Natural killer (NK) cell-mediated cytolysis of *Plasmodium falciparum*-infected human red blood cells *in vitro*

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ABSTRACT. The ability of human NK cells to inhibit the growth *in vitro* of the asexual blood stages of *Plasmodium falciparum* was tested. Purified NK cells from donors with no prior exposure to malaria significantly inhibited parasite growth after 48 hours of co-culture in the presence of human immune serum. This inhibition was completely abrogated by pre-treatment of the NK cells with an anti-CD95 (anti-Fas) monoclonal antibody and human Fas-Fc soluble protein. The level of growth inhibition was also substantially reduced by pre-treatment with an anti-CD56 antibody. These two antibodies caused reductions, to varying levels, of the amounts of NK cell-derived granzyme B (GrB) and pro-inflammatory cytokines, but only the anti-CD95 antibody affected the production of soluble Fas ligand (sFasL). Direct destruction of parasite-infected red cells by NK cells, in the absence of serum, was also observed in a standard ^{51}Cr cytotoxicity test, during which N-carboxybenzoxy-L-lysine thiobenzil ester (BLT esterase) activity, which catalyzes serine protease granule release, was detected. The results obtained are indicative of a novel mechanism of NK cell-mediated cytotoxic activity against *Plasmodium falciparum*-infected red cells, which is mediated in part by both Fas and by GrB.

Keywords: human, NK cells, *Plasmodium falciparum*, cytotoxicity, innate immunity

INTRODUCTION

Natural killer (NK) cells represent an important first line cellular effector mechanism of the human innate immune system [1]. They are currently defined as cytotoxic cells with a predominant morphology of large granular lymphocytes (LGL), but whose surface membrane does not express the CD3 complex or any of the known T-cell receptor chains (TcR) α/β or γ/δ. Most human NK cells constitutively express CD16 (FcγRIIIA), and CD56 (NCAM) antigens. NK cells also express NK receptor antigens (NKR), which belong to two families of molecules: i) human killer cell immunoglobulin-like receptors (KIR), and leukocyte immunoglobulin-like receptors (LIR), which are immunoglobulin-like receptors; and ii) CD94-NKG2 heterodimers, that resemble type C lectins [2-5]. NK cell activities such as cytotoxicity, proliferation and cytokine production are regulated by various cytokines both *in vitro* and *in vivo*. Interferon alpha (IFN-α) [6] and interleukin (IL)-2 [7] are the most powerful inducers of NK activity, but IL-12 also plays a role in regulating these activities [8-9]. IL-2 induces granzyme B (GrB) release by NK cells, and human granzyme M shows strong constitutive expression in NK cells and γδ-T cells but not in $CD4^+$ or $CD8^+$ T cells. The effect of IFNγ on NK cells is particularly noteworthy. Indeed, IFNγ acts on NK cell activation in both a paracrine fashion (when produced by other mononuclear cells), and in an autocrine fashion (when produced by NK cells themselves). Activated NK cells thus themselves secrete IFNγ[10, 11], as well as soluble Fas ligand (sFasL), perforin [12] and granzymes A, B and M [13, 14]. In the human immune response to malaria, it has been recently reported that live parasites induce much stronger IFNγ production from NK cells than do dead schizonts [15].

In the study described here, we therefore attempted to elucidate the possible role of NK cell-mediated cytotoxicity on erythrocytes infected *in vitro* with *P. falciparum*. We analyzed their effect on parasite growth, together with their cytotoxic activity against infected red blood cells (iRBC) and the profiles of cytokines and other mediators released specifically during their interaction with iRBC.

MATERIALS AND METHODS

Parasite culture

A laboratory-adapted culture of *P. falciparum* referred to as BINH was used in this study. The culture was originally

Abbreviations: BLT: N-Carboxybenzoxy-L-lysine thiobenzil ester; DNTB: [5-5 dithio bis (2-nitrobenzoic acid)]; GIA: growth inhibition assay; RBC: red blood cells; iRBC: infected-red blood cells; SGI: specific growth inhibitory index

derived from a traveller returning to Germany from Kenya. Parasites were cultured according to a modification of the method of Trager and Jensen [16]. Briefly, parasites were routinely cultivated with freshly prepared blood-bank-derived O^+ erythrocytes in complete medium comprising RPMI-1640 (Sigma, Deisenhofen, Germany), with 4 g/L glucose, 25 µg/L gentamicin (Gibco-BRL, Gaithersburg, MD, USA), 32 µM hypoxanthine (Sigma), 5.94 g/L HEPES (Seromed, Berlin, Germany), 2.33 g/L $NaHCO_3$ and supplemented with 0.5% Albumax II™, a lipid-rich bovine albumin with a minimal IgG concentration (Gibco-BRL), in an atmosphere containing 5% CO_2, 5% O_2 and 90% N_2 The culture medium was endotoxin-free and was changed every other day, and 400 µl of O^+ erythrocyte suspension were added to subcultures. Synchronous erythrocytic stages of *P. falciparum* in culture were obtained by suspending cultured parasites in 5% D-sorbitol as previously described [17]. The *P. falciparum* parasite culture was routinely tested for mycoplasma contamination with a PCR-based technique originally described by Spaepen *et al.* [18].

