welche bei NK92-Zellen nicht gefunden wurden (81). Die Microarray-Ergebnisse der Zelllinie suggerierten den Einfluss von NKG2C, was sich zwar durch qRT-PCR aber nicht mittels Durchflusszytometrie bestätigen ließ, d.h. dass zwar auf transkriptioneller Ebene eine verstärkte Expression zu verzeichnen war, die aber nicht in erhöhte Proteinoberflächenexpression übersetzt wurde. Es bedarf also noch fortführender Analysen

auch der anderen regulierten Gene, um eine endgültige Aussage bezüglich der Bedeutung der beobachteten veränderten Genexpression nach iRBZ-Kokultur zu treffen.
Obwohl im Kokultursystem ein Einfluss der NK-Zellen auf die Parasitämie beobachtet wurde, konnten unspezifische Effekt wie Substratmangel nicht ausgeschlossen werden, weil auch ausgelöst durch immunologisch inaktive Zellen wie z.B. Hela-Zellen eine ähnliche, wenn auch verminderte Reduktion der Parasitämie zu verzeichnen war.

4.2 IL-6-Familierezeptoren werden durch Zytokine reguliert

IL-6-Familierezeptoren scheinen keine Rolle in der direkten Interaktion zwischen NK-Zellen und iRBZ zu spielen. Allerdings könnte ihre Regulation der Rezeptoren durch Zytokine, die bekanntermaßen im Rahmen der Infektion von myeloischen Nachbarzellen freigesetzt werden, eine indirekte Beeinflussung durch den Parasiten zur Bedeutung haben. Interessanterweise führten iRBZ oft zu einer verringerten Expression der Rezeptoren.

4.3 Hsp70 als neue Zielstruktur auf iRBZ stimuliert NK-Zelleffektormechanismen

Schließlich konnte eine neue Theorie der Erkennung von seneszenten und infizierten Erythrozyten durch NK-Zellen erstellt werden. Solche Erythrozyten exponieren demnach Hsp70 an ihrer Oberfläche, welches von NK-Zellen erkannt wird und zur Ausschüttung von GzmB und schließlich zur Eryptose der RBZ führen kann.
NK-Zellen können durch das TKD-Peptid sensibilisiert werden, Hsp70-positive Zielzellen effektiver abzutöten (55) und daraufhin auch verstärkt Hsp70-positive iRBZ nach Stimulation erfolgreicher eliminieren. Dieses Modell ist besonders interessant, da *ex vivo* TKD stimulierte NK-Zellen bereits zur Therapie verschiedener Krebsarten in Form von Antigen-Hsp70-Fusionsgenen (82) oder auch als Fusionsproteine (83) in klinischen Studien eingesetzt wurden (84;85). Allerdings konnte nicht geklärt werden, welche Rezeptoren in der untersuchten Interaktion von NK-Zellen mit iRBZ membranassoziiertes Hsp70 erkennen könnten. CD94 ist ein möglicher Kandidat, weil er immer auf NK-Zellen präsent ist. Seine Bedeutung als Co-Rezeptor bei MICA/B-abhängiger Interaktion wurde berichtet (58). Jedoch konnte bei iRBZ keine Beteiligung der stressinduzierten NK-Zellliganden MICA/B gezeigt werden. Es wäre auch denkbar, dass es zu einer wechselseitigen Aktivierung („cross-talk“) zwischen DZs, iRBZ und NK-Zellen kommt. Qiao et al. haben gezeigt, dass durch extrazelluläres Hsp70 MICA/B auf DZs hoch reguliert wird (57). Als NKG2D-Ligand könnte es die NK-Aktivität

erhöhen und zur vollständigen IFN-γ-Sekretion stimulieren. Zusätzlich wäre es interessant aufzuklären, inwieweit Hsp70 ein Gefahrensignal („danger signal“) darstellt oder ob es eher dem Transport immunogener Peptide dient.

Obwohl verstärkte GzmB-Ausschüttung nach iRBZ-Kontakt und TKD-Stimulation zu verzeichnen war, bleibt aus den bisherigen Untersuchungen unklar, ob GzmB tatsächlich in die iRBZ aufgenommen wird oder ob es extrazelluläre Funktionen ausübt. In einer anderen Studie wurde gezeigt, dass während einer Malariainfektion GzmB-Level im Serum von Patienten erhöht waren (86). Außerdem wurde kürzlich eine Verknüpfung zwischen dem Komplementweg und GzmB gezeigt. Die Serinprotease konnte dabei über Aktivierung von C3 und C5 die Komplementkaskade starten. Erythrozyten haben einen Komplementrezeptor 1, über welchen C3b gebunden werden kann, was zur Opsonisierung und schließlich Erythrophagozytose durch Makrophagen führt. Auf diese Weise könnten NK-Zellen den Apoptoseweg mit der Komplementkaskade verknüpfen und zu einer effektiveren Entfernung von infizierten RBZ führen (87;88). Falls GzmB in die iRBZ aufgenommen wird, könnte so deren Eryptose eingeleitet werden. Dass eine Perforin unabhängige NK-Zell vermittelte Apoptose von Zielzellen möglich ist, wurde bereits gezeigt (43). Obwohl Erythrozyten weder Nucleus noch Mitochondrien aufweisen und dadurch einige charakteristische Merkmale der Apoptose wie DNA-Kondensation oder mitochondriale Depolarisation nicht aufweisen, können sie trotzdem in einen programmierten Zelltod eintreten (89;90). Dieser als Eryptose bezeichnete Zelltod ist gekennzeichnet durch Schrumpfen der Zellen, Vesikelabschnürung und Exposition von Phosphatidylserin (89;91). Durch Ca^{2+}-Eintritt steigt die zytosolische Ca^{2+}-Konzentration. Es werden Prostaglandin E_2 abhängig Ca^{2+}-sensitive Kationenkanäle geöffnet, was eine Zellmembranhyperpolarisation und durch austretendes Wasser aufgrund osmotischen Drucks das Schrumpfen der Zelle zur Folge hat (92). Es wurde bereits nachgewiesen, dass die Eryptose bei osmotischem Schock, oxidativem Stress, Energiemangel, Fieber und physiologischen Bedingungen wie Sichelzellanämie, β-Thalassämie, Glukose-6-phosphatdefizienz, Eisenmangel, Diabetes und Malaria erhöht ist. Auch *Plasmodium* induziert oxidativen Stress, wodurch die Ca^{2+} permeablen K^+-Kanäle aktiviert werden (93).

4.4 Bedeutung und Anwendbarkeit des postulierten Mechanismus

Es zeigte sich, dass NK-Zellen in unstimulierter als auch TKD aktivierter Form einen antiparasitären Effekt ausüben, was durch morphologisch veränderte Parasiten nach 24h Kokultur mit Lymphozyten sichtbar wurde. Dass diese Parasiten wahrscheinlich sterben,

wurde deutlich durch erhöhte Produktion und Freisetzung des Effektormoleküls GzmB durch die NK-Zellen, welche direkt durch Parasiten aber auch durch TKD-Peptid gesteigert werden konnte. Allerdings ist noch unklar, ob durch TKD-Behandlung die Infektion vollständig elimiert oder gar zukünftige Episoden verhindert werden könnten. Jedoch ist es bedeutsam, die Realisierbarkeit neuartiger Applikationen zu evaluieren. Durchführbarkeit, Sicherheit und Toxizität von e*x vivo* TKD-stimulierten, autologen NK-Zellen wurde bereits in einer Phase-I-Studie mit Lungen- und Kolonkrebspatienten belegt (94). Zusätzlich wurden weitere klinische Studien bei verschiedenen Krebsarten durchgeführt (95). Einerseits kamen dabei Hsp-basierte Vakzine als auch Hsp70-Peptidkomplexe zum Einsatz, welche in der Lage sind, auch andere Immunzellen wie beispielsweise DZ zu aktivieren. Antigenpräsentierende Zellen internalisieren Hsp70-Peptidkomplexe und präsentieren sie über MHC-Moleküle T-Zellen (96). So könnten über Hsp70 sowohl angeborene als auch adaptive Prozesse stimuliert werden. Es muss jedoch bedacht werden, dass TKD nicht wie zur Krebsbehandlung eingesetzt werden kann. Bei einer akuten Krankheit wie Malaria ist direkte Behandlung unabdingbar. Außerdem ist es momentan unwahrscheinlich *ex vivo* TKD-stimulierte NK-Zellen zum Einsatz zu bringen, da hierfür teuere GMP-Einrichtungen in Ländern benötigt würden, die zwar die höchste Infektionslast, aber auch schwache Finanzkraft aufweisen. Es wäre allerdings denkbar, das Peptid direkt zur *in vivo*-Stimulation zu injizieren. Durch in letzter Zeit aufgekommene neue Erkenntnisse, dass auch NK-Zellen ein immunologisches Gedächtnis entwickeln können (97;98), ergibt sich die Möglichkeit TKD/Hsp70 als Immunostimulans bei der Impfstoffentwicklung einzusetzen. Allerdings wurden diesbezügliche Experimente vorwiegend in Mäusen durchgeführt und spezifische Marker von NK-Gedächtniszellen des Menschen fehlen bisher zum Nachweis. So ist beispielsweise CD57 bereits auf 30-60% der zirkulierenden NK-Zellen präsent (99) und erscheint, obwohl als Marker propagiert (97), eher ungeeignet. Zusätzlich sollte bedacht werden, dass permanente Stimulation des Immunsystems mittels Hsp70/TKD zur Überlastung der involvierten Mechanismen führen könnte (immune exhaustion).

Der postulierte Mechanismus muss durch weitere Experimente bestätigt werden und auch ob er sich für andere intrazelluläre Parasiten als relevant erweist. Insgesamt eröffnen sich durch die gewonnenen Erkenntnisse aber interessante Perspektiven für die Impfstoff- bzw. Medikamententwicklung, die dringend notwendig sind.

4.5 Fazit

Zusammenfassend wurde deutlich, dass NK-Zellen allein durchaus von iRBZ aktiviert werden können. Allerdings äußert sich die Aktivierung nicht in IFN-γ-Produktion sondern in erhöhter Zytotoxizität vermittelt über GzmB, die in Eryptose der iRBZ mündet und somit auf eine Rolle der NK-Zellen bei der direkten Elimination des Parasiten hindeuten. Mittels eines limitierten Modells mit NK-Zelllinien kann nicht die ganze Vielfalt der Interaktion mit iRBZ untersucht werden. Solch ein Modellsystem muss deshalb mit äußerster Vorsicht verwendet werden und liefert nur begrenzte Information. Auch wenn in dieser Arbeit nicht die direkte Liganden-Rezeptor-Interaktion aufgeklärt werden konnte, wurde jedoch die Rolle von Wirts-Hsp70 bei der Erkennung von iRBZ aufgedeckt. Stimulation mit dem C-terminalen Epitop TKD-Peptid von Hsp70 ermöglicht eine Erhöhung der Produktion und Freisetzung von GzmB durch NK-Zellen. Ob die ausgelöste Eryptose auch *in vivo* eine Benefit für den Patienten darstellt, muss in zukünftigen Studien geklärt werden.

5 EIGENANTEIL

Hiermit erkläre ich, dass ich der alleinige Autor dieser Dissertation bin, die zunächst unter der Leitung von Prof. Dr. Jürgen F.J. Kun und später von Dr. Meral Esen am Institut für Tropenmedizin der Universität Tübingen durchgeführt wurde. Die im Folgenden aufgelisteten experimentellen Arbeiten wurden von mir selbst durchgeführt und sind in die beschriebenen Veröffentlichungen eingeflossen.

Veröffentlichung I:

- Parasiten- und Zellkultur
- Rosetting-Experimente
- Kokultur von iRBZ und NK92-Zellen
- RNA-Extraktion von NK92 für Mikroarrays
- Analyse der Mikroarrays, qRT-PCR-Validierung
- HRP2-Assay und Durchflusszytometrie (teilweise)

Veröffentlichung II:

- Parasiten- und Zellkultur
- Kokultur von iRBZ und NK92-Zellen mit/ohne Stimulation
- Proteinextraktion, Westernblots
- RNA-Extraktion, cDNA-Synthese, qRT-PCR
- Durchflusszytometrie, Analyse der Oberflächenmarker

Veröffentlichung III:

- Parasiten- und Zellkultur, NK-Zellisolation
- Kokultur von *Pf*iRBZ und NK-Zellen mit/ohne Stimulation
- Proteinextraktherstellung von u/iRBZ-Membran/Zytosol, Westernblots
- RNA-Extraktion, cDNA-Synthese, qRT-PCR
- Durchflusszytometrie, Analyse von Liganden auf iRBZ und NK-Zellrezeptoren
- Granzym B-Elispot
- Eryptoseassays

Erstellung und Durchführung der Studiendesigns, Datenanalyse sowie Verfassen der Manuskripte erfolgte weitgehend eigenständig. Veröffentlichung I wurde in Zusammenarbeit mit Elisandra Grangeiro de Carvalho angefertigt, die gleichberechtigter Koautor dieser Publikation ist. Teile dieser Ergebnisse sind auch in ihre Dissertation mit eingeflossen.

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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

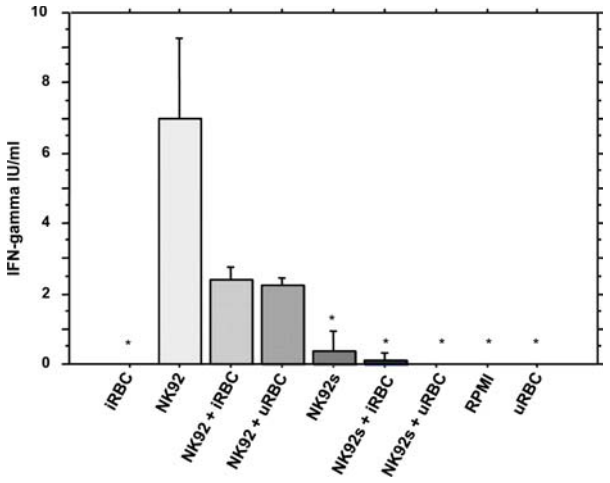

Figure 1 IFN-γ release of NK92 cells after co-culture with iRBCs. NK92 cells previously cultured with rIL-2 and without rIL-2 (24 hours starvation; NK92s) were co-incubated with 3D7 schizont-iRBCs and uninfected erythrocytes in RPMI-1640 medium (24 hours - 37°C - 5% CO_2) and supernatants were subsequently 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).

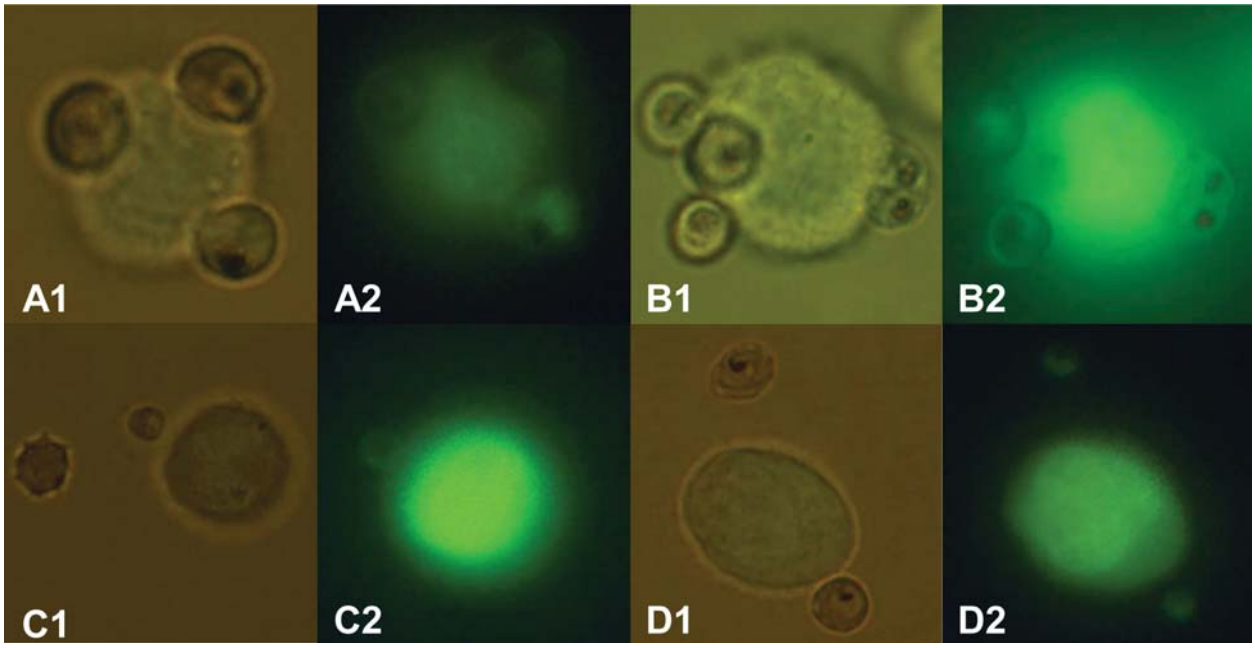

Figure 2 Rosettes of NK92 and FCR3-CSA parasites. NK92 were co-cultured with FCR3-CSA-iRBCs in 1:3 ratio respectively, for 1 h at 37°C/5% CO_2. After incubation, a sample of the co-culture was dyed with acridine orange and analysed in a fluorescence microscope. **A1 and B1**. Rosettes of 1 NK cell with 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.

