The interplay between *Leishmania* promastigotes and human Natural Killer cells *in vitro* leads to direct lysis of *Leishmania* by NK cells and modulation of NK cell activity by *Leishmania* promastigotes

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SUMMARY

NK cells represent one of the first lines of defence in the immune reaction after invasion of *Leishmania* parasites. Depletion of mouse natural killer (NK) cells dramatically enhances susceptibility of normally resistant mice. In this study we evaluated the fate of NK cells and parasites after contact formation. The hydrophilic fluorescent dye CMFDA (chloromethylfluorescin diacetate) that allows analysis of cytotoxicity in flow cytometry and microscopy was used. Furthermore, these findings were confirmed with scanning and transmission electron microscopy. Direct contact points were found between *Leishmania* promastigotes and naïve human NK cells. These contacts were associated with transfer of cytosol by membrane bridges and cytotoxicity of NK cells against *Leishmania*. However, in contrast to other target cells which allow repeated exocytosis of lytic granules, contact with *Leishmania* causes immediate destruction of NK cells in a non-apoptotic way. Our results give a reasonable explanation for *ex vivo* observations of reduced NK cell numbers and impaired NK response in patients with acute cutaneous leishmaniasis. Animal models have clearly shown that NK cells play a key role in the induction and direction of the immune response. Thus inhibition of NK cells at the onset of infection would be advantageous for the survival of the parasite.

Key words: cutaneous leishmaniasis, human NK cells, direct recognition, lysis.

INTRODUCTION

Cutaneous leishmaniasis (CL) following *Leishmania major* or *Leishmania aethiopica* infections manifests in human as localized ulcers that usually heal after induction of a potent Th1 immune response. However, in some patients after *L. aethiopica* infection the parasites spread all over the body causing lesions that may last several years, referred to as diffuse CL, DCL (Convit and Kerdel-Vegas, 1965; Bryceson, 1969) or in the case of *L. major* the parasites distribute over the lymphatic system in so-called non-healing CL (Habibi *et al*. 2001). Studies aimed at understanding the different clinical manifestation have shown that non-healing DCL is associated with increased levels of IL-10 in the affected patients (Akuffo *et al*. 1997). In addition, patients with *L. aethiopica*-induced CL have significantly diminished numbers of NK cells in the blood during the active phase of infection while cured individuals have levels in the range of healthy donors (Maasho *et al*. 1998).

NK cells are known to release interferon gamma (IFN-γ) within hours after infection with respective protozoan parasites in diseases such as Chagas disease, malaria, toxoplasmosis and leishmaniasis (reviewed by Korbel *et al*. 2004). In the mammalian host *Leishmania* multiply in phagocytic cells, and the activation of infected macrophages by cytokines such as IFN-γ has been identified as key for parasite killing (Belosevic *et al*. 1989; Laskay *et al*. 1995). Following infection with a number of *Leishmania* spp. there is an initial peak of NK cells and *L. major* infection models have shown that the NK cells direct the immune response to a Th1 phenotype within the first 3 days of infection (Scharton and Scott, 1993).

Human NK cells, which make up 5–20% of the peripheral blood lymphocytes, are classically defined as $CD3^-$ and $CD16/56^+$. Functionally, NK cells can be subdivided into 2 populations: (1) $CD16^{dim}/CD56^{bright}$ NK cells with potent

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cytokine producer ability, and (2) $CD16^{bright}$/$CD56^{dim}$ NK cells, comprising 90% of blood NK cells, which exhibit strong cytotoxic activity (Cooper *et al*. 2001). NK cell function is governed by a fine-tuned balance of signalling from inhibitory and activating receptors expressed on NK cells, such as the killer immunoglobin-like receptors (KIR) (Cerwenka and Lanier, 2001). These bind to MHC class I molecules on target cells. The lack of MHC class I molecules increases target cells susceptible for NK cell cytotoxicity, initially described in the missing-self theory (Karre, 2002). Additionally, it has been described that NK cells can be activated through exposure to Toll-like receptor (TLR) ligands, leading to induction of (IFN)-γ production (Becker *et al*. 2003). Furthermore, recognition of parasites by NK cells can lead to direct lysis if the surface molecules of the parasites interact with the NK cells (Lieke *et al*. 2004).