Isolation of human NK cells

Cellular effectors, NK cells were isolated by positive selection with the Miltenyi Biotec MACS system (Macs Cell Sorting of Human Leukocytes, Gladbach, Germany) from blood samples of nine healthy volunteers with no clinical or serological evidence of contact with *Plasmodium*, who donated blood at the Tübingen University Hospital blood bank. Positive selection by binding of CD56 mAb-coated magnetic microbeads has been shown not to affect NK cytotoxicity [19, 20]. After sampling, the blood was diluted 1:1 in RPMI-1640, deposited on a Biocoll density gradient (Biochrom, Berlin, Germany), then centrifuged at 2000 rpm for 20 minutes. The peripheral blood mononuclear cells (PBMC) were removed and washed twice in RPMI 1640 medium. After the second wash, the cell pellet was re-suspended in 500 µl of MACS labelling buffer [PBS containing 2 mM EDTA and 0.5% fetal calf serum (FCS) (Gibco-BRL)] and incubated consecutively with magnetic beads coated with various antibodies (anti-CD56 for NK cells, anti-CD14 for monocytes anti-CD4 for T-helper cells and anti-CD8 for cytotoxic T-cells, all Miltenyi). After 20 minutes of incubation with each antibody, the cells were washed twice to eliminate unbound antibodies, then passed through a Super MACS™ (Miltenyi) column placed in a magnetic field to retain labelled cells for subsequent elution. The eluted cells were again washed, re-suspended in phosphate-buffered saline (PBS: 0.15 mM sodium chloride/10 mM phosphate sodium, pH 7.4), pre-incubated for 20 minutes with fluorescent antibodies (see following section), then analyzed in a flow cytometer to determine their numbers and viability. Cells obtained after Biocoll processing and positive selection on Super MACS™ were labelled with the different antibodies coupled to fluorescein (FITC) or phycoerythrin (PE), to determine their numbers and viability. These controls were based on double labelling of each cell type. The following antibodies were used: CD56-PE clone B159, and CD16-FITC clone 3G8 for NK cells; CD14-FITC and CD16-PE clone 3G8 for monocytes; CD4-FITC clone RPA-T4 and CD3-PE clone UCHT1 for T-helper lymphocytes; CD8-FITC clone RPA-T8 and CD3-PE clone UCHT1 for cytotoxic T-lymphocytes. All these antibodies were from BD-Pharmingen (Heidelberg, Germany). The negative isotype controls were the IgG1-FITC/IgG2a-PE mixture of mouse dual TAG™ clone MOPC-21/UPC-10 (Sigma). Cells (10^6) were incubated at 4°C in 250 µl of PBS containing 1% FCS and an optimal antibody concentration, according to the manufacturer's instructions. After 20 minutes of labelling, cells were washed three times in PBS then directly analyzed. FACStar analysis of $CD3^-/CD56^+/CD16^+$ NK cells, $CD4^+$-T cells, $CD8^+$-T cells and $CD14^+$ cells showed that the population of NK cells, helper T-cells, cytotoxic T-cells and monocytes thus obtained were > 99% pure.

P. falciparum *growth inhibition test*

P. falciparum growth inhibition was tested in triplicate in flat-bottomed, 96-well plates (Costar®) at a hematocrit of 2% with a 1:1 NK/iRBC ratio with NK cells from three European malaria-naïve donors. The specific growth inhibitory index (SGI), which takes into account the possible inhibition induced independently by effector cells or by sera used as controls in each experiment, was calculated as previously described [21-23] as follows:

$$SGI = 100 \times \left[1 - \frac{(\%\text{ parasitaemia with effector cells and IS} / \%\text{ parasitaemia with IS alone})}{(\%\text{ parasitaemia with effector cells and NIS} / \%\text{ parasitaemia with NIS alone})}\right]$$

where IS = immune serum and NIS = non-immune serum

The pooled immune serum donors comprised eight adult residents of Lambaréné, Gabon, an area where *P. falciparum* is hyperendemic and perennially transmitted [24]. They had spent most of their lives in this locality and had a history of repeated malaria attacks. All were above 18 years of age and had parasite-negative Giemsa-stained thin blood smears at the time of blood collection. Individual consent was sought and given prior to venesection of blood into sterile tubes. Serum obtained from the blood samples by centrifugation after clotting was aliquotted and stored at – 70°C prior to use. Pooled, non-immune serum from non-exposed AB^+ European donors, for use as control, was obtained commercially (PAA Laboratories, Linz, Austria). Serum was used at 10% in assays. The action of NK cells on *P. falciparum* growth was assessed after 48 hours, using mature schizonts at 1-2% parasitaemia in synchronized cultures. Parasitaemia was estimated on routinely prepared Giemsa-stained thin films from triplicate wells, by microscopic observation of at least 10,000 erythrocytes.

Blockade of growth inhibition

To determine if the inhibition of *P. falciparum* growth observed in infected red cell-NK cell co-cultures was due to preliminary activation of NK cells, the latter were pre-incubated with a saturating concentration of two mouse anti-human monoclonal antibodies: anti-CD95 purified clone ZB4 [(Upstate, Lake Placid, NY, USA), which does not induce apoptosis in culture], since NK cells express sFasL [12], anti-CD56 purified, and human Fas-Fc soluble protein [a chimeric protein containing the extracellulair region of human Fas and the Fc portion of human IgG1 (R & D Systems, Minneapolis, MN, USA)] or with control isotype of the two antibodies (from BD-Pharmingen), for 1 hour at 4°C. NK cells were then washed three times to eliminate unbound antibodies before performing the

above-described inhibition tests. The results were analyzed after 48 hours.