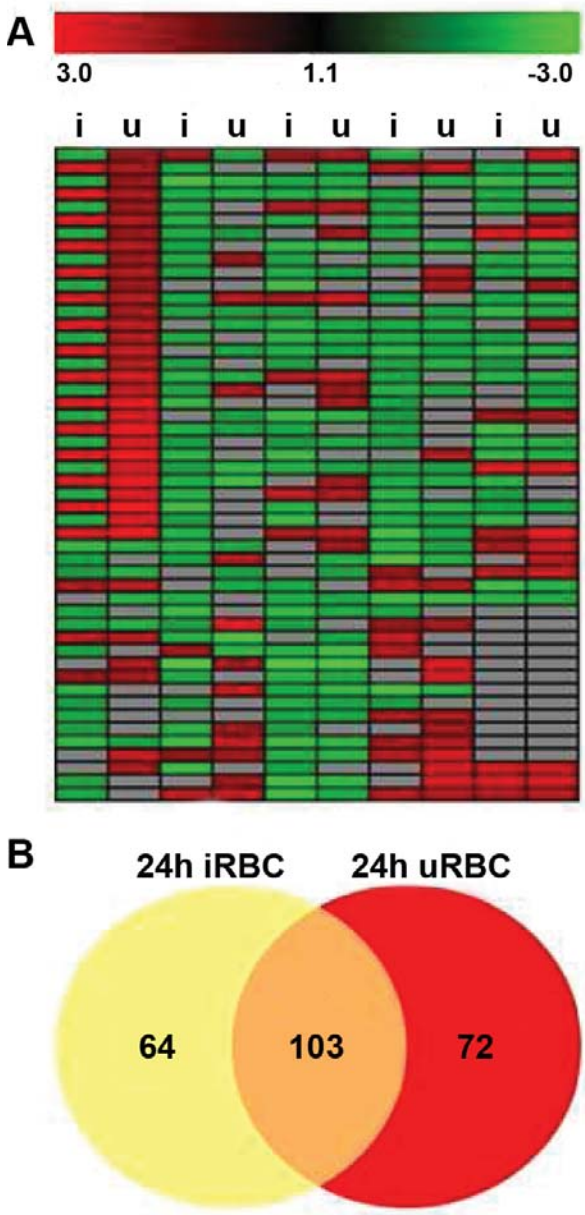

Figure 3 Gene expression patterns in NK92 after 24 h co-culture with *P. falciparum*-iRBCs. Normalised expression patterns were derived from microarrays of NK92 cells after 24 hours co-culture with 3D7-iRBCs or uRBCs (1:3). Data were obtained from 3 biological replicates **A**. Heatmap of altered genes 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

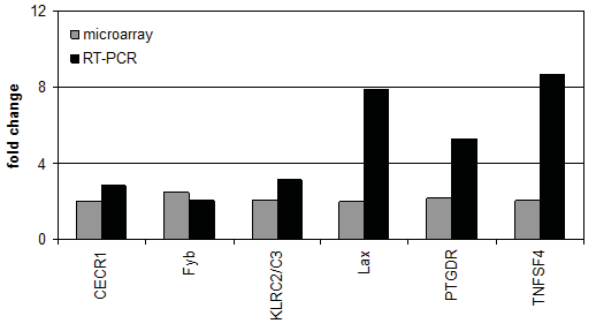

Figure 4 RT-PCR validation of microarray results. Fold change in gene expression of NK92 cells after 24 hours of 3D7-iRBCs co-culture comparing microarray and qRT-PCR results for 6 genes of interest (CECR1, Fyn-binding protein, KLRC2/C3, LAX, PTGDR, and TNFSF4 (Ox40L)) linked to priming of adaptive immune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

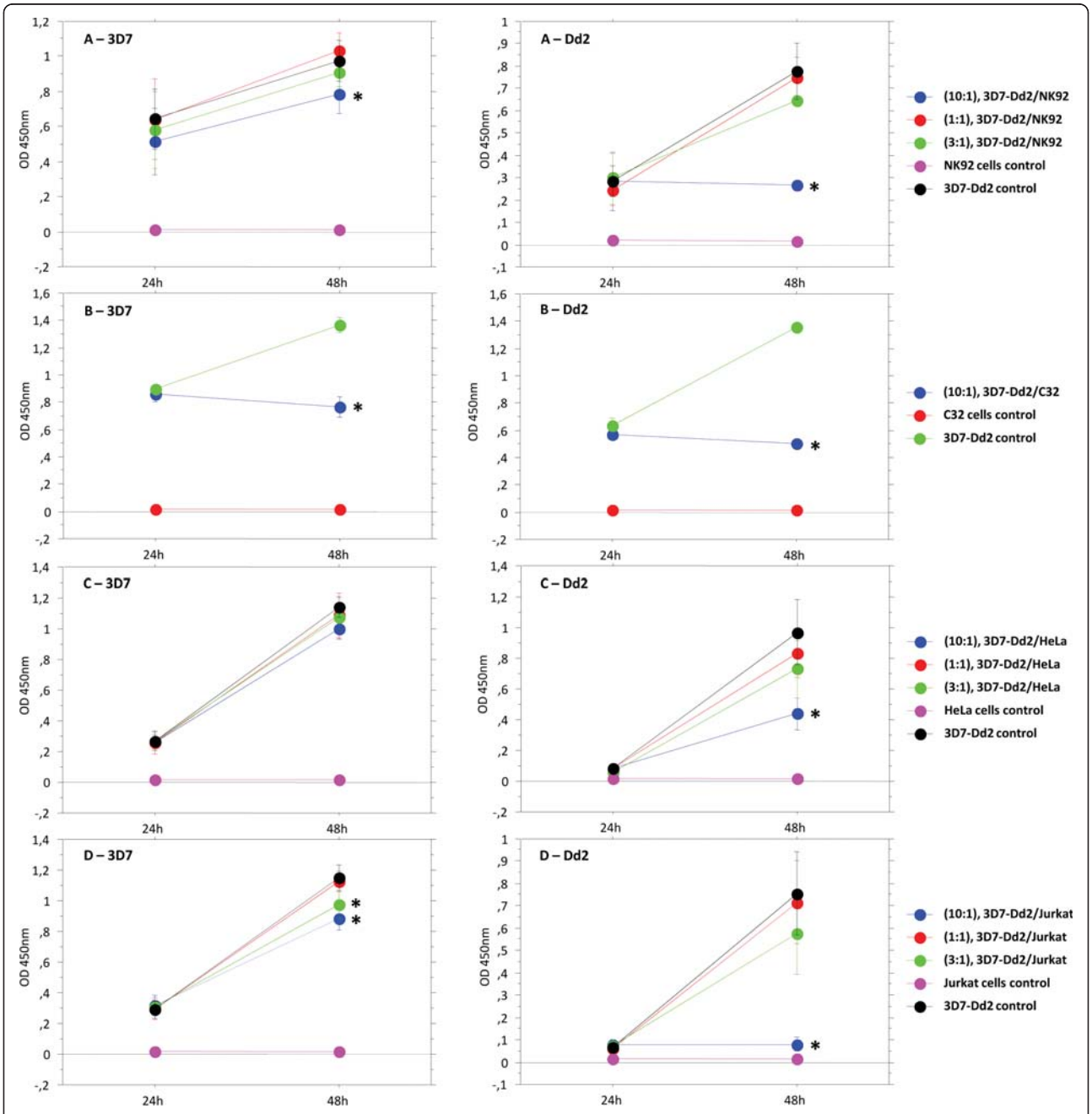

Figure 5 Parasite growth inhibition assay. 3D7 (left column) or Dd2 (right column) sorbitol-synchronized ring-stage-iRBCs were co-incubated with NK92 (A) in different ratios (10:1, 1:1, 3:1 - parasites:cells) in parasite growth medium. Starting parasitaemia was 0,125% in 1% haematocrit. As controls, 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

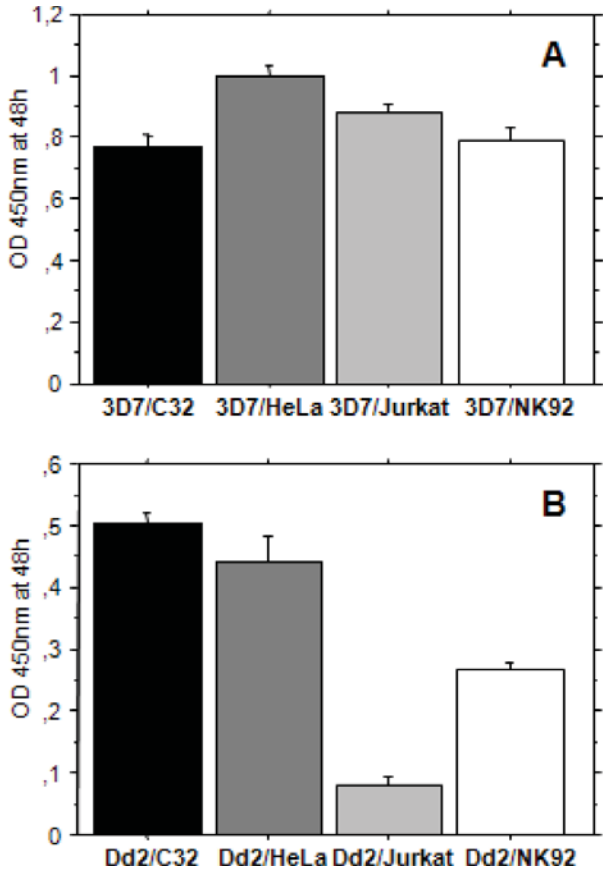

Figure 6 3D7 and Dd2 growth inhibition induced by different cell lines. 3D7 or Dd2 sorbitol-synchronized ring-stage-iRBCs were co-incubated with NK92, C32 cells, HeLa cells and Jurkat cells in the same conditions. After 24 to 48 hours of incubation, parasite growth inhibition was quantified by measuring 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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IL-6 family receptors in NK92 cells are regulated rather by cytokines than by *Plasmodium falciparum*-infected erythrocytes

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Abstract

A balanced pro-inflammatory cytokine response due to *Plasmodium ssp.* infection is crucial for the disease outcome. In order to gain deeper understanding on the regulation of three IL6-family receptors (IL-6R, CNTFR-α and gp130) in Natural Killer (NK) cells in the context of malaria, we conducted gene expression and protein abundance analysis in NK92 cells in response to several cytokine stimuli and *P.falciparum*-infected erythrocytes after 1 and/or 24 hours of stimulation. Analysis of gene expression revealed a surprisingly low effect of IL-6 on IL-6 receptor presentation on the surface of NK92 cells as well as on transcriptional changes. According to our data, IL-6 might act in a negative feedback loop to stop signal transduction by down-regulating IL-6R expression. Furthermore, the non-signalling α-receptors were regulated by a mixture of IL-2, -12 and -18. On the other hand, the signal-transducing β-receptor gp130 was not influenced by IL-6 but by IFN-α. *P. falciparum* alone could not stimulate NK cells to modulate the investigated receptors´ expression. Our results show that the IL-6 family signalling in the NK cell response to malaria is mainly influenced by cytokines derived from accessory cells but not by the parasite itself.

Key words: IL-6R family receptors, Natural Killer cells, cytokines, *Plasmodium falciparum*

1. Introduction

Natural killer (NK) cells as effector lymphocytes of the innate immunity are important key players in early responses to infection with a broad variety of pathogens such as viruses [1;2], protozoa [3;4], bacteria [5] and tumor-transformed cells. Initially, NK cells were described as cytotoxic lymphocytes able to kill tumour and virus-infected cells without prior sensitization. During the last years this picture has become more complex, and several studies have shown involvement of signals received from other cells to regulate their activity [6-8]. Especially dendritic cells (DCs) but also monocytes and macrophages provide a number of signals, including IL-12, IL-15 and IFN-α that lead to detectable effector functions. Furthermore, a need of direct contact between NK and accessory cells is reported [9;10].

Interleukin-6 (IL-6) is one of those NK cell-stimulating cytokines with pleiotropic functions involved in processes like hematopoesis, regulation of immune responses and acute-phase-reactions. Its receptor IL-6R is expressed on many different tissues and due to its diverse functions found on many cell types such as bone marrow cells, hepatocytes, PBMCs as well as leukemic blasts [11]. IL-6 belongs to the helical neuropoetic IL-6-family, which comprises IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), and cardiotrophin-like cytokine (CLC) [12]. These cytokines are involved in the modulation of inflammation, immune responses, heart development, and fertility, among other processes. All members share the same common β-receptor CD130, which associates with the respective α-receptor (IL-6Rα, CNTFR-α, etc.). While expression of the associated specific α-receptor is strictly regulated, CD130 is found almost ubiquitously in most tissues. The signal-transducing receptor was described initially in mice as a membrane-bound glycoprotein termed gp130, absent on resting

lymphocytes but present on blasting T cells [13]. The receptor consists of a cytoplasmic region, a single transmembrane segment, and an extracellular domain [12]. After binding of its α-receptor, CD130 will associate, form homo- or heterodimers and become phosphorylated by non-covalently bound receptor-associated janus kinases (Jak1, Jak2 or Tyk2) at specific tyrosine residues of the cytoplasmic domain. IL-6-related cytokines preferentially signal via STAT1 and STAT3 [14], or additionally STAT5 [15].

Cytokines are recognized as playing an important role in immune responses to *P. falciparum.* In severe malaria, production of pro-inflammatory cytokines (IFN-γ, IL-12, IL-6 and TNF-α) is crucial for activation of immune responses and their levels correlate with disease severity. Pro-inflammatory cytokines produced in response to *Plasmodium spp*. are thought to contribute to the systemic and organ-related malaria illnesses [16-21]. Several clinical manifestations such as periodic fever, rigors and chills are caused by peak levels of pro-inflammatory cytokines produced by the cells of the innate immune system in response to high doses of parasite components released during schizont burst [19-21]. Analysis of plasma levels of patients revealed that in those with cerebral malaria or kidney failure IL-6 and IL-6R levels were significantly higher compared to control groups. It was hypothesized that reduction of IL-6R after therapy and low CNTF expression cause or lead to cerebral malaria or renal failure [22].

Since also soluble receptors of IL-6 and CNTF are present, and given the ubiquitous expression of CD130, it seems that virtually all cells can respond to IL-6. It has been shown that CD130 present on both hematopoietic and non-hematopoietic cells varies significantly among different PBMC subpopulations depending on the cell's activation status. It was absent in resting NK cells [16-18], but on the other hand, a role in NK

cell activation measured as presence of CD54 and CD69 in response to IL-27 secreted by dendritic cells (DCs), could be assigned for CD130.