The surface coat of infectious promastigotes of *Leishmania* species consist mainly of lipophosphoglycan (LPG), proteophosphoglycan (PPG) and GPI-anchored protein with the prominent glycoprotein (gp) 63 (Ilgoutz and McConville, 2001). We have previously reported that promastigotes induce cytokine production by purified NK cells (Nylen *et al*. 2003), and have also shown that gp63 binds to a subset of human NK cells resulting in modulation of NK receptor expression and suppression of proliferation (Lieke *et al*. 2008).

In this study, we show direct interactions of human NK cells with *Leishmania* promastigotes leading to lysis of the parasites coupled with a significantly decreased number of NK cells with the remaining NK cells exhibiting signs of exhaustion. The results are descriptive and we acknowledge that they do not mimic the *in vivo* situation. However, the implications may still be relevant *in vivo* since NK cells might get into contact with *Leishmania*-derived molecules from the surface coat of promastigotes if not with whole *Leishmania* promastigotes.

MATERIALS AND METHODS

Parasites and donors

Leishmania aethiopica (isolated in Ethiopia from a patient with localized cutaneous leishmaniasis, LCL) and the wild type *L. major* strain NIH S (MHOM/SN/74/Seidman) clone A2 (A2WF) were propagated as previously described (Maasho and Akuffo, 1992). For infection of monocytes the *L. major* Friedlin strain was used. Live promastigotes harvested at stationary growth phase were used in all assays.

Blood was collected from healthy laboratory workers resident in Sweden with no history of leishmaniasis or history of travel to leishmaniasis-endemic areas. Buffy coat cells from blood donors at the Karolinska Hospital, Stockholm, were also used. Informed consent and ethical approval was received to perform these studies.

Preparation, isolation and stimulation of NK cells

PBMC were isolated from defibrinated or heparinized blood on a Ficoll gradient as previously described (Boyum, 1968; Nylen *et al*. 2001). NK cells and T cells were isolated by negative selection using MACS NK cell isolation kit II and MACS Pan T cell isolation kit II (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. $CD14^+$ monocytes were isolated by positive selection using MACS CD14 MicroBeads (Miltenyi Biotech). The purity of isolated NK cells, T cells and $CD14^+$ cells was checked for each preparation using flow cytometry and found to be $\geqslant$90% $CD16/56^+ - CD3^-$ and $CD14^+$, respectively.

Cells were incubated in RPMI medium supplemented with L-glutamine, antibiotics and 10% heat-inactivated human serum (AB+ serum, Karolinska Hospital).

Surface marker expression

Freshly isolated or cultured PBMC were stained for surface marker expression using anti-human CD16-APC, CD56-PE (clone B159), CD14-PE, NKG2D-PE and CD3-PerCP (all from BD Biosciences, San Diego, CA, USA).

Determination of parasite to cell ratio for each assay

The parasite to cell ratio used for each of the assays was determined following dose-response tests. The optimal ratio and cell numbers varied from assay to assay. The optimal ratio and cell numbers are indicated for each assay.

Labelling of promastigotes with CMFDA

For quantification of cytotoxic activity, 1×10^8 culture-derived promastigotes of *L. aethiopica* and *L. major* were washed in serum-free medium and viable cells were tracked through labelling with 4 μM CellTracker Green (5-chloro-methylfluorescin diacetate (CMFDA); Molecular Probes, Eugene, Oregon, USA) for 20 min at 26 °C. CMFDA is initially a non-fluorescent molecule able to pass the cell membrane and be cleaved in viable cells into the fluorescent, strongly hydrophilic form building a bulky water sheath averting its release through the intact cell membrane of viable cells. However, in cells where there is membrane leakage and loss of the cytosol, CMFDA exits the cell with the cytosol and lysed cells become non-fluorescent. Thus CMFDA acts as a marker for viability. Promastigotes were subsequently washed in RPMI medium warmed to

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room temperature (20 °C) containing 10% human serum and were further incubated for 30 min at 26 °C. After a single final wash labelled *Leishmania* were incubated with lymphocytes.

Assessment of viability of Leishmania *promastigotes by flow cytometry or fluorescent microscopy*

Samples of 1×10^6, 5×10^5 and 1×10^5 CMFDA-labelled parasites were incubated with 1×10^6 freshly purified NK cells for 4 h at 26 °C in the dark. Cells were washed in PBS once, fixed with 2% paraformaldehyde, and analysed with a FACS-Calibur (Becton Dickinson, Mountain View, CA, USA). For assessment of differences in fluorescence of the promastigotes 20 000 events were analysed.