Target cells

Red cells infected *in vitro* by the BINH strain were used as targets in ^{51}Cr cytotoxicity tests (see below) and enzyme assays of cytotoxic granule secretion. These infected red cells were mature schizontes. K562 cells, a myeloid tumor cell line obtained from a patient with chronic myeloid leukemia in blastic phase [25], was used as positive control (K562 cells were a generous gift from Professor E. Vivier, CIML, Marseille-Luminy, France). These cells were maintained in RPMI-1640 complete culture medium containing 10% FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM glutamine and 1 mM sodium pyruvate (Gibco-BRL) in cell culture flasks (Polylabo, Strasbourg, France); culture medium was changed every 2 days.

^{51}Cr labeling of target cells

Target cells (3×10^6) were incubated with 200 µCi of ^{51}Cr (aqueous solution of sodium chromate: $Na_2{}^{51}CrO_4$) (NEN, Germany) in 1.5 mL of complete medium for 1 hour at 37°C in humidified 5% CO_2/95% air. On the basis of ^{51}Cr decay, volumes of radioactivity varied from 80 to 250 µl according to the reference date. At the end of the incubation period, cells were washed three times in complete medium and adjusted to a density of 5×10^4 cells/ml. For each test, 100 µl of this suspension (equivalent to about 5000 cells) was used.

Cytotoxicity

For NK cytotoxicity tests, target cells (erythrocytes infected *in vitro*, iRBC) were incubated for 1 hour at 37°C with 200 µCi of $Na_2{}^{51}CrO_4$, washed twice and deposited (5,000 cells per well) in round-bottomed, 96-well plates. Effector cells (NK cells) were then added in triplicate, in a final volume of 200 µl. The plates were centrifuged for 2 minutes at room temperature to favor the formation of NK-iRBC conjugates. Cytotoxic activity was assessed at effector:target cell ratios of 40:1; 20:1; 10:1; 5:1 and 1:1. After 4 hours of incubation at 37°C the plates were centrifuged and the supernatant (100 µl) of each well was collected and placed directly in a gamma counter (Cobra Auto-Gamma, Packard Cambera Company) to determine radioactivity. Maximal chromium release was determined by adding 1% sodium dodecyl sulfate (SDS) (Sigma); spontaneous release was determined in wells in which target cells were incubated with culture medium alone. Spontaneous release never exceeded 20% of maximal release. Specific cytotoxicity was calculated using a standard. Percentage specific marker release was calculated as follows:

% specificity = [(cpm. Exp. – cpm Spont./cpm Max. – cpm Spont.)] × 100.

BLT esterase assay

Esterase activity was measured as previously described by Sayers *et al.* [26]. Briefly, 50 µl of test sample was added to 100 µl of 1 mM DNTB [(5-5 dithio bis (2-nitrobenzoic acid)](Sigma), prepared in PBS containing 1 mM $CaCl_2$ and 2 mM $MgCl_2$ at pH 7.2. The reaction was initiated by adding 50 µl of 2 mM BLT [(N-carboxybenzoxy-L-lysine thiobenzyl ester)](Sigma). The duration of the experiment depended on the time required for the emergence of optimal coloration (approximately 30 min). Absorbance was measured at 420 nm on an ELISA plate reader piloted by Mikrowin software, using a 620-nm reference filter. Controls (DNTB alone, BLT alone, or DNTB + BLT) were always used to take non-specific effects into account. To block specific signaling by GrB, inhibition of esterase activity was performed by incubating NK cells for 4 hours at 37°C with 100 µM GrB inhibitor, Z-AAD-CH_2Cl (Oncogene, Boston, MA, USA).

Cytokine assays

Cytokine assays were performed on supernatants of NK-iRBC co-cultures performed in the absence of immune serum, to determine cytokine production by NK cells. NK cells (10^5), obtained as described above, were co-cultured with iRBC, in 96-well, flat-bottomed plates in a total volume of 200 µl per well. The medium was RPMI-1640. After incubation at 37°C for 48 hours, supernatants were aspirated and stored frozen at –30°C before testing by ELISA for IFNγ, TNFα, IL-2, IL-12p40 (Biosource, Nivelles, Belgium), sFasL (BD, Pharmingen) and GrB, (Chemicon, Germany). Results are presented as means ± SEM of triplicate wells.

Statistical analysis

Statistical analysis was done using JMP 4.0 software (JMP 4.0, SAS Institute Inc, Cary, NC, USA, software). Levels of significance were determined by *t*-tests and values of $p < 0.05$ were considered significant.

RESULTS

NK cells inhibit **P. falciparum** *growth in vitro in the presence of immune serum*

Since NK cells express FcγRIII (CD16), the low-affinity receptor for IgG1/IgG3, we wished to determine the effects of inclusion of serum from malaria semi-immune, African adults in this system i.e. the potential for antibody-mediated enhancement of NK cells' lytic activity for iRBC. The addition of purified NK cells at a 1:1 effector:target cell ratio resulted in specific growth inhibition, as reflected by an SGI of 25% (Figure 1A). The combination of NK cells with $CD4^+$ T-cells with immune serum resulted in an SGI of 27%, whilst the strongest inhibition of parasite growth (44%) was obtained when iRBC were incubated with monocytes and immune serum (Figure 1A). No effect was found in the absence of immune serum (data not shown).