Because NK cells communicate with other PBMCs they should respond to signalling molecules provided by accessory cells due to infection. General importance of IL-2, -12 and -18 for proper NK cell activation is well-reported. Furthermore, it is acknowledged that NK cells are crucial for early immune responses to *Plasmodium ssp.* infection. However, little is known about the impact on expression of receptors of the IL-6-family in this cell type in the context of malaria and also apart from that. Reported CD130 cross talk with interferon signalling suggested influence of IFN-α on IL-6 signalling [23]. These observations led us to investigate whether NK cells can be stimulated to CD130, IL-6R and/or CNTFR-α expression directly by *P.falciparum*-infected erythrocytes or by cytokines normally provided by accessory cells during infection to determine their respective role in malaria in either balancing or supporting effects of pro-inflammatory cytokines, especially IL-6. To test our hypothesis we utilized NK92 cells as a repeatable model system.

2. Results

2.1. NK92 up-regulate transcription and surface expression of gp130 after IFN-α stimulation

To detect surface expression of gp130, NK92 cells were analysed by flow cytometry. Examples of representative flow cytometry diagrams are shown in Figure 1. Not stimulated cells (ns) were compared to NK92 stimulated with IL-2/-12 and -18 (IL), IL-6, IFN-α (IFNa) or u/iRBC.

After 24 hours, expression of the β-receptor gp130 was only significantly up-regulated after IFN-α-treatment compared to not stimulated NK cells (Fig.1, paired student´s t-test, $p < 0.05$). For all other treatments no significant changes in gp130 surface expression could be observed. Since IL-6 signals via gp130 this result was at least not expected for IL-6. Following incubation for 24 h in normal growth medium without IL-2, between 0.8 and 4.4 % of NK92 cells expressed gp130 (mean = 2.2 ± 1.5 %, n = 4). Gp130 expression levels did neither increase with IL-mixture (mean = 3.6 ± 2.7 %, n = 4), IL-6 (mean = 4.1 ± 3.9 %, n = 4) nor with u/iRBCs (means = 6.1 ± 6.5 % and 5.4 ± 4.7 %, n = 4). However after IFN-α stimulation 14.65 ± 8.2 % of NK92 cells (n = 4) were stained positive for gp130 surface expression (Fig. 2).

Since a quick effect of IL-6 stimulation is discussed, levels of gp130 surface expression were determined also after 1 h cytokine or RBC treatment. Gp130 is already up-regulated after 1 h with IL-6 (500 U/10^6 cells), but not with IL-2/-12/-18 or u/iRBC. After IFN-α stimulation NK cells show a tendency to express more surface gp130 compared to control. However, this up-regulation turned out to be not significant. Similar results were obtained using FCR3CSA-iRBC for stimulation (data not shown).

To verify whether the detected effects would also be reflected on transcription level, RNA was extracted from 24 h stimulated and not stimulated NK cells and reverse-transcribed. Expression of gp130 was compared to the house-keeping gene β-Actin by qRT-PCR. As shown in Tab.1, almost no up-regulation was found after 24 h stimulation with different interleukins (IL-2/-12/-18 = 1.56 ± 0.39-fold and IL-6 = 1.34 ± 0.08-fold). Following incubation with iRBC a 2.13 ± 0.23-fold up-regulation was detected. However, also uRBCs increased gp130 expression 2.44 ± 0.18-fold. For IFN-α, a 2.30 ± 0.20-fold increase was observed. These results confirm on the one hand the flow cytometry measurements, as 24 h incubation with IFN-α but not with IL-6 led to higher expression of gp130 in NK cells. On the other hand, an equivalent effect of non-autologous RBCs could be observed, which seems to slightly decrease when those erythrocytes are infected with *P.falciparum*.

Tab. 1: fold-change in gene expression of gp130 in NK92 cells after 24 hours stimulation

stimulation	Fold change
IL	1,56 ± 0.39
IL-6	1,34 ± 0.08
IFN-α	2,30 ± 0.20
uRBC	2,44 ± 0.18
iRBC	2,13 ± 0.23

Abbreviations: IL: IL-2 (10ng7ml), IL-12/-18 (100U/ml); IL-6 (500 U/10^6 cell)s; IFN-α (500 U/10^6 cells); u/iRBC: un/infected erythrocytes (1:3 NK:RBC)

2.2. Up-regulation of CNTFR-α on the surface of NK92 after interleukin stimulation

In contrast to gp130, CNTFR-α was significantly up-regulated on the NK92 surface after 24 h stimulation with IL-2, -12 and -18 (50.13 ± 23.15 %, n=3, $p<0.05$) compared to control (16.43 ± 5.58 %, n = 3) and iRBC-stimulated cells (14.30 ± 8.32 %, n = 3). After 24 h treatment with the IL-mix approximately half of the NK92 were provoked to express CNTFR-α at their surface. IL-6 and IFN-α also led to more surface expression of CNTFR-α however, these up-regulations were not significant (IL-6: 42.43 ± 20.83 %, n =3; IFN-α: 30.87 ± 23.33 %, n = 3). Interestingly, again a slight reduction after iRBC contact was detected for the α-receptor of CNTF, while uRBC yielded a higher expression. In the case of stimulation with *P.falciparum*-iRBC, 14.3 % were stained positive, while for the uRBC control 23.50 ± 12.80 % could be assigned positive for CNTFR-α surface expression (Fig. 3).

2.3. Effects on cytosolic abundance of CNTFR-α in NK92 cells after cytokine stimulation

After 24 h stimulation, NK92 cells were lysed and 10 µg cytosolic protein extract was separated by gelelectrophoresis. As demonstrated in Fig. 4A, the NK cells generated more cytosolic CNTFR-α when stimulated with IFN-α, less with IL-mix and equal amounts with IL-6. The obtained result reflects the surface expression, since it seems to be *vice versa* to the detected amounts in the NK cell cytosol. All 3 stimulations resulted in more gene expression of CNTFR-α, but not necessarily more protein exposed at the surface. We conclude that the transport to the cell surface might be differently regulated for the applied stimuli. While with IFN-α, the protein is somehow

retained inside the cells, after interleukin stimulation, especially with the mix of IL-2, -12 and -18 more protein is transported to the cell surface.

For determination of the influence of *P.falciparum*-infected erythrocytes on the abundance of CNTFR-α in the NK92 cytosol, a time kinetics experiment was performed. Cytosolic protein extracts of untreated NK92 and NK92 stimulated with IL-12/-18, uRBC or iRBC were obtained after 3, 6, 12 and 24 h incubation. Compared to control, no differences in abundance could be detected until 12 h of stimulation. After 24 h, a slight up-regulation of CNTFR-α inside the NK cells was observed for the samples treated either with IL-12/-18 or iRBCs (Fig. 5).

2.4. IFN-α induces significant up-regulation of CNTFR-α transcription levels but not increases surface expression

As stated above, flow cytometry analysis of 24h-stimulated NK92 cells demonstrated no significant changes in surface expression of CNTFR-α. However, gene expression levels between IFN-α stimulated NK cells were significantly higher compared to NK cells incubated with IL-2, -12 and -18 or iRBC. Surprisingly, again non-autologous uRBC had a significant influence on CNTFR-α transcription. For this situation a mean of a 2.2-fold change ($n=4$, $p< 0.01$) was detected. NK cells stimulated with IL-2, -12 and -18 for 24 hours reduced transcription almost 4-fold ($n=3$, mean = 0.28, $p<0.01$). This seems interesting since more CNTFR-α is presented at the surface after IL treatment. Somehow, the expression points to a stopping of signal which will occur before 24 h of stimulation. Transcription levels decrease to a lesser degree after challenge with *P.falciparum*-infected erythrocytes ($n=3$, mean = 0.41, $p<0.01$). In contrast to the interleukin mix stimulus, this does not represent a termination of

response to parasite-induced signal transduction, because also reduced surface expression was observed compared to untreated NK cells (Fig. 4B).

2.5. IL-2, -12 and IL-18 but not IL-6 change surface expression pattern of IL-6R

Similar to CNTFR-α, surface expression of IL-6R is only significantly altered after 24h stimulation with a mix of IL-2, -12 and -18. Again, a slight trend to higher surface expression was detected for one day of IFN-α challenge. However surprisingly, IL-6 neither after 1 or 24 h nor iRBC resulted in altered surface expression of the α-receptor on NK92 cells (Fig. 6A). This could be an effect of already sufficient expression, so that no further up-regulation is required for exerting IL-6 signalling.

2.6. Transcription levels of IL-6R are highly reduced after cytokine and RBC stimulation

Determination of changes in transcription revealed that with all the given stimuli IL-6R expression was diminished, ranging from around 1.85-fold after 24 hours co-culture with *P.f.*-infected erythrocytes or IFN-α stimulation up to 9-fold down-regulation after IL-6 treatment (Fig. 6B). The strongest effect was observed for IL-6, while also stimulation with the other interleukins yielded in 3.2-folded down-modulation of transcription levels of IL-6R. It appears that if signalling events are transduced via IL-6R the provoked signal will be terminated already far before 24h. This seems logical since gp130 is only up-regulated after 1h of IL-6 stimulation, and so otherwise abundance of the β-receptor would be limiting.

3. Discussion

If we summarize our results, we first conclude, that the α-receptors are similarly regulated by the several applied cytokines. IL-6 has surprisingly little, while IFN-α and the IL-mix had greater impact. Constitutive IFN-α cross talk for proper IL-6 signalling and also proximal localization of IFNAR-1 and gp130 was reported by Milani et al., what could explain this phenomenon [23]. The IL-mix increases surface expression but declines cytosolic abundance and transcription. This may point to a negative feed-back loop to stop the provoked signal. Response of the β-receptor gp130 is similar, but unfortunately, no cytosolic data is available for gp130 and IL-6R, since we were unable to perform western blot experiments with the commercially available antibodies. Overall, the tested cytokines have some but no great impact. They mainly induce either up- or down-regulation of ~ 2-fold, what is barely about a sensible background level of 1.5-fold. But maybe we did not depict the right time point. Here we just report a snapshot picture after 1 and 24 hours stimulation. It would be interesting to perform a time kinetics experiment. However, we chose 24 hours since proper NK cells activation measured as IFN-y production is well reported for this incubation period.

Secondly, we show that uRBC and *Pf*iRBC have even less influence thereby demonstrating that NK cells do not react to *Pf*iRBC by up-regulating receptors of the IL-6 family. Moreover, IL-6R and CNTFR-α were slightly down-regulated, however, to a lesser extent than after cytokine stimulation. In our experiments we used schizont-iRBCs to stimulate NK92 cells, what implies an erythrocyte burst and thereby release of merozoites during the course of the experiment. It is known that schizonts lead to activation of NK cells [24] and iRBC burst is related to severe side-effects in malaria correlated with peak levels of pro-inflammatory cytokines. Since balance of pro-

inflammatory and anti-inflammatory cytokines is important for the disease outcome, it was interesting to study the effects of several pro-inflammatory cytokines on NK cells. Our observations suggest that IL-6 acts in an auto-regulatory manner, stopping the induced signalling cascade itself through negative feed-back mechanisms. Interestingly, some confounding regulation due to non-autologous uRBCs needs to be taken into consideration since they manipulate results that are not directly linked to *Plasmodium falciparum* infection, but when not run as a control could mislead interpretation of results. This effect was observed in other studies, too, that are currently performed (own observations).

Conflicting results exist regarding IL-6R expression in NK cells. Oberg *et al.* describe the complete lack of IL-6R and gp130 on resting NK cells of healthy donors [16]. The same was reported by a different study that found that IL-6 receptor expression was not detectable on NK cells in normal blood of rhesus monkeys [11]. This is in contradiction to our presented results, where we were able to detect IL-6R on the surface of NK cells, either stimulated or not. Apart from that, Rabinowich *et al.* reported response of NK cells to IL-6 [25]. Additionally, there are some reports demonstrating that IL-6 stimulates NK activity of human CD3-LGL in the absence of T cells [26,27] and LAK activity of IL-2 cultured human PBMC [28]. Luger et al. concluded that IL-6 enhanced NK cells´ cytotoxicity by subsequent IL-2 production [26]. That we could show, but other authors not, the presence of IL-6R can be due to our approach of using a cell line, which in some aspects will not behave as a normal resting NK cell. NK92 cells already produce high amounts of IFN-γ and express the activation marker CD25 on their surface under normal culture conditions [29]. This implies that they are somehow pre-activated. Furthermore, the lack of detection could be an effect of low template amounts for RT-PCR, since we had to adjust to proper starting quantities in order to obtain amplification.

In summary, we show some new insights into the regulation of 3 different receptors of the IL-6 family in NK cells after 1 and/or 24 hours of stimulation with several cytokines important in malaria and the disease-provoking agent *Plasmodium falciparum*. Apparently, IL-6 itself has no great effect on surface expression of gp130, IL-6R and CNTFR-α. Moreover, it mainly seems to act as a cytokine leading to termination of induced signalling events by down-regulating IL-6R expression. Furthermore, the non-signalling α-receptors were regulated by a mixture of IL-2, -12 and -18, which are activating cytokines for NK cells derived from accessory cells during early malaria infection. Additionally, the signal-transducing β-receptor gp130 was not influenced by IL-6 rather than by IFN-α. *P. falciparum* alone could not stimulate NK cells to modulate the investigated receptors´ expression. So, if these receptors play a role in the NK cell response to malaria, probably this should be effect of the tested accessory signals. To complete the picture it could be interesting to test different time points. But as in this study the main focus was on *P. falciparum*-provoked effects on NK cells, an incubation time of 24 hours seemed most interesting. Apparently, there is a balance between the pro- and anti-inflammatory cytokines. The pro-inflammatory interleukins act before IFN-α in first up-regulating IL-6 family receptors and followed by consecutive down-modulation of first, the α-receptor and later also gp130. These novel insights will help to deepen our understanding on NK cell regulation in the context of malaria and could be of interest for other infections where the tested cytokines play also a role.

4. Materials and methods

4.1. NK Cells

NK92 cells (DSMZ, Braunschweig, Germany) were grown in 75% alpha-MEM supplemented with 12.5% fetal bovine serum and 12.5% horse serum, 2 mM L-glutamine, 10ml/l penicillin – streptomycin, 10 ng/ml recombinant human interleukin-2 (rIL-2) and 5 µg/ml Plasmocin to prevent *Mycoplasma* contamination. Cultures were kept at 0.2-0.6x10^6 cells/ml up to maximum 12^{th} passage and routinely shown to be free from *Mycoplasma* by PCR.

4.2. P. falciparum culture

The *P. falciparum* strain 3D7 was maintained in continuous culture as described previously [30]. Parasites were grown in 0^+ human erythrocytes (Blood bank, University Hospital Tübingen, Germany) in RPMI 1640 supplemented with 25 mM HEPES, L-Glutamin, Gentamycin and 50 ml Albumax II. Cultures were constantly synchronized with 5% sorbitol and mature schizont-infected erythrocytes (iRBCs) were harvested by magnetic cell sorting LD columns (MACS; Miltenyi Biotec). Parasite cultures were routinely shown to be free from *Mycoplasma* by PCR.

4.3. NK92 activation with different stimuli

To exclude prior activation, NK92 cells were kept in cell medium without rIL-2 overnight. NK92 cells were either co-cultured with schizont-iRBCs and uRBCs (1:3, NK cell to RBC) or stimulated with IFN-α (500 U/10^6 cells), IL-6 (500 U/10^6 cells) or a mix of IL-2/-12/-18 (10 ng/ml IL-2, 100 ng/ml IL-12 and -18) for 1 or 24h. After the indicated time of incubation at 37ºC and 5% CO_2, lysis buffer (BD) was added to all cultures to lyse the RBCs.