Alternatively, viability was analysed by fluorescent microscopy. For this, 1×10^6 *L. aethiopica* and *L. major* A2WF promastigotes (labelled with 10 μM CMFDA) were incubated with $2–3\times10^5$ purified NK cells at 26 °C in the dark (optimal range of parasite to NK cells determined by preliminary experiments). Twenty μl of this culture were transferred to 15-well glass slides and air-dried overnight in the dark. Samples were fixed in methanol-acetone (1:1) for 10 min and dried again. After washing 3 times with PBS, samples were stained with anti-LPG antibodies (a kind gift from Dr Sam Turco, College of Medicine, University of Kentucky, USA) for 30 min. Samples were washed twice and stained with goat-anti-mouse-Cy5 antibodies (Jackson, Soham, Cambridgeshire, UK) and with HOECHST 33342 (Pierce, Fisher Scientific AB, Goteborg, Sweden) for nuclear staining. Slides were covered and preserved wet with a DABCO/glycerol anti-fading solution.

For assessment of interactions of NK cells with infected monocytes, 2×10^6 purified $CD14^+$ cells were incubated overnight in 8-well Lab-Tek Chamber Slides (Nunc, Wiesbaden, Germany) and infected with promastigotes (MOI 1) of *L. major* Friedlin strain for 2 days before 5×10^5 purified CMFDA-labelled NK cells were added for 4 h. Cells were fixed with 4% PFA for 2 h at room temperature and air-dried overnight. The procedure followed the protocol for staining of promastigotes and NK cells.

Assessment of NK cell viability by flow cytometry or light microscopy

Freshly purified NK cells were incubated with *Leishmania* promastigotes for 4 h as described above and the number of live NK cells was evaluated by Trypan Blue exclusion using light microscopy. In addition, the proportion of dead NK cells after 12 h of co-culture with promastigotes was assessed by propidium iodide (PI) uptake (BD Biosciences) using flow cytometry analysis.

NK cell cytotoxicity assay

For functional analysis of general NK cytotoxicity after incubation with promastigotes NK cells were incubated with human target cell line K562 and murine P815 cells, respectively. P815 cells are commonly not recognized by human NK cells but, transfected with Fc-γRIII receptor and coated with antibodies, these cells served as read-out for reverse antibody-dependent cellular cytotoxicity (rADCC).

For radioactive labelling cells were used in exponential growth state. Cells were washed once and incubated with 0·1mCi for 90 min in medium at 37 °C. Cells were washed 3 times with warm medium and counted.

For rADCC, murine P815 were either untreated after radioactive labelling or were coated with 5 μg/ml anti-human CD16 or anti-human NKG2D for 30 min at room temperature in FCS-free medium. Cells were washed twice in FCS-supplemented medium to remove unbound antibodies.

For the cytotoxicity assay, 1×10^5 NK cells were co-cultured with 5×10^4 *L. major* promastigotes for 24 h. Then the cells were collected, stained for characteristic NK receptors or incubated in variable effector:target ratios with ^{51}Cr-labelled human MHC class I deficient K562 cells or antibody coated P815 cells for measurement of cytotoxicity. After incubation for 4 h at 37 °C, a 100 μl sample of the supernatant was analysed for ^{51}Cr. For spontaneous and maximum release of ^{51}Cr, target cells were incubated with medium alone or with 1% Triton, respectively. Specific lysis was calculated from triplicates using the formula cpm (sample) – cpm (spotaneous)/cpm (maximum) – cpm (spontaneous).

Scanning and transmission electron microscopy

For scanning electron microscopy (SEM), 3×10^5 highly purified NK cells (purity over 98%) were incubated with 3×10^5 *L. major* promastigotes for 4 h at 26 °C. Cells were subsequently fixed with 2% glutaraldehyde in 100 mM sodium cacodylate buffer. The samples were critical-point dried and applied to poly(L-lysine)-coated cover slides (Cellocate; Eppendorf). Slides were washed after 2 h and fixed again with 1% osmium oxide in 100 mM sodium cacodylate buffer for 30 min at 4 °C. After repeated washing, cells were dehydrated with increasing ethanol concentrations and dried thoroughly. Samples were spotted with gold and analysed in a scanning electron microscope (PSEM 500; Philips, Hamburg, Germany). For transmission electron microscopy (TEM), 1×10^6 bulk PBMC were incubated with 1×10^6 *L. major* promastigotes for 4 h at 26 °C. The cells were treated as described for SEM, dehydrated with graded ethanol solutions and propylene oxide. The cells were embedded using the AGAR-100 kit (Plano, Wetzlar, Germany), 70-nm

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ultrathin sections were cut (Ultra Cut E; Reichert/Leica, NuBlock, Germany) and counter-stained with uranyl acetate and lead citrate. Sections were examined with a Philips CM 10 transmission electron microscope at an acceleration voltage of 80 kV.