Anti-CD95 antibodies prevent **P. falciparum** *growth inhibition by NK cells*

Next we investigated the possible involvement of the perforin/granzyme and/or Fas/FasL lytic pathway in this system. Pre-treatment of NK cells with a non-apoptotis-inducing anti-Fas antibody (ZB4), human Fas-Fc soluble protein and Z-AAD-CH_2Cl a GrB inhibtor completely abrogated their parasite growth inhibitory capacity resulting in an SGI of –10% and – 8% respectively (Figure 1B). An anti-CD56 antibody had a similar abrogating effect

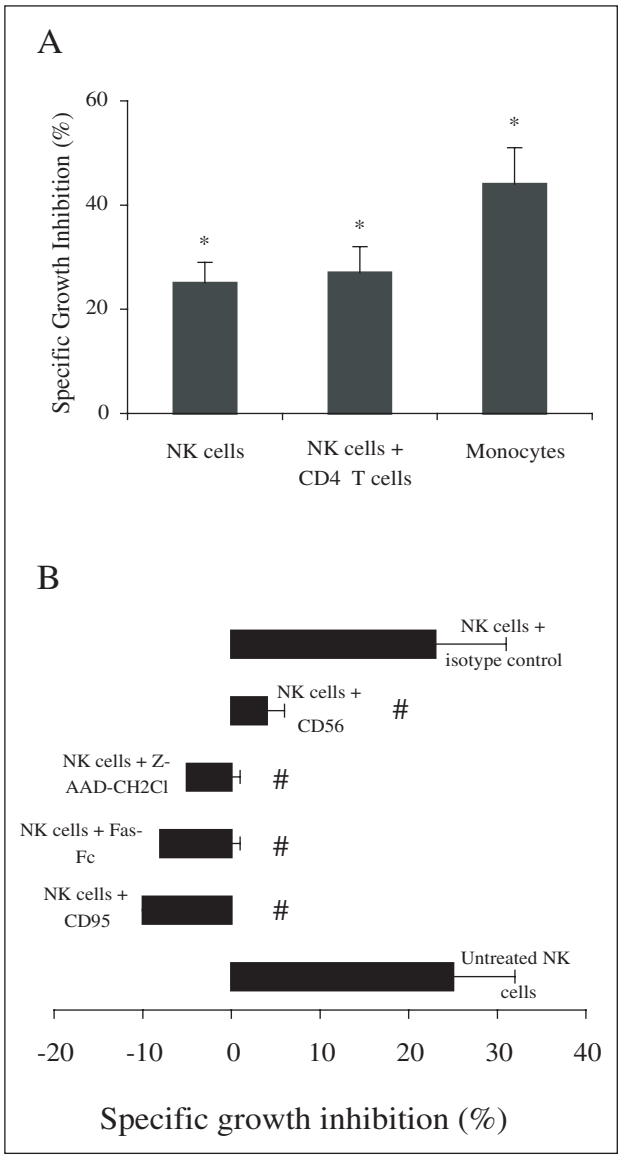

Figure 1
***Plasmodium falciparum* growth inhibition by NK cells, monocytes and CD4⁺ T cells.**
A) Inhibition of *Plasmodium falciparum* growth *in vitro* by NK cells, by NK cells + $CD4^+$ T-cells or by monocytes. The growth inhibition was determined by calculation of the Specific Growth Inhibition index (SGI). Results are expressed as mean ± SD of individual values of three separate experiments. B) Blockade of parasite growth inhibition by NK cells pre-treated with antibodies to CD56 (1 µg/ml) CD95 (1 µg/ml), isotype control (1 µg/ml), human Fas-Fc soluble protein (15 µg/ml) or Z-AAD-CH_2Cl (100 µM). The blockade of growth inhibition was determined by calculation of the Specific Growth Inhibition index (SGI). Results are expressed as mean ± SD of individual values of three independent experiments. * $p < 0.001$ for comparison with control; # $p < 0.001$ & † $p = 0.03$ for comparison with NK cells.

with an SGI of 4.5% (Table 1). No inhibition was found with isotype control. (A negative SGI reflects parasite growth relative to the control).

NK cells exert direct activity on P. falciparum-*infected erythrocytes*

To determine if the observed growth inhibition of *P. falciparum* was at least partly due to a direct cytotoxic action of NK cells on iRBC, we performed conventional ^{51}Cr cytotoxicity tests. Four hours' incubation of radioactively-labelled iRBC with NK cells, in the absence of immune serum, showed target cell destruction compared with uninfected control red cells (Figure 2). Five different effector-:target cell ratios were used. The cytotoxic action of NK cells on iRBC was specific and dependent on the effector cell density, as illustrated in Figure 2.

NK cells activate BLT on contact with infected erythrocytes

As the cytotoxic activity of CTL and NK cells involves enzyme activation leading to the release of cytotoxic granules by effector cells, we examined the enzyme activation status of NK cells incubated with iRBC. The same E:T ratios as those used for cytotoxicity tests were used for these enzyme analyses, and confirmed the specificity of the reaction. At effector:target ratios greater than 5:1 the combination of NK cells with either the NK cell-sensitive K562 cell line or with iRBC resulted in BLT-associated enzyme activity significantly higher than background (NK with RBC, Figure 3, $p < 0.01$). In the case of the K562 cell line, this activity increased significantly at higher ratios ($p < 0.001$), but with iRBC this activity reached a plateau at a 10:1 ratio (Figure 3). When used to pretreat NK cells, Z-AAD-CH_2Cl dramatically reduced the BLT activity pointing to the involvement of granzyme release by the effector cells.