4.4. Cell surface staining for flow cytometry

Cells were stained for 30 min at 4ºC in the dark with fluorochrome-conjugated antibodies to the surface markers CD56 (FITC), CD3 (PE), CD130 (PE), CD126 (PE), PE isotype control (BD Biosciences). Cells stained in the dark with mouse anti-human CNTFR-α (1 µg $IgG_{2a}/10^6$ cells) for 20 min on ice, were washed twice with FACS buffer (PBS, 1% FBS, 0.1% sodium azide) and then stained in the dark with goat anti-mouse IgG_{2a}-FITC (1 µg $IgG_{2a}/10^6$ cells, Santa Cruz) for another 20 min on ice. Dead cells were excluded by 7-AAD staining performed directly before measurements. Flow cytometry measurements were carried out using a FACScanto flow cytometer (BD Biosciences) and analysed with BD FACS Diva 6.0 software. Gating was done based on forward and sideward scatter. A total of 10.000 events were collected from each sample.

4.5. Western Blot

1 x 10^7 cells were lysed in 1 ml lysis buffer (20 mM HEPES, 250 mM NaCl, 20 % Glycerin, 1mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 % Np-40 (IGEPAL), 1 mM Dithiothreitol, and proteinase inhibitor (1 tablet complete protease inhibitor / 50ml), incubated 30 min on ice and subsequently, rotated at 13000 x *g* for 15 min at 4℃. 10 µg of total protein were boiled at 95℃ for 10 min in reducing loading buffer, separated on a 7.5 % SDS-PAGE and blotted 45 min onto a nitrocellulose membrane (Bio-Rad). Blocking was performed overnight with 4 % Bovine Serum Albumin (BSA) in 1 % PBS-Tween-20 (PBST). After washing twice with PBST the membrane was incubated with a mouse anti-human CNTFR-α monoclonal antibody (AN-H6, Becton Dickinson, 0.5 µg/ml), followed by 1h incubation with goat anti-mouse IgG linked to horseradish peroxidase (Cell Signalling) at a 1:3000 dilution. Conjugated proteins were visualized with ECL reagent (Amersham Corp.) according to manufacturer's

instructions. For control of equal protein loading, membranes were incubated in stripping buffer (62.5 mM Tris-HCl pH 6.7, 100 mM β-mercaptoethanol, 2% SDS) at 50℃ for 30 min under occasional agitation. After washing with PBST, blocking with BSA-PBST was repeated as described above and membranes were incubated with a mouse monoclonal antibody against the house-keeping protein β-actin (0.5 µg/ml ACTBD11B7, Santa Cruz), followed by immunoblot as described before.

4.6. Real-time quantitative analysis of CD130, CNTFR-α and IL-6R expression

Total RNA was extracted from 3 x 10^6 cells with RNeasy MiniKit (Qiagen) according to manufacturer's instructions. 0.5 µg RNA was converted into cDNA using Quantitect Reverse Transcription Kit (Qiagen), which includes a DNA elimination step prior to reverse transcription. For Real-time PCR the following pre-established Quantitect Primer Assays (Qiagen, Hildesheim, Germany) were used: QT00091294 (gp130), QT00022505 (CNTFR-α), QT00023660 (IL-6R), QT01192646 (β-Actin). Reaction mix was prepared according to the standard protocol of Rotor-Gene SYBR Green RT-PCR Kit (Qiagen Hildesheim, Germany). RT-PCR was carried out with Rotor Gene 3000 Cycler. The thermal profile was set as follows: 5 min at 95℃ for initial denaturation, 40 cycles of 95℃ for 10 sec and 60℃ for 15 sec with a final amplification step at 60℃ for 7 min. To exclude contamination, a non-template as well as a water control was run in parallel to each performed experiment. Specificity of products was verified by melting curve analysis and agarose gelelectrophoresis. Measurements were done in triplicate. For analysis of the obtained amplification curves, the ΔΔCt-method was applied. Ct-values above 30 were excluded and a threshold of 0.01 was set.

4.7. Statistical analysis

Analysis was performed using JMP for Windows 5.0.1 (SAS Institute Inc., Cary, North Carolina) running on Windows XP (Microsoft Corp., Redmond, Washington). The level of significance was set at p-value <0.05.

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Figures

Figure 1: Surface expression of gp130 on NK92 cells after 24 hours stimulation. NK92 were co-cultured with infected/uninfected erythrocytes in a 1:3 ratio or stimulated with IL-6 (500 U/ml), IFN-α (500 U/ml) or a mixture of IL-2 (10 ng/ml), IL-12 (100 ng/ml) and IL-18 (100 ng/ml). Surface expression was analysed by flow cytometry and compared to untreated cells (NA). As shown, only IFN-α treatment leads to significant up-regulation of gp130 surface presentation. Experiments were done in triplicate or quadruplicate.

Figure 2: Representative flow cytometry diagrams of NK92 cells after 24 hours stimulation. NK92 were co-cultured with infected/uninfected erythrocytes in a ratio of 1:3 or stimulated with IL-6 (500 U/ml), IFN-α (500 U/ml) or a mixture of IL-2 (10 ng/ml), IL-12 (100 ng/ml) and IL-18 (100 ng/ml). Surface expression of gp130 was analysed by flow cytometry and compared to untreated cells (NA). NK cells were gated according to SSC, FSC and absence or presence of CD3 and CD56, respectively. Autofluorescence and isotype-matched controls wer run in parallel to determine negative and positive populations. As shown, only IFN-α treatment leads to significant up-regulation of gp-130 surface presentation. Experiments were done in triplicate or quadruplicate.

Figure 3: Surface expression of CNTFR-α on NK92 cells after 24 hours stimulation. NK92 cells were co-cultured with infected/uninfected erythrocytes or stimulated with IL-6, IFN-α or a mixture of IL-2, IL-12 and IL-18. Surface expression was analysed by flow cytometry and compared to untreated cells (NA). As shown,

only IFN-α treatment leads to significant up-regulation of gp-130 surface presentation. Experiments were performed in triplicate.

Figure 4: Influence of cytokines and *P.falciparum* on cytosolic abundance and gene expression of CNTFR-α in NK92 cells after 24 hours stimulation. (A) NK92 cells were left untreated (ns) or cultured with different cytokines such as a mixture of IL-2 (10 ng/ml), IL-12 (100 ng/ml) and IL-18 (100 ng/ml), IL-6 (500U/ml), IFN-α (500U/ml). 10 µg of cytosolic protein extract of untreated and stimulated NK cells were separated on a 7.5% SDS-PAGE and blotted onto a nitrocellulose membrane that was incubated 1h with a mouse anti-human monoclonal antibody (clone AN-H6) against CNTFR-α, followed by 1h incubation with goat anti-mouse IgG-HRP. After 24 hours stimulation the amount of CNTFR-α is significantly increased compared to non-stimulated NK cells. For the other treatments no effect was detected. (B) NK92 cells were incubated with different cytokines such as a mixture of IL-2 (10 ng/ml), IL-12 (100 ng/ml) and IL-18 (100 ng/ml), IL-6 (500U/ml), IFN-α (500U/ml) or co-cultured with u/iRBC (ratio 1:3). RNA was isolated after 24 hours and reverse-transcribed. RT-PCR was performed for the gene of interest CNTFR-α and actin-β as housekeeping gene. Fold changes were calculated in comparison to untreated cells applying the $\Delta\Delta C_T$ method.

Figure 5: Cytosolic abundance of CNTFR-α in NK92 cells after 0, 6, 12 and 24 hours stimulation. NK92 cells were left untreated (NA), co-cultured with infected(i)/uninfected erythrocytes (u) in a ratio 1:3 or stimulated with a mixture of IL-12 and IL-18 (IL). 10 µg of cytosolic protein extract of untreated and stimulated NK cells were separated on a 7.5% SDS-PAGE and blotted onto a nitrocellulose membrane, that was incubated 1h with a mouse anti-human monoclonal antibody

(clone AN-H6) against CNTFR-α, followed by 1h incubation with goat anti-mouse IgG-HRP. Only after 24 hours a slight difference in protein abundance is seen for IL and iRBC stimulation compared to unstimulated NK cells. No effect of uRBC was observed.

Figure 6: Influence of cytokine and *P.falciparum* stimulation on surface and gene expression of CD126 (IL-6R) in NK92 cells after 24 hours stimulation. NK92 were co-cultured with infected/ uninfected erythrocytes in a 1:3 ratio or stimulated with IL-6 (500 U/ml), IFN-α (500 U/ml) or a mixture of IL-2 (10 ng/ml), IL-12 (100 ng/ml) and IL-18 (100 ng/ml). (A) Surface expression was analysed by flow cytometry and compared to untreated cells (NA). As shown, results are quite heterogenous ranging from 10-90% surface exposure under normal condition. However, in all 3 experiments an up-regulation was seen after 24 hours IL mixture stimulation. (B) Transcriptional changes due to applied stimuli revealed significant down-modulation of IL-6R expression for all treatments ranging from around 1.85-fold after 24 hours co-culture with *P.f.*-infected erythrocytes or IFN-α stimulation up to 9-fold down-regulation for the IL-6 treatment.

FIGURE 1

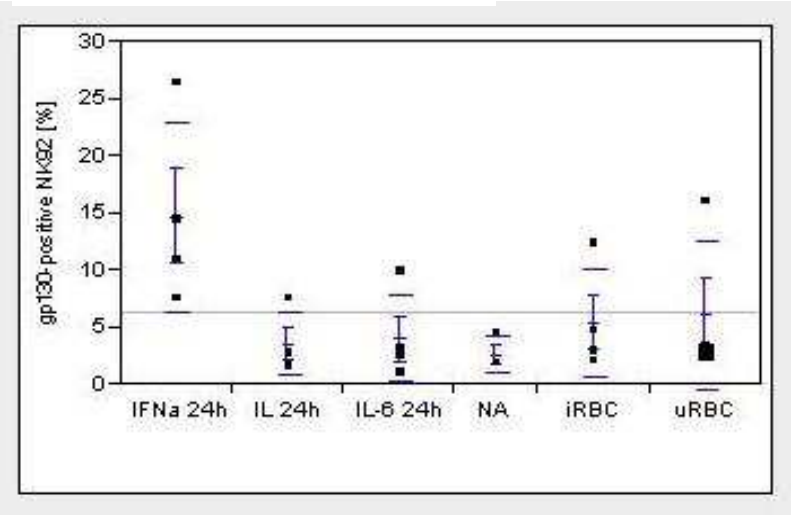


FIGURE 2

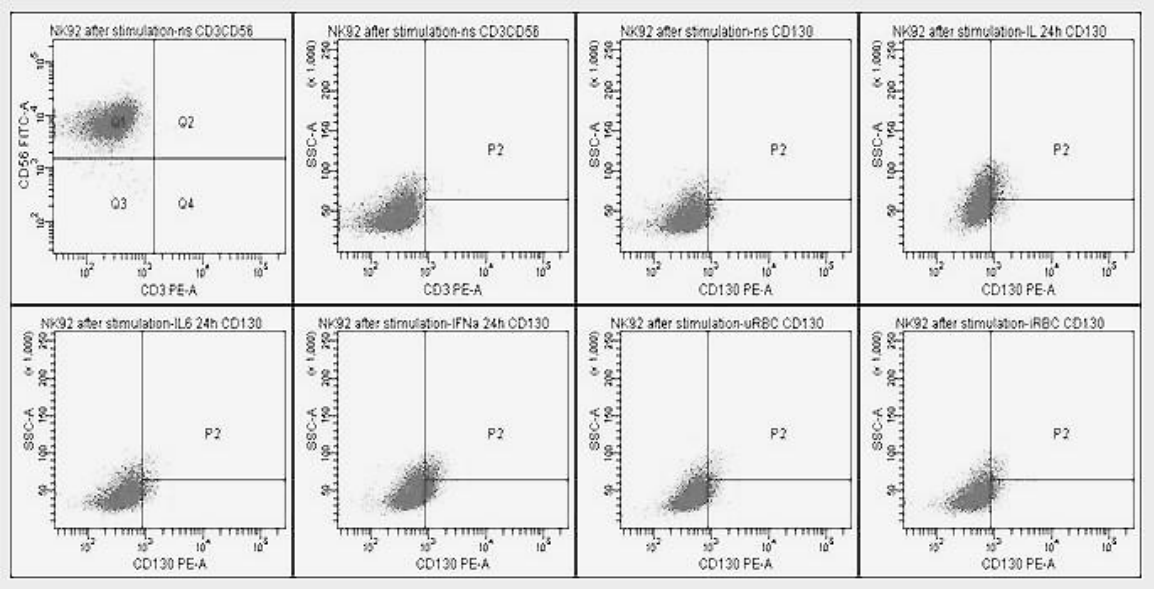


FIGURE 3

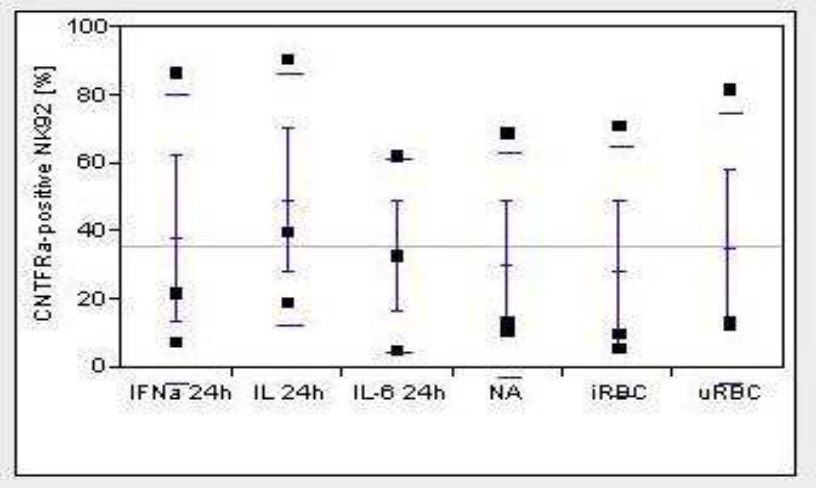


FIGURE 4

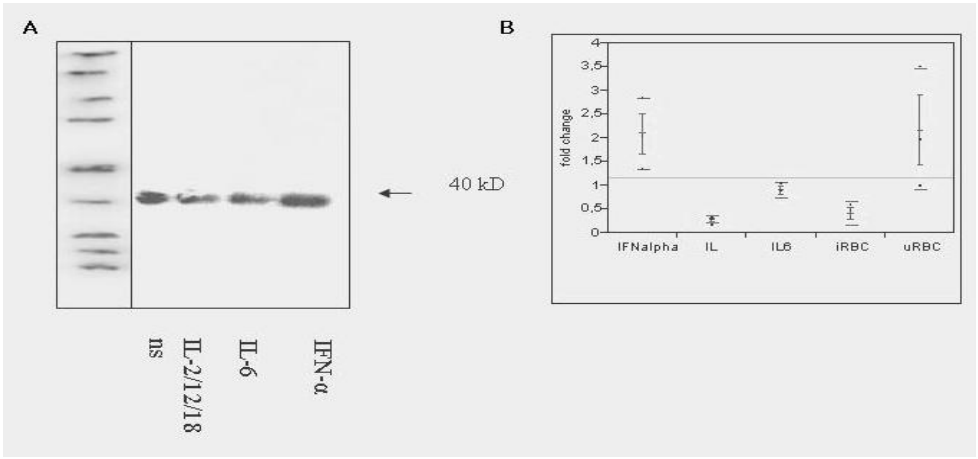


FIGURE 5

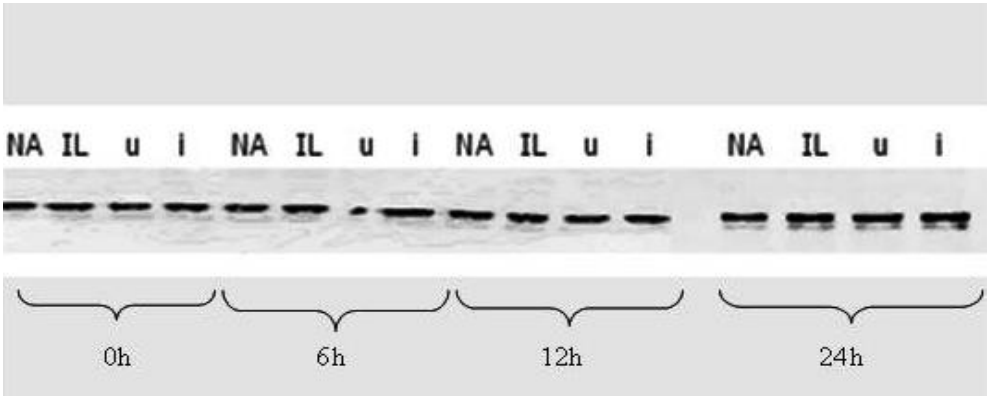
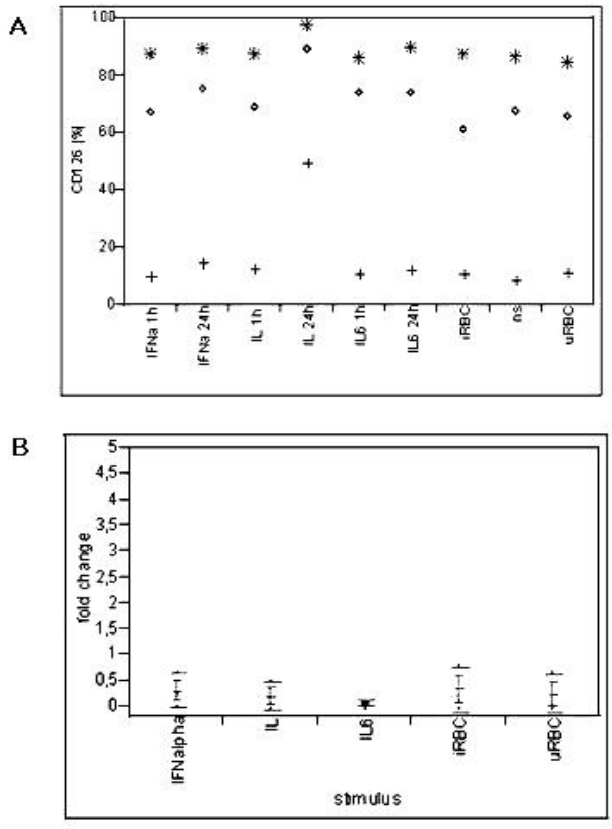