Detection of cytokines and soluble mediators in NK-IRBC co-culture supernatant

The supernatants of 48 hour NK cell-IRBC co-cultures performed in the absence of immune serum contained substantial amounts of IFNγ, IL-12 and TNFα (Figure 4A, B, C). Pre-treatment of the purified NK cells with monoclonal antibodies showed marked reductions of all three cytokines with both antibodies, with the most pronounced effect exerted by the anti-CD95 antibody (Figure 4A, B, C). The production of sFasL was significantly reduced only following NK cell pre-treatment with the anti-CD95 antibody (Figure 4D), whereas GrB production was inhibited to varying extents by both antibodies (Figure 4E). Pre-treatment of NK cells with GrB inhibitor Z-AAD-CH_2Cl before co-cultivation completely abolished the release of GrB (Figure 4F). No IL-2 was detected in the NK-iRBC co-culture supernatants (data not shown).

Fas antigen expression by Plasmodium falciparum-*infected erythrocytes*

Fas is a major member of the death receptor family [27]; cross-linking by its natural ligand (sFasL) or by agonistic antibodies leads to sequential targeting of caspase [28], a family of cysteine proteases specific for aspartate, whose activation is required to propagate the biochemical events responsible for the induction of cell death by apoptosis [29]. As our results showed that anti-CD95 prevented *P. falciparum* growth inhibition by NK cells, and that high sFasL levels were produced in the coculture supernatants of NK cells and iRBC, we used flow cytometry to analyze the surface expression of CD95 antigen by erythrocytes. Red cells infected *in vitro* by *P. falciparum* expressed CD95 at their surface (Figure 5), and this expression appeared to be functional, given the capacity of CD95 to bind antibodies *in vitro* (Table 2).

Table 1
Growth inhibition assay with immune serum

Sample N°	NK cells alone	CD95	Fas-Fc	Z-AAD-CH_2Cl	CD56	Isotype control
1	26	– 23	– 18	– 9	7	19
2	31	– 16	– 5	4	3	26
3	35	3	– 13	– 9	4	39
4	14	– 6	9	– 10	2	19
5	21	– 12	– 7	3	6	24
6	24	– 7	– 14	– 9	5	11
Mean ± SD[a]	25 ± 7	– 10 ± 8	– 8 ± 9	– 5 ± 6	4.5 ± 2	23 ± 8

Values given are percentage parasite growth inhibition of growth inhibition assay relative to appropriate controls. Percentages with negative values represent enhancement of parasite growth. Parasitaemia at start: 0.5%; mean (± SD) parasitaemia of control after 48 hours: 5.2 ± 0.7%. See materials and methods for details of calculations.
[a] Mean ± SD of all samples tested.

DISCUSSION

The results of the *in vitro* studies presented here define an entirely novel mechanism of NK cell-mediated inhibition of the growth of the asexual blood stages of *P. falciparum*, which we conclude involves sFasL-Fas interactions at the effector:target (NK:iRBC) level, as well as cytolytic GrB activity. The fact that these growth inhibitory effects were observed both in the presence and in the absence of parasite antigen-specific antibodies implies that NK cells may represent an active but as yet unrecognised arm of the human innate immune response to *P. falciparum* infection in non-immune individuals. It is important to note in this context that all these observations were derived from experiments conducted with non-activated NK cells purified from non-exposed donors.

Antibody-dependent monocyte-mediated cellular inhibition (ADCI) of the growth of *P. falciparum* asexual stages *in vitro* depends on opsonization of merozoites by parasite antigen-specific IgG antibodies, leading to the release of parasitistatic effector molecules [23, 30]. We have confirmed here that the combination of monocytes with immune serum leads to substantial inhibition of parasite growth. The similar effect we observed with NK cells, presumably involving opsonisation and CD16-mediated interactions, is consistent with the fact that antibody-dependent cytotoxicity is a recognised pathway of NK cell-mediated killing [31]. Thus, the addition of serum served to enhance NK cell-mediated iRBC killing. In an attempt to define the mechanism(s) potentially involved in this process, we compared the outcome of pre-treatment of NK cells with antibodies directed to different surface-expressed molecules. A potential role for both sFasL and GrB was suggested by the results of pre-treatment with an anti-CD95 antibody and Fas-Fc soluble protein as well as

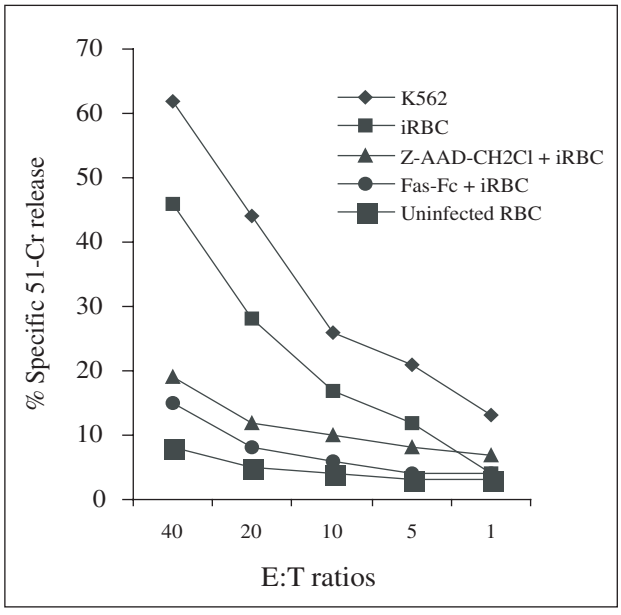

Figure 2
Cytolytic activity of NK cells from non-immune individuals against *Plasmodium falciparum*-infected red blood cells
Lysis of the indicated target cells (10^4/well) was determined in a 4-h ^{51}Cr-release assay. SD for all data were less than 3%. This experiment is representative of three performed with similar results. K562: NK-cell sensitive cell line. iRBC: infected red blood cells. RBC: red blood cells.

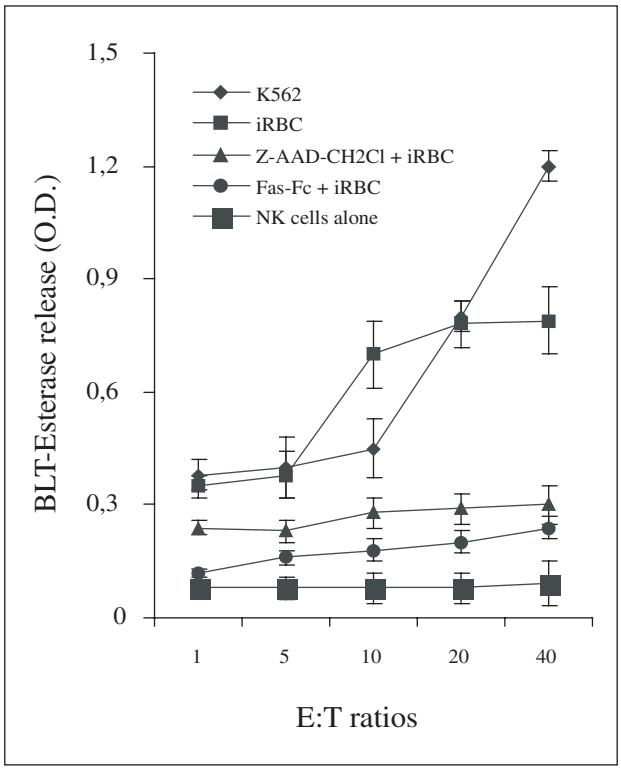

Figure 3
The release of cytolytic granules.
The release of cytolytic granules was determined in a 4-h BLT esterase release assay, performed as described in Materials and Methods. This experiment is representative of four independent experiments performed with similar results. The increases in BLT are significant between the various E:T ratios and between the various group compared to control.