FIGURE 6



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PLoS one

Plasmodium falciparum-Infected Erythrocytes Induce Granzyme B by NK Cells through Expression of Host-Hsp70

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Abstract

In the early immune response to *Plasmodium falciparum*-infected erythrocytes (iRBC), Natural Killer (NK) cells are activated, which suggests an important role in innate anti-parasitic immunity. However, it is not well understood whether NK cells directly recognize iRBC or whether stimulation of NK cells depends mainly on activating signals from accessory cells through cell-to-cell contact or soluble factors. In the present study, we investigated the influence of membrane-bound host Heat shock protein (Hsp) 70 in triggering cytotoxicity of NK cells from malaria-naïve donors or the cell line NK92 against iRBC. Hsp70 and HLA-E membrane expression on iRBC and potential activatory NK cell receptors (NKG2C, CD94) were assessed by flow cytometry and immunoblot. Upon contact with iRBC, Granzyme B (GzmB) production and release was initiated by unstimulated and Hsp70-peptide (TKD) pre-stimulated NK cells, as determined by Western blot, RT-PCR and ELISPOT analysis. Eryptosis of iRBC was determined by Annexin V-staining. Our results suggest that presence of Hsp70 and absence of HLA-E on the membrane of iRBC prompt the infected host cells to become targets for NK cell-mediated cytotoxicity, as evidenced by impaired parasite development. Contact of iRBC with NK cells induced release of GzmB. We propose that following GzmB uptake, iRBC undergo eryptosis via a perforin-independent, GzmB-mediated mechanism. Since NK activity toward iRBC could be specifically enhanced by TKD peptide and abrogated to baseline levels by blocking Hsp70 exposure, we propose TKD as an innovative immunostimulatory agent to be tested as an adjunct to anti-parasitic treatments *in vivo*.

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Introduction

Malaria still remains one of the most devastating infectious diseases world-wide causing ~655.000 deaths per year, regardless of great efforts in the past years. Immunity against *P. falciparum*, the most dangerous malaria-provoking agent, develops with age over the course of multiple infections and it has been shown that humoral immunity is crucial for protection from severe disease. It is known that Natural Killer (NK) cells play a pivotal role in early innate immune responses by secreting interferon-gamma (IFN-γ) but also via cross-talk and priming of adaptive immunity [1,2]. Effector functions are triggered by cytokine secretion from and through direct contact with myeloid accessory cells [3,4]. Originally, NK cells were described as non-specific cytotoxic effector cells killing their target cells without prior sensitization [5,6]. Nevertheless, during the last years it became apparent that NK cells are more sophisticated. Their function is regulated by numerous inhibiting and activating receptors [7] interacting with a set of different ligands, e.g. stress proteins and MICA/B [8]. Furthermore, it is well-known that they become activated by cells missing MHC class-I molecules on their cell surface [9]. Interestingly, mature erythrocytes do not express MHC, but usually are not eliminated by NK cells. Therefore, either additional signals triggering NK cell-mediated cytotoxicity towards infected erythrocytes (iRBC) must exist or erythrocytes express further inhibiting ligands. Since erythrocytes are host cells for *P. falciparum* and are crucial for replication and growth of the parasite during bloodstage infection investigation of the interplay of iRBC and NK cells may be important to discover protective factors during the first phase of infection especially in age groups where semi-immunity has not yet developed. Several, studies showed that cross-talk between NK cells and iRBC results in activation of NK cells [10–12]. Therefore we address the question how NK cells sense the intracellular parasite and how they react after recognizing iRBC.

Recruitment of host-Hsp70 to the RBC membrane by *P. falciparum* was shown previously [13]. Hsp70 was demonstrated to represent a potent activator for NK cell cytotoxicity, especially when expressed on cancer cells [14–16]. Intracellularly, it serves as a chaperone to assist proper folding of aberrant and nascent proteins, thereby preventing apoptosis [17]. Other groups recently proposed extracellular Hsp70 as a cytokine and danger signal [7,18]. Hsp70 was suggested to either serve as an antigen-presenting cell-activating cytokine or as a carrier for antigenic peptides to the cell surface [17]. Multhoff *et al.* discovered that a 14-amino acid oligomer (TKD peptide), localized in the C-

terminal domain of Hsp70, represents an epitope recognized by activated NK cells [19]. Binding of NK cells to this epitope results in GzmB-mediated but perforin-independent apoptosis of tumor target cells [20]. Based on these findings, we addressed the questions whether iRBC express Hsp70 or other activating NK cell ligands on their cell surface, and whether iRBC are eliminated by NK cells in a GzmB-mediated manner by erythrocytic cell death. Therefore, we firstly investigated the expression of Hsp70, MICA/B, and HLA-E present on the surface of iRBC, and secondly whether the presence of one or more of these ligands impacts the expression of activating receptors such as CD94/NKG2C on NK cells. We were also interested to test whether NK cells respond to iRBC by an up-regulated expression and release of GzmB, whether perforin is involved, and finally, if NK cell activity can be further enhanced by prior stimulation with TKD and abrogated by blocking Hsp70-membrane presence.

Results

Co-culture of NK cells and iRBC induces growth delay of *P. falciparum*

As determined by co-culture experiments, NK cells could delay the growth or induced crisis forms of parasites, represented by packed dot-shaped forms (Figure 1C). However, in the absence of lymphocytes Plasmodia developed normally from schizonts (Figure 1A) into ring-stage trophozoites after 24 h of incubation (Figure 1B). When NK cells were pre-incubated with TKD peptide, no further visible effect was observed (Figure 1D). As shown in Figure 1E, non-stimulated PBMCs had minor influence on the development of the parasite within a 24 h co-culture period. The majority of the parasites developed normally into ring-stage parasites. Interestingly, a 5-day pre-stimulation of PBMCs with TKD peptide also induced crisis forms of Plasmodia (Figure 1F). Figure 1G shows that predominantly NK cells had an effect on parasitemia and parasite development during the 24 h co-culture period. Crisis forms were significantly induced by co-culturing iRBC with NK cells alone compared to co-culture with PBMCs ($p \leq 0.001$, student's t test, $n = 3$). A significant difference could also be detected between PBMCs ($8.9 \pm 3.6\%$) as well as untreated iRBC ($5.0 \pm 8.7\%$) compared to PBMCs pre-stimulated with TKD (PBMC+TKD; $27.8 \pm 8.9\%$, $p \leq 0.001$, student's t test, $n = 3$). The proportion of crisis forms of iRBC co-cultured with unstimulated NK cells was already very high ($85.7 \pm 4.9\%$) and could not be significantly enhanced by using pre-activated NK cells (NK+TKD; $94.0 \pm 4.3\%$) (Figure 1E).

Hsp70 but neither HLA-E nor MICA/B is present on the membrane of ring-stage infected and senescent RBC

Since we could demonstrate that NK cells had a direct influence on parasite growth, we were interested in identifying the interaction partners of NK cells and iRBC. Therefore, the expression of Hsp70, HLA-E and MICA/B was determined by flow cytometry on iRBC and uRBC following co-culture with NK92 cells. On iRBC neither MICA/B nor HLA-E was present on the membrane (Figure 2A+B). To investigate the presence of Hsp70 on RBC, membranes of i/uRBC were stained with cmHsp70.1-FITC and analyzed by flow cytometry. Parasite DNA was stained with Hoechst or Hydroethidine to distinguish iRBC from uRBC. Hsp70 was detectable on ring-stage iRBC (stained with Hydroethidine) by flow cytometry as demonstrated in Figure 2E, but not on uRBC (Figure 2C). On schizont-iRBC (stained with Hoechst) the Hsp70 signal was not as prominent as on ring–stage iRBC (Figure 2D).

To confirm these results, we assessed if host-Hsp70 is present in membrane lysates of iRBC. Protein extracts were derived from the cytosol and membrane of iRBC and uRBC. In a first attempt senescent uRBC were used. Surprisingly, Hsp70 was detectable in both infected and uninfected membrane preparations of senescent RBC (Figure 3A). When using fresh RBC only iRBC presented Hsp70 on their membrane (Figure 3B). This finding resulted in the exclusive use of fresh RBC for all other experiments.

Characterization of cell surface markers on NK92 cells

In search for interaction receptors on NK cells for iRBC, the expression of surface receptors on NK92 cells was investigated. As shown, NK92 cells in the absence or presence of iRBC do express CD94 (Figure 4B) but not the activatory co-receptor NKG2C (Figure 4A) on their cell surface. Uninfected RBC had no impact on the surface expression of CD94 or NKG2C in NK92 cells. A slight up-regulation of NKG2C was observed when NK92 cells

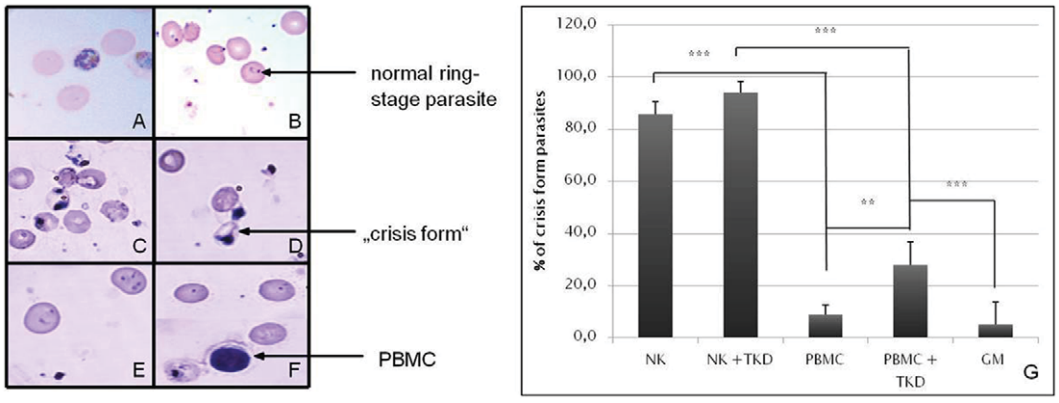

Figure 1. Growth delay in *Plasmodium falciparum* development after NK cell contact. A: Representative figure of 3D7-iRBC before the start of co-cultures. Parasites were synchronized by magnetic cell sorting columns for late stages. B: Representative figure of 3D7-iRBC after 24 h of incubation. The parasites have developed into normal ring-stage forms that are expected for this time point. Infected RBC were either co-cultured 3:1 with autologous NK cells (C), NK cells+TKD (pre-stimulated for 5 days before co-culture with 2 μg/ml TKD peptide) (D), PBMCs (E), or PBMCs stimulated with TKD peptide (F). Normal parasite development of a control culture of parasites without leukocytes was observed in parallel. A blood smear was prepared before and after 24 hours of incubation and stained with 10% Giemsa. Experiments were repeated with RBC from 3 different donors.

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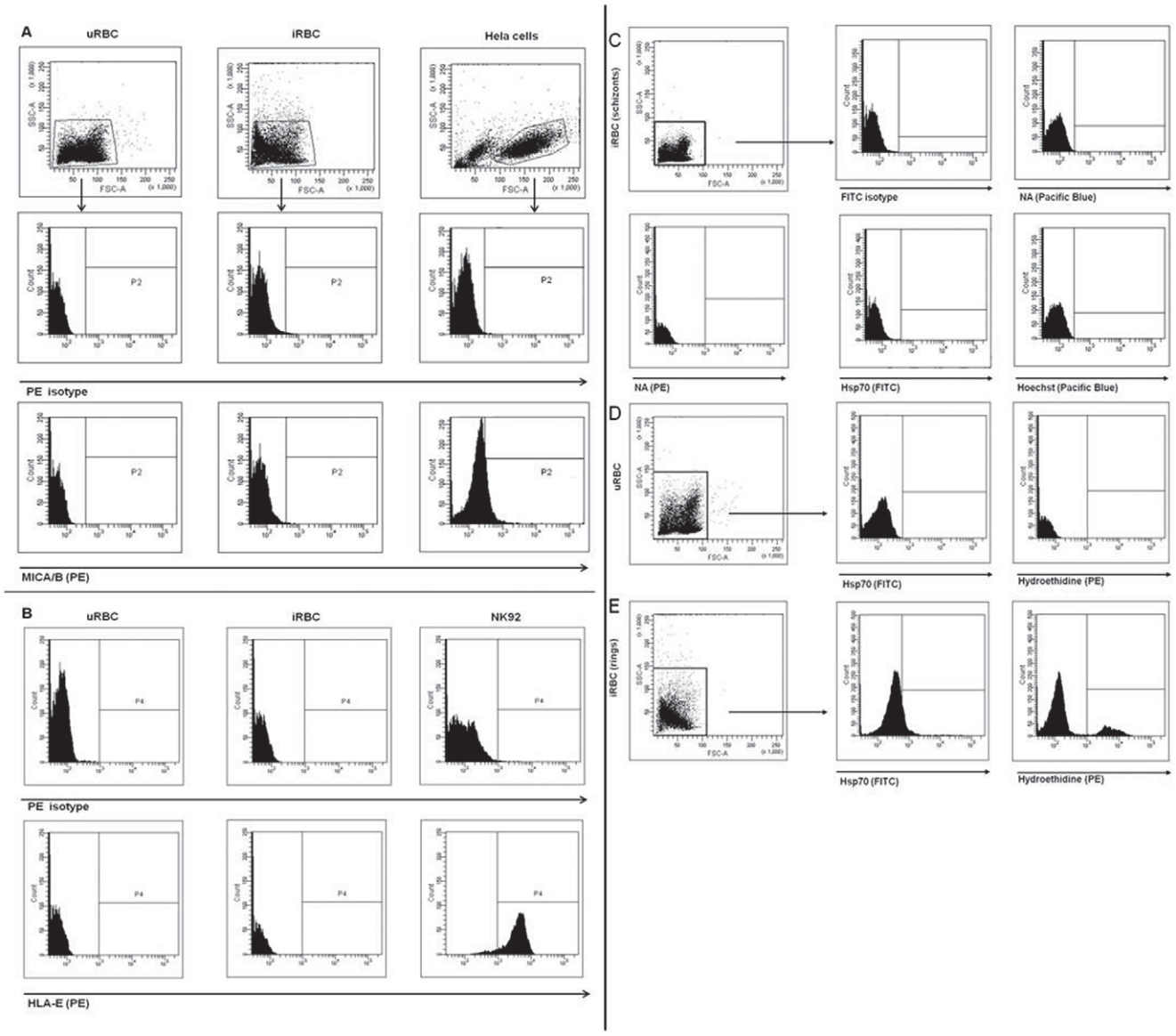

Figure 2. Flow cytometry analysis of erythrocytes for possible NK cell ligands. 0.5×10^6 i/uRBC or Hela cells were stained with anti-hMICA/B-PE (A), anti-hHLA-E-PE (B) or the respective isotype control. As a control, 0.5×10^6 iRBC, uRBC or NK92 cells were also stained with anti-hHLA-E-PE (B) or a PE-isotype control. The presence of Hsp70 on uRBC (C) and iRBC (D, E) was determined with anti-Hsp70 mAb cmHsp70.1-FITC compared to FITC isotype. Parasite DNA was stained with Hoechst dye (schizonts, D) that is detected in the Pacific blue channel or Hydroethidine (rings, E) that is detected in the PE channel. Experiments were repeated 3 times.
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were incubated with IL-12/IL-18 (3.9% $NKG2C^+$-NK92, MFI: 191) compared to cells cultured in growth medium alone (0.4% $NKG2C^+$-NK92, MFI: 104). In addition, stimulation with IL-12/IL-18 also induced elevated CD94 expression compared to unstimulated cells (75.8% $CD94^+$, MFI: 5317 vs. 36.5% $CD94^+$, MFI: 3518).