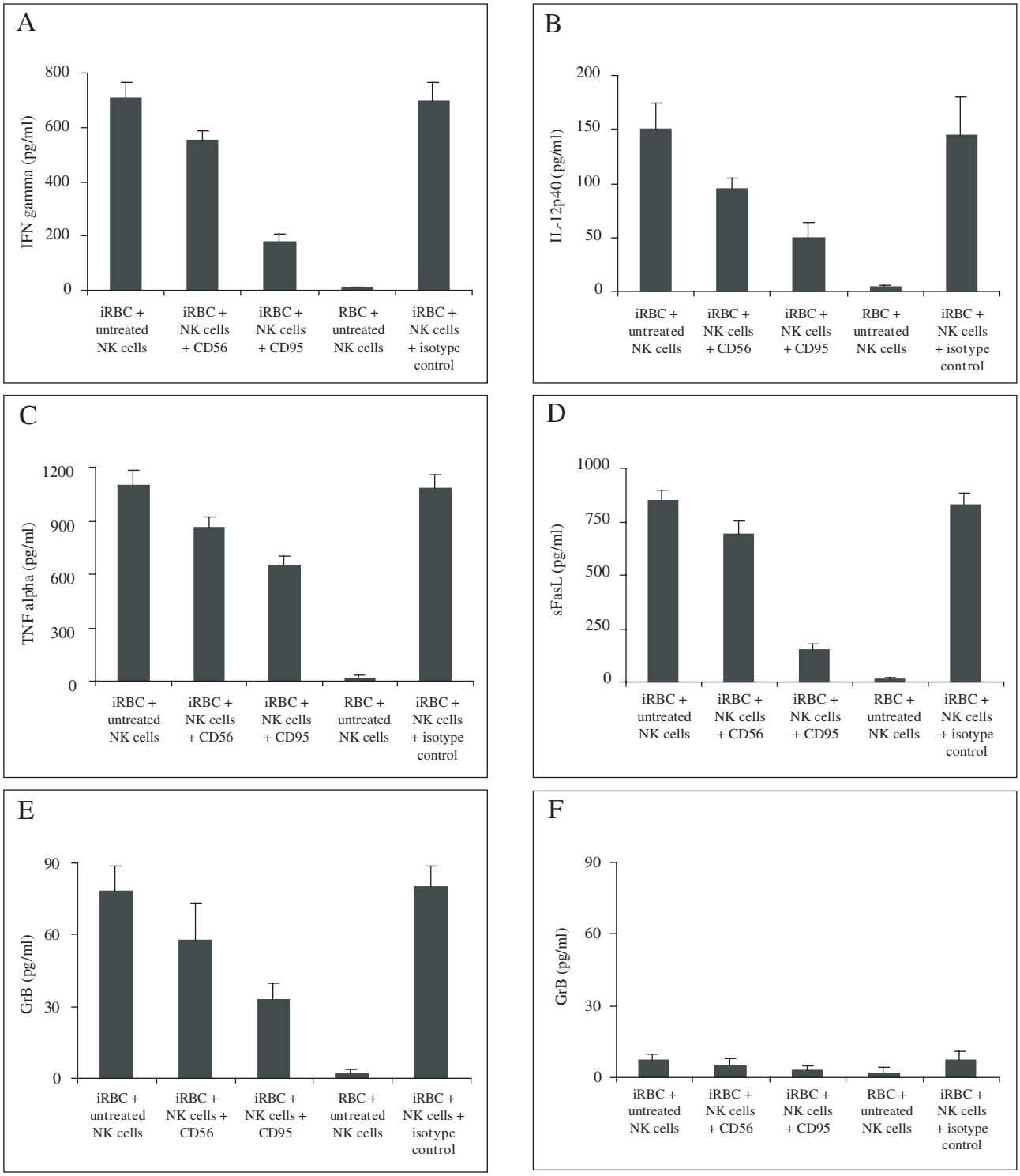

Figure 4
Cytokines, sFasL and granzyme B detection in co-culture supernatant.
Detection of (A) IFNγ, (B) IL-12p40, (C) TNFα, (D) sFasL, (E) GrB in supernatants of NK:IRBC co-cultures and (F) Inhibition of granzyme B release by prior incubation of NK cells with 100 μM GrB inhibitor Z-AAD-CH_2Cl ($p < 0.001$). Results from three donors are expressed as mean ± SEM of triplicate wells.

the GrB inhibitor Z-AAD-CH_2Cl, which led to a reduction of the levels of both molecules and was associated with complete abrogation of parasite growth inhibition. Growth inhibition was, however, also reduced following pre-treatment with an anti-CD56 antibody, which was associated with a less marked effect on sFasL or GrB levels compared with anti-CD95. We speculate that this may result from interference with appropriate effector:target cell contact mediated by CD56, although the requirement for direct cell contact in this system remains to be established [32]. Parasite growth inhibition could be maintained via antibody-CD16 interactions in the presence, as we observed, of appropriately high levels of sFasL and GrB. In the same context, the release of cytokines we observed is an indication that NK cells are strongly activated during co-culture with infected red blood cells. NK cells are

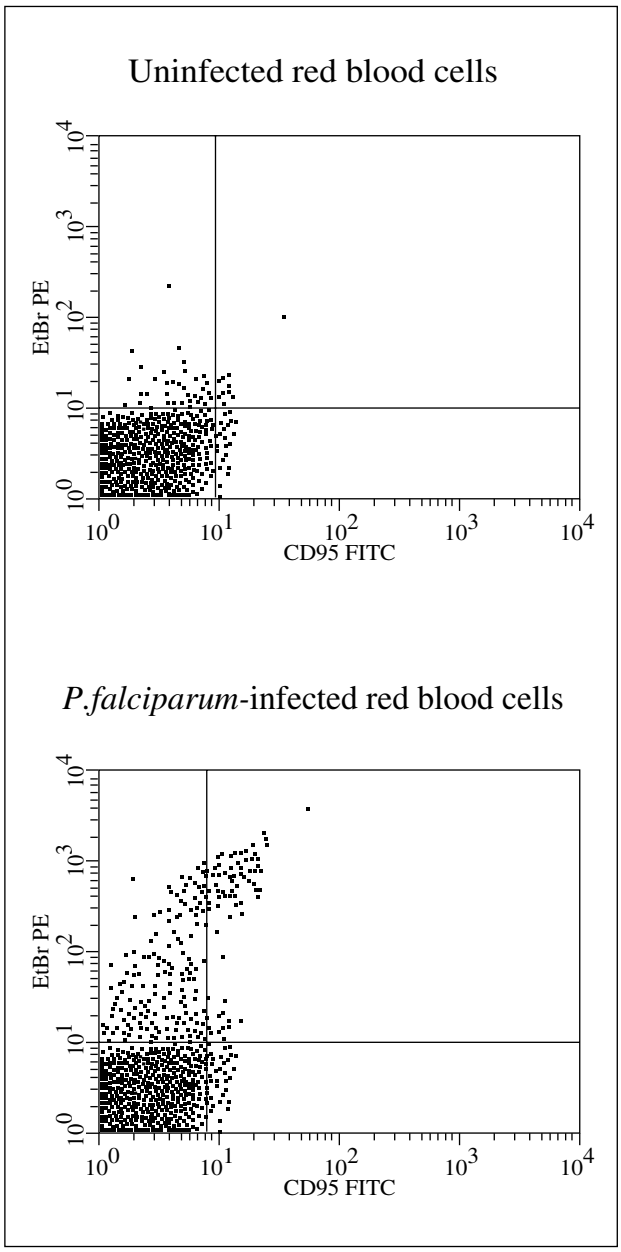

Figure 5
Up-regulation of CD95 expression on the surface of *Plasmodium falciparum*-infected red blood cells.
After liquid culture for 48 hours, uninfected (upper panel) or *Plasmodium falciparum*-infected red blood cells, 5% parasitaemia (lower panel) was harvested and stained for CD95 and EtBr. *P. falciparum*-infected red cells are shown in dotplots, with EtBr staining on the Y-axis and CD95 staining on the X-axis. Similar results were obtained in 3 separate experiments and 10,000 events were analysed in each experiment. Percentages of staining are presented in Table 2.

known to be major IFNγ producers [33], and since IFNγ acts in both an autocrine and a paracrine fashion, we assume that the TNFα, IL-12p40, sFasL and GrB detected in our co-culture supernatants were produced by NK cells as a result of initial IFNγ activation. Nevertheless, the maintenance of parasite growth inhibition in the face of the low levels of both IFNγ and TNFα observed, following antibody pre-treatment, suggests that these cytokines have little, if any, direct effect on parasite growth in this *in vitro* system.

Table 2
Percentages of single and double staining red blood cells with CD95 and EtBr

Markers	Uninfected red blood cells		*P. falciparum*-infected red blood cells	
CD95	0.14 ± 0.01	(0.15)[a]	0.14 ± 0.03	(0.17)
EtBr[b]	0.33 ± 0.29	(0.63)	2.38 ± 1.18	(3.56)
CD95/EtBr	0.03 ± 0.02	(0.05)	1.17 ± 0.03	(1.20)
IgG1a	0.01 ± 0.00	(0.01)	0.01 ± 0.01	(0.01)
IgG2a	0.01 ± 0.00	(0.01)	0.01 ± 0.01	(0.01)

[a] In parenthesis, the maximum percentage of measured responses.
[b] Ethidium bromide

Our results clearly imply a role for sFasL/Fas activity in the NK-iRBC interaction, which raises the question of how CD95 might be involved. A precise role for CD95 in immunity to *P. falciparum* has yet to be defined, but serum from patients with acute *P. falciparum* malaria does contain elevated levels of sFasL [34]. Human NK cell cytotoxicity is mediated by sFasL [35] and CD95 plays a role in the immunoregulation of some pathological states, especially of the liver and heart [36]. Erythroblasts exhibit apoptogenic receptors such as TNFRI, TNFRII and Fas [37, 38] and anti-Fas or anti-TNFR antibody can opsonize murine erythrocytes, suggesting that Fas and TNFR are expressed on erythrocytes [39]. Recent studies show that mature human erythrocytes express caspase-3 and caspase-8 [40] and can undergo a rapid self-destruction process which shares several features with apoptosis [31]. Furthermore, two CD95 signaling pathways have been identified in two cell types: type I cells have developed a way to bypass mitochondrial functions as they activate caspase-8 at the DISC followed by caspase-3, independent of mitochondrial activity. Only type II cells seem to depend on mitochondria during induction of apoptosis [42]. We speculate, therefore, that sFasL interactions with Fas on the erythrocyte surface may represent one possible mechanism of NK cell-mediated cytolysis of *P. falciparum*-infected human red blood cells.

The ability of NK cells to directly lyse *P. falciparum*-infected erythrocytes, as we describe here, has been the subject of some controversy. Coleman and colleagues [43] reported that mouse splenic lymphocytes exhibited cytotoxic activity against red cells infected with *P. berghei*, but experiments with human cells, suggested that red cells infected with *P. falciparum* were poor targets for either NK or CTL [44]. The latter study, however, used a mixed population of mononuclear cells rather than purified NK cells, resulting in considerably lower effector:target cell ratios compared to those we used here. The absence of activity observed by Theander and colleagues [45] could also be explained by their methodology. To analyze NK cytotoxic activity they used a heterogeneous cellular population (PBMC) at a density of between 4 and $16x10^6$ cells/ml. [NK cells only represent 5 to 15% of PBMC]. Moreover, their target cells (5×10^5 cells/ml) were pre-incubated with 0.1 μCi/ml ^{51}Cr, i.e. approximately 2000 times less than the concentration generally used in conventional ^{51}Cr cytotoxicity tests; this could explain the low level of Cr uptake and re-release, as conventional tests necessitate 100 to 300 μCi of radioactive chromium [46]. Others have shown that $CD3^-$ $CD16^+$ and $CD3^-CD56^+$ cells do exhibit lytic activity for schizonts and that the

cytotoxicity of $CD3^{-}CD56^{+}$ cells is increased by treatment with IFNα and/or IL-2 [47], in keeping with our results. We have shown, in addition, that NK-iRBC co-culture resulted in elevated levels of BLT esterase, a protease whose activity is preferentially mediated by granzymes [48]. BLT esterase and granzyme A can induce the destruction of cell matrix components [49]. Thus, it seems reasonable to speculate that iRBC-induced NK cell activation leads to the release of protease granules, and thus to cytotoxic effects on neighboring cells. Identification of the substrates, through the use of specific inhibitors or neutralizing monoclonal antibodies, is now necessary to determine the precise role of serine proteases in NK cell biology during *P. falciparum* infection.

To summarize, our findings establish a role for NK cell-mediated cytolysis of *P. falciparum*-infected erythrocytes, and indicate that the mechanism involves, at least partly, sFasL and GrB. The results with perhaps greatest relevance to *in vivo* conditions are those obtained in the presence of immune serum. The ability of NK cells to interact with transplacentally-transferred maternal antibodies may represent an important first line of defense for neonates, and is an aspect we are actively investigating. Studies with murine models of malaria do lend strong support to the idea that NK cells contribute to protective immune responses against plasmodia [50-52]. Ongoing work in our laboratory seeks to further delineate human NK cell responses both in non-exposed individuals and during and after naturally acquired *P. falciparum* infections in exposed populations.

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