Cytosolic GzmB is increased on protein but not on transcriptional level in NK92 cells after contact with iRBC

To assess how NK cells respond to iRBC, changes in mRNA levels of GzmA, GzmB and perforin were analyzed. NK92 cells were stimulated either with IL-2/IL-12/IL-18, IFN-α or co-cultured with i/uRBC for 24 hours (1:3). Compared to untreated NK cells, GzmB up-regulation was clearly induced when NK cells were cultured in the presence of IL-2/IL-12/IL-18 ($p<0.01$, student's *t* test), whereas increased expression of perforin was especially observed upon incubation with IFN-α ($p<0.05$, student's *t* test). Slight up-regulation of GzmB was also observed after contact of NK92 cells with iRBC (1.5-fold change). Although this effect was significantly different to that of unstimulated NK92 cells ($p<0.05$, student's *t* test), it cannot be considered as such since no significant differences were observed between iRBC and uRBC. None of the stimuli used did significantly affect the transcription of GzmA (Figure 5A).

To further explore effects of *P. falciparum* on the protein levels of GzmB in NK cells, cytosolic protein extracts of NK92 cells were generated after a 24 h co-culturing period with i/uRBC or following stimulation with IL-12/IL-18. Compared to unstimulated NK cells, a clear up-regulation of GzmB protein levels was detected upon stimulation of NK92 cells with IL-12/IL-18 and to

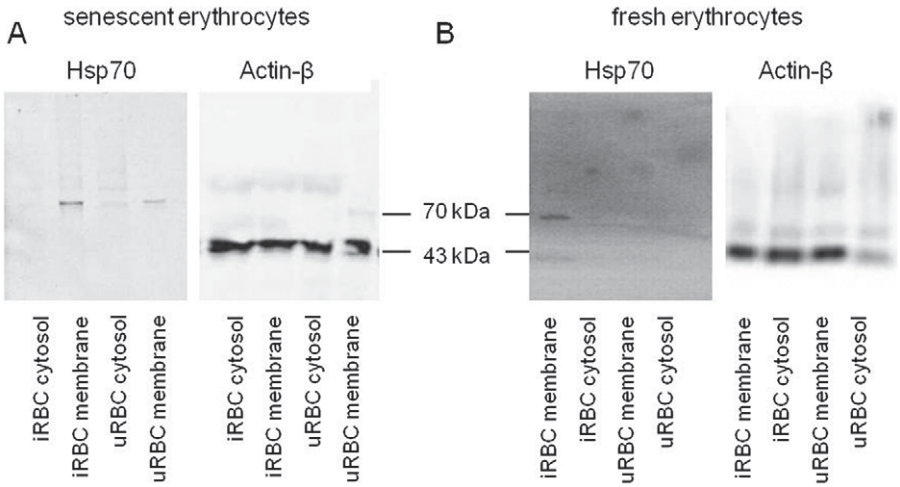

Figure 3. Presence of Hsp70 in the membrane of iRBC or senescent uRBC. Cytosolic and membrane protein extracts were prepared from iRBC and uRBC and submitted to SDS-PAGE. Western blots were incubated with anti-Hsp70 antibody or anti-β-Actin to control protein loading. Experiments were repeated 3 times. A: representative immunoblot of 3–4 week old erythrocytes B: representative immunoblot of fresh blood erythrocyte extracts.
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a lesser extent after contact with iRBC but not with uRBC (Figure 5B). No significant influence on GzmA and perforin could be detected for the different treatments at a translational level (Figure 5B).

Contact of NK cells with iRBC results in increased GzmB release which is inhibited by blocking Hsp70-surface expression

Considering that activated NK cells kill Hsp70 membrane positive tumor cells by GzmB-mediated apoptosis and having observed up-regulation of GzmB protein expression, we assessed the effect of parasitized erythrocytes on GzmB release of NK cells. Freshly isolated NK cells, PBMCs or NK92 cells kept for 5 days in the presence of IL-2 +/− TKD peptide were co-cultured for 24 h with i/uRBC at two different ratios, 1:3 or 10:1 (NK:RBC). Previously, it was shown that NK cells exert their cytotoxic action towards Hsp70 membrane positive tumor cells in a conjoint manner (G. Multhoff, unpublished results). However, in *in vitro* culture of *P. falciparum* a ratio of 1:3 seems to be sufficient for proper activation of NK cells. Hsp70-antibody blocking studies were performed to confirm the target specificity of the killing. In a first set of experiments, we observed that NK92 cells significantly increased GzmB release compared to untreated cells after 5 day pre-incubation with TKD peptide ($p \leq 0.001$, student's *t* test, $n = 6$) irrespectively of further stimuli such as iRBC or uRBC (Figure 6A). Similar results were obtained for primary NK cells (Figure 6B). However, the difference of NK92 cells co-cultured with iRBC was not significantly altered in comparison to untreated cells. No significant differences in GzmB release were seen with PBMCs after an identical treatment regimen (data not shown). However, if experiments were repeated using freshly separated NK cells, significant differences could be detected (Figure 6B). Due to the allogenicity of the NK/RBC system, a basal release of GzmB by NK cells was observed, irrespectively of the co-cultivation with uRBC or iRBC (data not shown). In an autologous system, only iRBC led to a significant increase in GzmB release by autologous NK cells at both ratios compared to uRBC ($p \leq 0.05$, student's *t* test). GzmB secretion could be completely blocked down to basal levels after the addition of anti-Hsp70 blocking antibody at a ratio of 1:3 (NK:iRBC) for NK cells that were co-cultured with iRBC ($p \leq 0.05$, student's *t* test). The effect of TKD+iRBC was especially reversed by the anti-Hsp70 blocking antibody at a ratio of 10:1 ($p \leq 0.001$, student's *t* test). The specificity of the effect was verified with another set of experiments where additionally freshly isolated NK cells were pre-incubated with the scrambled NGL peptide (Figure 6C). No difference to untreated NK cells was observed. Furthermore, the specific action of the Hsp70 blocking antibody was evaluated by addition of an IgM isotype control 20 minutes prior to co-culture with u/iRBC (Figure 6C). Only when NK cells were pre-activated with TKD peptide and afterwards co-cultured with iRBC, did addition of the IgM isotype have a significant decreasing effect (student's *t* test, $p \leq 0.05$). However, the amount of GzmB-releasing cells was still significantly higher in comparison to TKD-pre-stimulated NK cells that were co-cultured with cmHsp70.2-treated iRBC (student's *t* test, $p \leq 0.05$). If no pre-stimulation was applied the abrogating effect of cmHsp70.2 was even more pronounced (iRBC+blocking mAb (ns) vs iRBC+IgM isotype (ns): student's *t* test, $p \leq 0.01$).

In summary, NK92 cells as well as freshly isolated NK cells displayed a significantly increased release of GzmB 5 days after stimulation with TKD peptide. Co-culturing of NK cells with iRBC, but not uRBC, further increased the release of GzmB. The addition of Hsp70 blocking antibody specifically abrogated the stimulatory effect induced by TKD and iRBC.

NK cells induce eryptosis of iRBC

Since we could show that Hsp70 membrane expression as well as GzmB release are important characteristics of the responses of NK cells towards iRBC, we were interested whether cell death is induced. Programmed death of iRBC was evaluated after 24 h of co-culture with either autologous PBMCs or NK cells that were pre-stimulated with/without TKD. Necrotic cells were excluded by propidium iodide staining. No spontaneous eryptosis of uRBC or iRBC was observed at the beginning of the experiment as well as 24 h later (Figure 7A). Our results demonstrate that iRBC undergo eryptosis but not necrosis following contact with NK cells (NK vs GM: $p \leq 0.001$). This effect was even more pronounced when TKD-stimulated NK cells were used (NK vs NK+TKD: $p \leq 0.01$). Also PBMCs had the capacity to induce eryptosis of

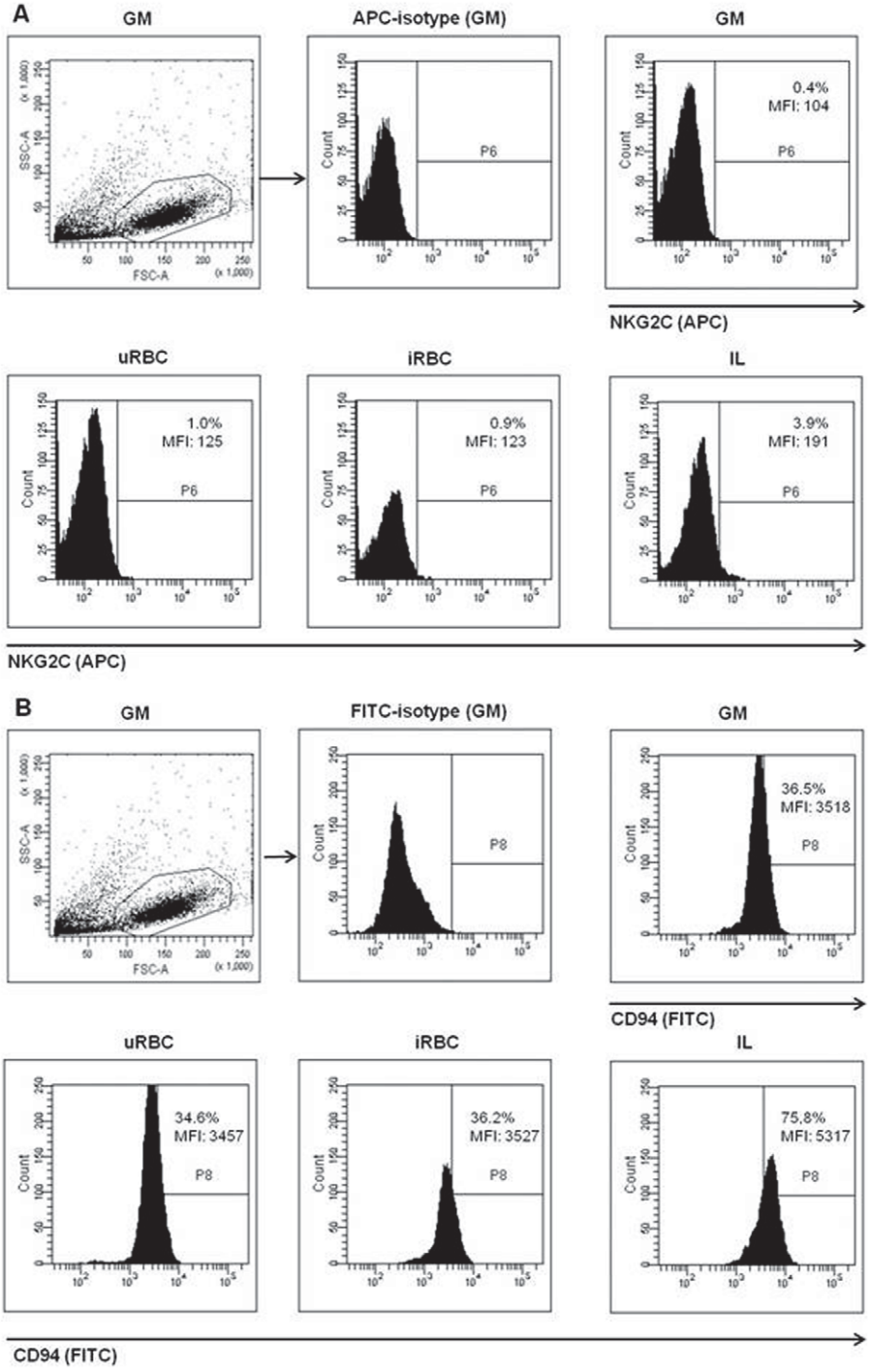

Figure 4. Surface expression analysis of possible recognition receptors of iRBC on NK92 cells. NK92 cells were analyzed by flow cytometry for surface expression of NKG2C and CD94. 0.5×10^6 NK92 cells were stained with anti-CD56-FITC, anti-CD3-PE and anti-NKG2C-APC (A) or anti-CD94-FITC (B) after 24 h incubation in growth medium (GM), with 1.5×10^6 iRBC, 1.5×10^6 uRBC, or IL-12/-18 (IL) and analyzed by flow cytometry; a total of 10,000 events was counted for each sample. $CD56^+/CD3^-$ cells were gated according to FSC/SSC properties in the unstained autofluorescence control and compared to the respective isotype controls. Experiments were repeated three times.
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iRBC, however, at a lower level (Figure 7B, $p\leq0.01$). Signs of eryptosis could also be detected by smaller cell size compared to untreated iRBC measured as reduced FSC properties of erythrocytes after co-culturing with NK cells (Figure 7C). Again, stimulation with TKD peptide enhanced the effect significantly ($n=6$, $p\leq0.05$). Addition of PBMCs or PBMCs stimulated with TKD did not significantly alter cell shrinkage (Figure 7C). The number of necrotic iRBC was always low and ranged between $0.8\pm0.8\%$ in growth medium to $1.7\pm2.0\%$ after co-culture with PBMCs+TKD (Figure 7D).

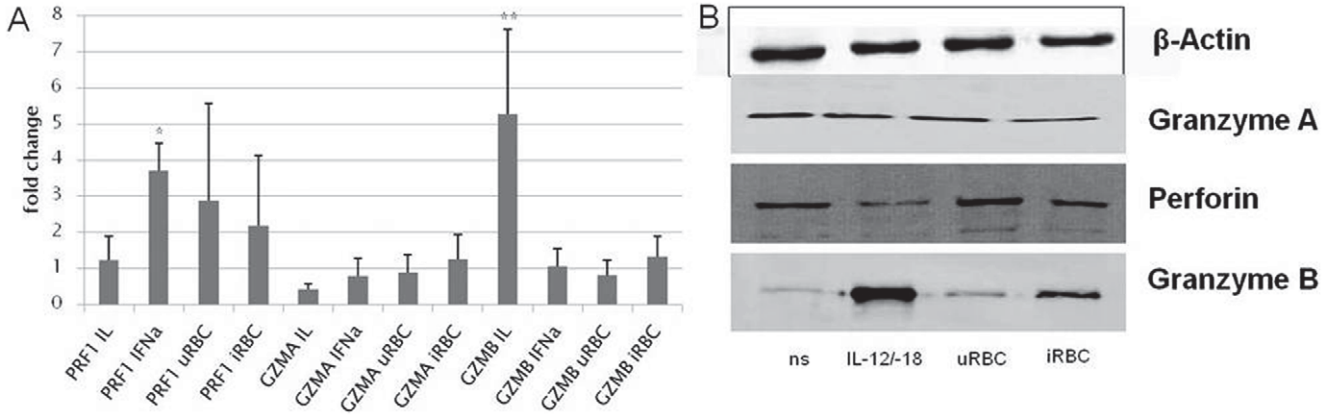

Figure 5. Transcriptional and translational changes of GzmA, GzmB and Perforin in NK92 cells after 24 hours stimulation. NK92 cells were left untreated (ns) or cultured with IL-12/-18 (IL), IFN-α, uRBC or iRBC (1:3) for 24 h. A: Changes on transcriptional level of stimulated cells in comparison to untreated cells were analyzed for GzmA, GzmB and Perforin. β-Actin expression served as house-keeping gene normalizer. Data are represented as mean ± SD (student's *t* test * $p<0.05$; ** $p<0.01$) and are representative of three independent experiments each performed in duplicate. B: After 24 h, 1×10^7 NK92 cells were lysed, incubated 30 min on ice and subsequently centrifuged at 13000× *g* for 15 min at 4°C. 10 µg of total supernatant protein were separated by 7.5% SDS-PAGE and blotted onto a nitrocellulose membrane. After blocking, membranes were incubated for 1 h with anti-β-Actin (lane 1), anti-hGzmA (lane 2), anti-hGzmB (lane 3), or anti-hPrf (lane 4).
doi:10.1371/journal.pone.0033774.g005

Discussion

In the present study, we investigated a new hypothetical pathway by which senescent (Fig. 8A) or iRBC (Fig. 8B) are recognized and marked for removal by NK cells. This model implies that upon infection with *P. falciparum* or when aged erythrocytes display Hsp70 on their surface. This results in GzmB release probably triggered by a yet unknown NK cell receptor. Pre-stimulation of effector cells with Hsp70-peptide TKD leads to an increased release of GzmB. Subsequently, GzmB will either become endocytosed, be up-taken with the help of an unknown receptor or concomitantly by internalization of Hsp70 by RBCs which finally will undergo eryptosis.

First insights into the investigated interaction were obtained by co-culture experiments. We have previously found that direct impact of NK92 cells on iRBC results in reduced parasitemia after 48 h of co-culture [21]. Additionally, we observed that NK92 cells suppress the expression of vital genes in the *P. falciparum* laboratory strain FCR3-CSA (unpublished results). However, in these experiments we could not exclude that those effects were a sign of nutrient depletion in the culture system. In the present study, we show that the observed impairment of parasite development is directly mediated by NK cells. The induction of parasite crisis form was much more pronounced in co-cultures of fresh iRBC with isolated NK cells compared to those where PBMCs were co-cultured. Because NK cells represent only 5–15% of PBMCs it seems that the number of NK cells within the PBMC fraction may be too small to efficiently affect the parasite in the iRBC. However, when PBMCs were pretreated with TKD we also found a significant increase of crisis forms within the iRBC compared to PBMCs. This could be explained either by the fact that within the PBMC fraction also other effector populations than NK cells will be activated by TKD and affect the parasites or that NK cell activation is more efficient in the presence of other immune cells upon stimulation with TKD.

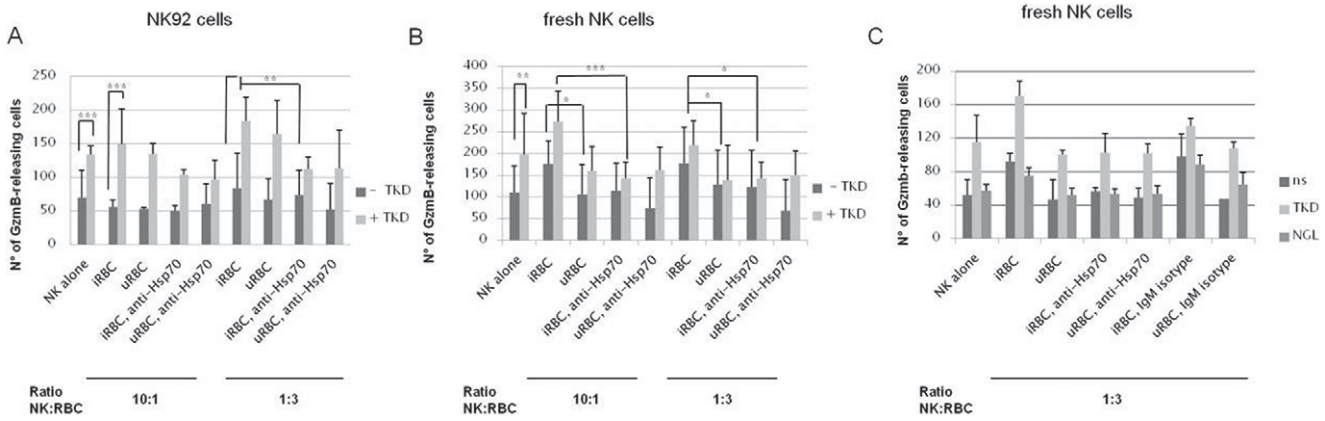

Figure 6. Granzyme B-Elispot of NK92 cells (A) or isolated NK cells (B,C) after 24 hours of co-culture with i/uRBC. Some cultures were pre-activated 5 days with TKD peptide or the scrambled NGL peptide and/or pre-incubated with blocking Hsp70 antibody (cmHsp70.2) or a blocking antibody IgM-isotype control for 20 minutes before the start of the experiment. After stimulation, 2000 NK cells were cultured either alone, with iRBC or uRBC (1:3 or 10:1) on a GzmB antibody-coated 96-well plate. GzmB-releasing cells were counted after 24 hours of incubation (* $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$, student's *t* test, n = 6).
doi:10.1371/journal.pone.0033774.g006

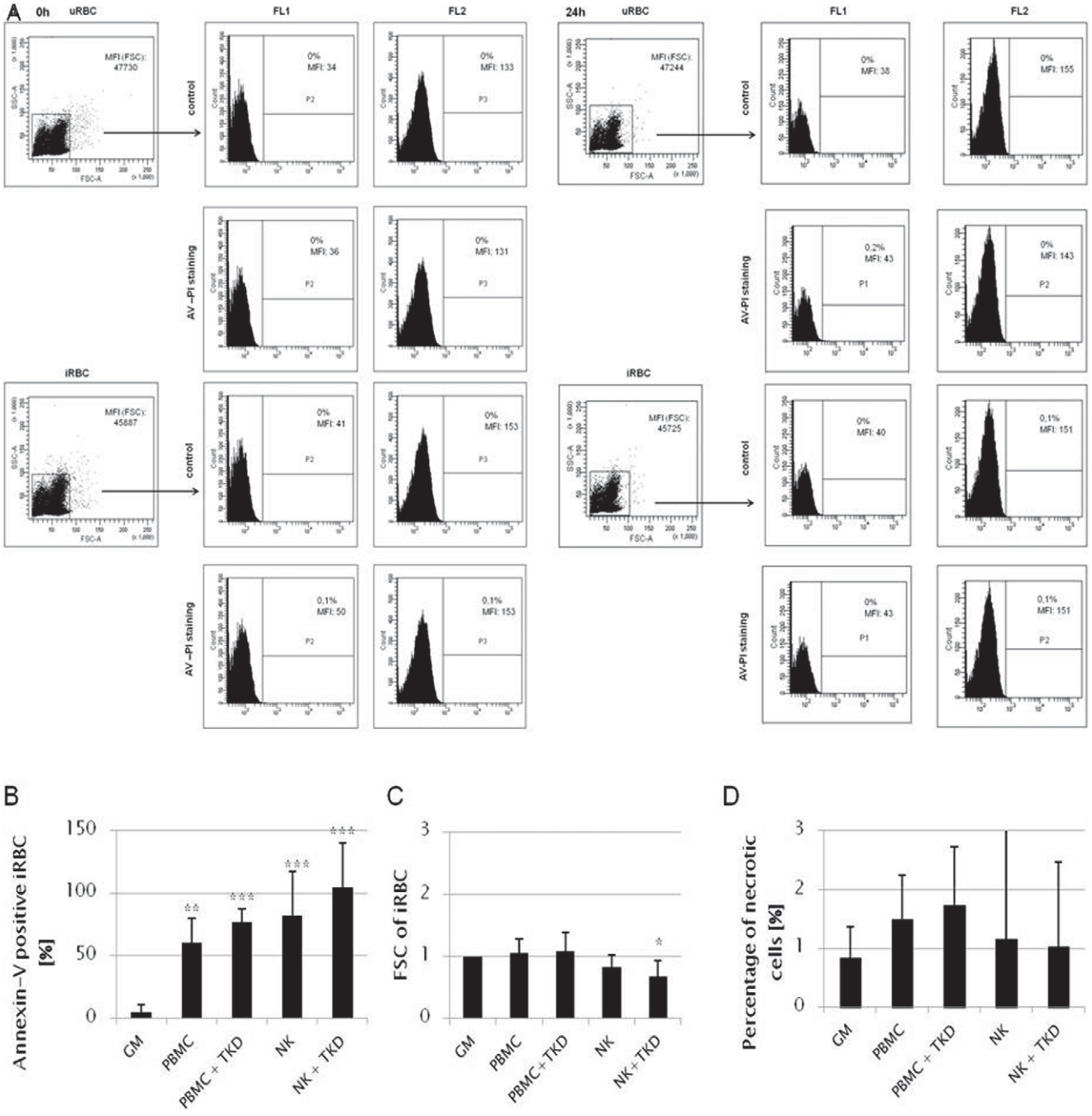

Figure 7. Eryptosis of iRBC after co-culture with PBMCs or purified NK cells. iRBC were co-cultured 3:1 for 24 hours with NK cells, NK cells+TKD (5 day stimulation), PBMC, PBMC+TKD (5 day stimulation) of the same donor. 0.5×10^6 erythrocytes were washed in 5 mM Ringer solution. Afterwards, cells were stained for 15 minutes with Annexin-V (1:500) and propidium iodide (1:50). Eryptotic cells were determined as Annexin-V-positive (AV^+) and propidium iodide-negative (PI^-). A: Baseline levels of eryptotic uRBC and iRBC at the start of the experiment and after 24 h of culture in growth medium without leukocytes. RBC were stained with AV+PI or left unstained. Gating was done based on FSC/SSC properties and the unstained control. AV was measured in the FL1 channel and PI in the FL2 channel. B: Percentage of AV^+/PI^--iRBC after co-culture in growth medium (GM) or with different effector cells in relation to starting parasitemia (** $p\leq0.01$, student's *t* test, n = 6). C: Normalized FSC of iRBC after co-culture in growth medium (GM) or with different effector cells (* $p\leq0.05$, student's *t* test, n = 6). FSC values of co-cultured iRBC were normalized to untreated iRBC (GM). D: PI^+ necrotic iRBC after culture in growth medium (GM) or with various effector cells. Numbers of necrotic cells were normalized to untreated iRBC (GM).
doi:10.1371/journal.pone.0033774.g007

That pre-treatment of lymphocytes with the Hsp70 TKD peptide had a significant impact on parasite development suggests that iRBC serve as targets for NK cells through their membrane expression of Hsp70. The origin of membrane Hsp70 remains unclear. It was only detected in small quantities in the cytosol of senescent but not of fresh RBC, although RBC do not have the protein machinery for *de novo* synthesis. Other authors could show presence of Hsp70 in the RBC cytosol [13] possibly because

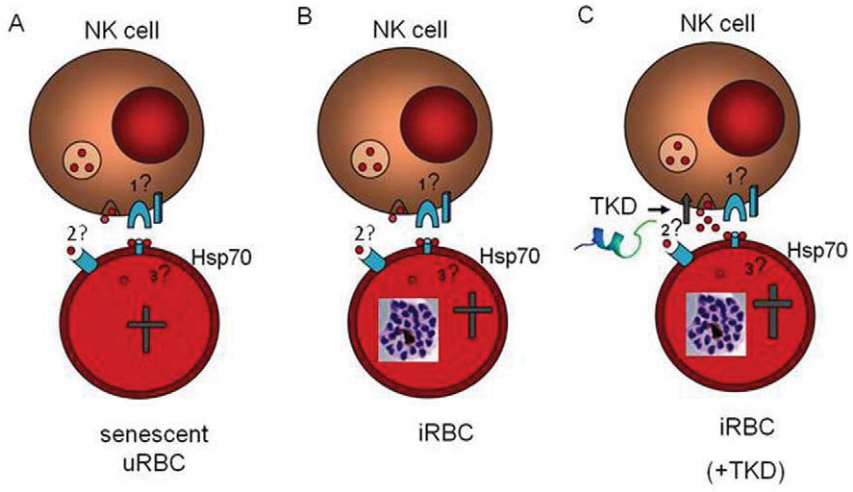

Figure 8. Hypothetical model of NK cell response to iRBC and senescent uRBC. If erythrocytes become senescent (A) or are infected with *Plasmodium falciparum* (B,C) host-Hsp70 will be recruited to the cell membrane. ?1 NK cells recognize Hsp70-exposure by yet unknown receptors, possibly CD94. Recognition of Hsp70 leads to GzmB release. ?2 GzmB will enter the target cell with either assistance of Hsp70, an unknown receptor or become endocytosed. ?3 Once inside the iRBC, GzmB induces eryptosis. Pre-stimulation with TKD peptide enhances both GzmB release and the amount of eryptotic iRBC (C).
doi:10.1371/journal.pone.0033774.g008

Hsp70 is a well-known chaperone residing in the cytosol. The reason that we could not detect sufficient cytosolic amounts might be due to antibody sensitivity as a long visualization time was required in order to detect Hsp70. Interestingly, we also found Hsp70 expression in senescent erythrocyte membranes. This suggests that senescent erythrocytes might also be targets for the cytolytic attack of NK cells. Up to now it remained enigmatic why MHC-negative erythrocytes are not target cells for the cytolysis of NK cells. We suggest that missing-self [10] is not sufficient to activate NK cells and assume that additional activatory signals are required to induce killing activity of NK cells. We could identify that membrane-expressed Hsp70, selectively expressed on iRBC but not on uRBC, acts as a key stimulatory factor for NK cell activation. However, if host-Hsp70 membrane exposure on iRBC has negative or beneficial effects for parasite survival *in vivo* remains to be elucidated. Perhaps Hsp70 is involved in the formation of crucial parasite-derived protein complexes at the RBC surface that are required for cytoadherence or immune evasion. As suggested by others, it may serve to transform the RBC membrane [22]. This seems likely since Hsp70 was only present in early but not late stages of parasite development when RBC membrane remodeling events mainly take place [23,24]. Hsp70 is expressed on senescent erythrocytes as well. Therefore, Hsp70 might represent a physiological removal sign such as aggregated band 3, which is formed rapidly during parasite growth within the iRBC [25]. On the other hand, Hsp70 could act as a danger signal or exert chaperone function by transporting immunogenic peptides to the surface of iRBC in order to alert the immune system to clear the parasite infection. The knowledge of potential antigens that are presented with the help of Hsp70 would therefore deepen our understanding of the complete mechanism involved.

Apart from Hsp70, we aimed to investigate additional ligands on iRBC and their corresponding receptors on NK cells. The balance of the presence and absence of Hsp70 and HLA-E were reported to be relevant for NK cell cytotoxicity [8,26]. Hence, HLA-E expression was analyzed on iRBC as well as presence of its receptor NKG2C on NK cells. Co-culture of NK92 cells with iRBC results in an up-regulation of NKG2C as previously determined in microarray assays [21]. However, in our experiments, neither MICA/B nor HLA-E was found to be present on the surface of iRBC. The co-receptor CD94 but not NKG2C was present on NK cells irrespectively of parasitic infection of RBC meaning that CD94 could be involved in the NK cell erythrocyte interaction. The expression density of CD94 is important for the cross-talk of Hsp70 membrane-positive tumor cells with NK cells and it was formerly reported to lead to higher anti-Hsp70-activity [27]. Moreover, NK cells with an elevated CD94/NKG2 expression were previously reported to produce high amounts of IFN-γ [12]. In the present study we have not identified whether CD94 is the interaction partner of Hsp70 or not and further studies have to be performed to solve this question. Human mature NK cells are heterogeneous for the expression of numerous receptors (NKG2 family, KIR family) or maturation markers such as CD57 and CD62L. Thus, detailed investigation of resting NK cells are required to evaluate the role of these different subsets in Hsp70-mediated recognition of iRBC.

In the context of malaria an involvement of accessory cells was assumed. In these studies activation of NK cells was mainly shown through their expression of CD25, CD69 and the production of IFN-γ [2,10]. Our findings support the importance of cytokines derived from accessory cells for an optimal stimulation of NK cells. Here we extend the role of IL-12/-IL-18 to trigger cytotoxicity via up-regulation of GzmB transcription and its cytosolic abundance. In our experiments iRBC enhanced cytosolic expression and release of GzmB, possibly mediated via the presence of Hsp70 as shown by Hsp70 blocking experiments, where GzmB release was inversed. This effect was also observed to a lesser extent using an isotype control. This suggests that other stress signals and/or activating NK receptors such as DNAM-1, NKp30 or NKp46 might be additionally involved in this process. The finding that only translation but not transcription of GzmB was up-regulated is in accordance with other investigations. High levels of mRNA but low protein expression of GzmB and perforin in resting NK cells or CTL but elevated protein expression after activation were observed in different T-cell subsets and NK cells [28,29]. Furthermore, a pre-existing pool of mRNA of GzmB and perforin that can be quickly translated into protein after stimulation was reported from a mice study [30]. In addition, translational

repression of GzmB/perforin by micro-RNA was recently demonstrated in human NK cells [31]. Thus, the result of elevated GzmB protein expression after co-culture implies that through iRBC contact translation might be activated. GzmB release in response to iRBC could be further raised by prior sensitization of NK cells with the Hsp70 TKD peptide. Whether such enhancement is beneficial or harmful for the host requires to be elucidated. It was previously shown that GzmB levels are elevated in individuals with severe malaria [32]. However, it remains unclear whether this is cause or result of the disease. In other studies, up-regulation of perforin and GzmA but not GzmB was detected in NK cells following PBMC co-culture with iRBC. The reason why we could not detect perforin or GzmA might be due to the fact that we were using isolated NK cells meaning that in other settings perforin or GzmA might be released because of accessory cells. Additionally, the fact that NK92 are transformed cells that already underwent differentiation also needs to be taken into account.

Recently it was shown that Hsp70 recognition by NK cells leads to apoptosis of tumor cells in a GzmB-dependent but perforin-independent manner [20]. Erythrocytes cannot undergo classical apoptosis characterized by DNA degradation since they are non-nucleated, highly specialized cells whose main function is oxygen transport. Nevertheless, many studies have observed a form of programmed cell death called eryptosis, which is characterized by membrane scrambling and cell shrinkage [33]. These authors also showed that eryptosis occurs during infection of erythrocytes with *P. falciparum* [34]. The mechanism, however, was not yet characterized. We could show that eryptosis was induced by co-culturing iRBC with different effector lymphocytes. In our experiments, we excluded spontaneous eryptosis of u/iRBC cultured without lymphocytes showing that eryptosis depends on cytotoxic cells which are co-cultured with the erythrocytes. Since parasitemia at the beginning of the co-culture varied from 2.1–19.0% the number of eryptotic iRBC varied as well in each experiment suggesting that only infected erythrocytes are affected by eryptosis. Furthermore, we could also detect eryptosis in iRBC co-cultured with PBMCs, which suggests that either other lymphocytes (e.g. $CD8^+$ T cells) may also be activated to elicit cytotoxicity or that a larger proportion of NK cells are properly activated due to signals delivered by other PBMCs.

If supported by further studies, our results could provide the basis for a new strategy to treat malaria. Our experiments show that NK cells as well as TKD-pretreated cells have an anti-parasitic effect but we do not know if this treatment completely clears the infection and more importantly, will prevent future infections with the parasite. Nevertheless, it is important to identify and to evaluate basic mechanisms occurring during *P. falciparum* infection in order to find adjuvants or drugs which act on specific cell types and to develop feasible applications for future settings. Feasibility, safety and toxicity of adoptively transferred *ex vivo* TKD-stimulated autologous NK cells were already proven in a phase I clinical trial in lung and colon cancer patients [35]. Several clinical trials with different types of cancers employing heat shock protein based-vaccines were already undertaken [36]. Furthermore, Hsp70-peptide complexes (Hsp70-PC) can also activate other immune cells such as antigen presenting cells (e.g. DCs), leading to maturation via NF-kB-activation. This activation results in stimulation of cytokine release (TNF-α, IL-1b and IL-6), expression of co-stimulatory molecules (B7.1, B7.2, CD40 und MHC class-II) as well as release of nitric oxide. Additionally, APCs are known to internalize Hsp70-PC and then present them as MHC-complexes which involve both the innate and the adaptive immune response [37]. All these mechanisms play a role in malaria for which reason it is conceivable that Hsp70 is important in this context. Apparently, NK cell activation by TKD peptide is not easily applicable in malaria, first, because in contrast to cancer, malaria is an acute disease which requires an immediate treatment. Moreover, it is at the moment unlikely to perform *ex vivo* TKD-stimulated NK cell treatment that requires cost intensive GMP-facilities in resource-poor countries where the burden of malaria is highest. However, one could imagine directly injecting the peptide into patients in order to stimulate NK cells, *in vivo*. Recently, several studies have proposed that NK cells have a memory [38,39]. If this is the case, administration of TKD peptide, maybe in combination with a standard vaccine would help to better respond to Hsp70-positive iRBC during *Plasmodium* infection and also to other modified cells in diseases like virus infection or cancer. How long does this effect last? How can we assure that the TKD peptide reaches NK cells in appropriate concentrations? Many issues remain to be solved. Nevertheless, our proposed model opens the way for new alternatives, which are urgently needed.

Methods

Ethics statement

Freshly isolated PBMCs and NK cells were obtained from six healthy malaria-naïve donors after written informed consent. The study was approved by the Ethical Committee of the Medical Faculty of Tuebingen University and the University Hospital of Tuebingen.

Cell line

NK92 cells (DSMZ) were grown at $0.2–0.6\times10^6$ cells/ml in 75% α-MEM, 12.5% fetal bovine serum (FBS) and 12.5% horse serum supplemented with 2 mM L-glutamine, 10 ml/l penicillin – streptomycin, 10 ng/ml recombinant human interleukin-2 (rIL-2). *Mycoplasma* contamination was prevented by addition of 5 µg/ml Plasmocin and routinely excluded by PCR.

PBMC and NK Cell isolation

Venous blood was collected into ammonium heparin tubes (10 IU/ml blood, Sarstedt GmbH, Nuembrecht, Germany), isolated by Ficoll separation (GE Healthcare), washed twice with 2% FBS in RPMI and resuspended at $2.5–5\times10^6$ cells/ml in RPMI supplemented with 6 mM L-glutamine, 5% FBS, 100 U/ml penicillin-streptomycin and 100 U/ml IL-2 (Invitrogen). NK cells were isolated with Dynabeads® Untouched™ Human NK Cells Isolation Kit (Dynal Invitrogen, Oslo, Norway), washed twice with isolation buffer (2% FBS in phosphate buffered saline (PBS) supplemented with 2 mM EDTA) and cultured in 24-well plates at a density of 2×10^6 cells/ml. Purity of NK cells (≥93.5%) was verified by CD3-PE/CD56-FITC staining (Becton Dickinson).

P. falciparum culture

P. falciparum parasites (strain 3D7) were maintained in continuous culture as described elsewhere [40]. Parasites were either grown in 0^{Rh+} human erythrocytes (Blood bank, University Hospital Tübingen, Germany) or in autologous RBC from the respective donor in RPMI 1640 supplemented with 25 mM HEPES (Sigma-Aldrich), L-glutamine (PAA), gentamycin and 50 ml Albumax II. Mature schizont-infected erythrocytes were harvested by magnetic cell sorting LD separation columns (MACS; Miltenyi Biotec). Parasite cultures were routinely screened for *Mycoplasma* contamination by PCR.

NK cell stimulation

To prevent prior activation, NK92 cells were kept in culture medium in the absence of rIL-2 overnight. NK92 or freshly isolated NK cells were either co-cultured with iRBC, uRBC (1:3) or stimulated with IL-12/IL-18 (100 ng/ml each) or IFN-α (100 U/ml) for 24 h at 37°C and 5% CO_2. Some cultures were stimulated five days prior to co-culture with 2 μg/ml TKD (multimmune GmbH, Munich, Germany) peptide (purity>96%; Bachem, Bubendorf, Switzerland), since it was previously demonstrated that this period is optimal for activating NK cells towards $Hsp70^+$ target cells [8,35].

Growth delay

To evaluate the effect of freshly isolated NK cells on parasite growth, NK cells or PBMCs were pre-stimulated with/without TKD for 5 days prior to experiment and co-cultured with iRBC (1:3) from the same donor. After 24 hours, blood smears were prepared from the pellets. Cells were fixed in methanol and subjected to 10% Giemsa staining for 20 minutes. Development of parasites was evaluated in comparison to untreated iRBC before and after co-culture. Photography was taken at 1000× amplification.

Cell surface staining for flow cytometry to detect possible interaction partners on the NK cell and iRBC surface

NK cells were characterized by flow cytometry using fluorescence labelled CD56 (FITC, BD Biosciences), CD3 (PE, BD Biosciences), CD94 (FITC, R&D Systems) or NKG-2C (APC, R&D Systems) antibodies. Both uRBC and iRBC were incubated with anti-hMICA (PE, R&D Systems), anti-hMICB (mouse monoclonal IgG_{2B}, clone 236511, R&D Systems), subsequently stained with goat anti-mouse IgG_{2B}-FITC (1 μg $IgG_{2B}/10^6$ cells, Santa Cruz), hMICA/B (PE, clone 6D4, eBioscience), or anti-hHLA-E (PE, eBiosciences). The presence of Hsp70 on RBC was determined with anti-Hsp70 (Alexa 488-conjugated, clone D69, Cell Signaling) or cmHsp70.1 (FITC, IgG_1, multimmune GmbH, Munich, Germany). Parasite DNA was stained with Hoechst dye for 30 minutes at 37°C in the dark or with Hydroethidine (0.014 mg/ml; Polysciences, Eppelheim, Germany). Dead cells were excluded by 7-AAD staining (BD). Flow cytometry measurements were carried out using a FACSCanto flow cytometer (BD Biosciences) and analyzed with BD FACS Diva 6.0 software. Gating was done based on forward and sideward scatter properties.

Real-time quantitative analysis of GzmB, GzmA, and Perforin expression

Total RNA was extracted from 3×10^6 NK92 cells with RNeasy MiniKit (Qiagen, Hildesheim, Germany). 0.5 μg RNA was converted into cDNA using Quantitect Reverse Transcription Kit (Qiagen, Hildesheim, Germany). For real-time PCR the following Quantitect Primer Assays (Qiagen, Hildesheim, Germany) were used: QT01001875 Hs_GZMB_2_SG (GzmB), QT00015575 Hs_GZMA_1_SG (GzmA), QT01869602 Hs_PRF1_2_SG (Perforin), and QT01680476 Hs_ACTB_2_SG (β-Actin). The reaction mix was prepared according to the standard protocol of Rotor-Gene SYBR Green RT-PCR Kit (Qiagen). RT-PCR was carried out in triplicate with a Rotor Gene 3000 Cycler with standard thermal profile. Transcriptional changes were calculated with the ΔΔCt-method, excluding Ct-values≥35 and applying a threshold of 0.01.

Western Blot

1×10^7 cells were lysed in 1 ml lysis buffer (20 mM HEPES, 250 mM NaCl, 20% glycerine, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% Np-40 (IGEPAL), 1 mM Dithiothreitol, and proteinase inhibitor (1 tablet complete protease inhibitor / 50 ml), incubated 30 min on ice and subsequently centrifuged at 13000× *g* for 15 min at 4°C. Supernatants containing the RBC cytosol were removed, pellets with the RBC membranes were washed twice with lysis buffer and the supernatants were combined. 10 μg of total protein were boiled at 95°C for 10 min in reducing loading buffer, separated by 7.5% SDS-PAGE and blotted 45 min onto a nitrocellulose membrane (Bio-Rad). Blocking was performed overnight with 4% bovine serum albumin (BSA) in 1% PBS-Tween-20 (PBST), membranes incubated for 1 h with either anti-hGzmA (GA6, Santa Cruz, 0.5 μg/ml), anti-hGzmB (Santa Cruz, 0.1 g/ml) or anti-hPrf (4E4, Santa Cruz, 0.5 μg/ml). Separated and blotted RBC extracts were incubated with anti-Hsp70 (D69, Cell Signaling, 1:300) and subsequently incubated with rabbit anti-mouse IgG linked to horseradish peroxidase (Cell Signalling, 1:3000). Conjugated proteins were visualized with ECL reagent (Amersham Corp.) according to manufacturer's instructions. For control of equal protein loading, membranes were incubated in stripping buffer (62.5 mM Tris-HCl pH 6.7, 100 mM β-mercaptoethanol, 2% SDS) at 50°C for 30 min under occasional agitation. Following washing with PBST, blocking with BSA-PBST was repeated as described above and membranes were incubated with mouse anti-hActin (0.5 μg/ml ACTBD11B7, Santa Cruz), followed by immunoblot as described before.

Determination of release of cytotoxic effector molecules using GzmB ELISPOT assay

A non-radioactive assay was chosen to assess GzmB release of freshly isolated NK cells or NK92 cells cultured with rIL-2 and with/without TKD peptide (2 μg/ml) or a 14-mer scrambled NGL peptide (NGLTLKNDFSRLEG) consisting of the same amino acid residues in a different order (2 μg/ml; Bachem, Bubendorf, Switzerland) for 4–5 days prior stimulation with i/uRBC (1:3 or 10:1). The same set of experiments was repeated using a blocking antibody directed against membrane Hsp70 by pre-incubating i/uRBC with 10 μg/ml cmHsp70.2 (IgM, multimmune GmbH, Munich, Germany) for 20 minutes or a control IgM isotype (clone MM-30, 10 μg/ml, Biolegend). Granzyme B release was elaborated using ELISPOT assays (Abcam, Cambridge, UK) following the standard protocol with 2000 effector cells (ratio 1:3 or 10:1 of NK cell to u/iRBC) in complete RPMI for 24 hours at 37°C. Spot formation was monitored and counted using an ImmunoSpot Series 5.0.9 Analyzer (CTL-Europe GmbH, Aalen, Germany).

Eryptosis assay

Previous reports showed eryptosis, an apoptosis-like cell death of erythrocytes triggered by a broad variety of stimulators such as temperature increase [41], and others [33]. To determine whether eryptosis of iRBC is induced due to NK cell contact, phosphatidylserine exposure was assessed by measuring Annexin-V-staining at an excitation wavelength of 488 nm and propidium iodide (PI) at an excitation wavelength above 600 nm; in addition forward scatter (FSC) was recorded. After 24 hours of co-culture with PBMCs or isolated NK cells (3:1) that were sometimes pre-incubated 5 days with TKD peptide (2 μg/ml) before co-culture, 0.5×10^6 erythrocytes were washed in 5 mM Ringer solution (NaCl, KCl, CaCl, HEPES, glucose). Afterwards, cells were stained for 15 minutes with Annexin V-FLUOS (1:500, Roche, Mannheim, Germany) and PI (1:50).

Statistical analysis

Statistical analysis was performed using JMP for Windows 5.0.1 (SAS Institute Inc., Cary, North Carolina), applying either student's *t* test, assuming unequal variances, or one-way ANOVA. Results were considered statistically significant at $P<0.05$.

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Author Contributions

Conceived and designed the experiments: EB JFJK. Performed the experiments: EB. Analyzed the data: EB GM JFJK ME. Contributed reagents/materials/analysis tools: EB GM JFJK. Wrote the paper: EB GM ME.

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