Statistical analysis

Most of the assays involved microscopic analysis and thus no statistics were performed. When numerical comparisons were made the Student's *t*-test and two-way Anova *F*-test analysis were performed.

RESULTS

Contact formation between human NK cells and Leishmania *promastigotes*

Previous reports from our group showed direct interactions of human NK cells with extracellular promastigotes of various *Leishmania* strains causing cutaneous leishmaniasis (Lieke *et al*. 2008; Nylen *et al*. 2003). In this study we aimed for visualization of these contacts using electron and fluorescence microscopy.

Scanning electron microscopy showed tight contacts between NK cells and *L. major* promastigotes leading to deformation of the parasite's cell membrane (arrows) and significant changes in size and shape (Fig. 1A). In transmission electron microscopy attachment of membranes over a large area was detectable, indicating highly ubiquitously expressed receptor-ligand counterparts. At some sites contacts appeared to be accompanied by fusion of the cell membranes of NK cells and promastigotes allowing exchange of cytoplasm (Fig. 1B, magnification of highlighted area).

To verify this impression in fluorescence microscopy we labelled promastigotes of *L. major* with CMFDA, a green fluorescent dye which is completely soluble in the cytosol thus can be used as indicator of cytoplasm flow and cytotoxic activity by loss of fluorescence due to membrane leakage. CMFDA labelling resulted in fluorescence of the complete body of *Leishmania* promastigotes (Fig. 1C i). Incubation of promastigotes with NK cells also revealed contact between the lymphocytes and parasites giving the impression of direct exchange of cytoplasm (Fig. 1C ii–iv, magnification of framed area) that appeared as fuzzy clouds of fluorescence around or within NK cells or as very distinct spots of fluorescence.

These observations were seen with both *L. major* promastigotes as well as promastigotes of *L. aethiopica* (Fig. 1D).

Expression of prominent promastigote surface molecules on infected monocytes

It is acknowledged that prolonged exposure of NK cells to extracellular promastigotes *in vivo* is unlikely. Thus, it is of interest to know if NK cells also interact with infected monocytes. However, contacts indicated recognition by NK cells of molecules on the surface coat of promastigotes. It has been reported that infected cells incorporate *Leishmania*-derived molecules in their cell membrane at the site of invasion (Descoteaux and Turco, 2002). Isolated $CD14^+$ monocytes were exposed to *L. major* metacyclic promastigotes and after 2 days the cells were stained with antibodies against LPG, one of the most prominent surface molecules on promastigotes. LPG staining was observed associated with intracellular amastigotes which were identified with nuclear staining by HOECHST 33342. In addition, merged pictures of fluorescent and bright light microscopy identified LPG occurrence on the surface of infected monocytes (Fig. 2A). CMFDA-labelled purified NK cells were incubated with infected monocytes in order to follow contact and interaction between the NK cells and infected monocytes. However, it was not possible to confirm whether either the monocytes or the NK cells were damaged due to these contacts (Fig. 2B). CMFDA-labelled NK cells were used to follow possible flow of cytosol from NK cells to monocytes since membrane bridges allow unselective flow in both directions. However, no adoption of fluorescence by the monocytes could be observed (Fig. 2B) and vice versa (data not shown).

Nevertheless, since one of the main characteristics of NK cells is cytotoxic activity, numerous experiments were performed to evaluate whether expression of *Leishmania*-derived molecules on monocytes leads to lysis of infected cells despite the protection of self-MHC which prevents NK cell-mediated cytotoxicity. However, neither radioactive nor fluorescence labelling of monocytes gave convincing results since the infection rate in the culture was too low to detect differences after exposure to NK cells in comparison to untreated infected cells. Consequently, to evaluate whether recognition of *Leishmania*-derived molecules elicits cytotoxic activity, assessment of exposure of *L. major* promastigotes to NK cells was chosen instead.

Lysis of Leishmania *promastigotes by human NK cells*

Promastigotes are resistant to radioactive labelling thus CMFDA was again used as the indicator of lysis of protozoan target cells (Lieke *et al*. 2004). To perform a statistically significant assessment of possible cytolytic activity of NK cells against *Leishmania* promastigotes, flow cytometry and promastigotes of *L. aethiopica* were used because, due to their size, promastigotes of this species can be separated from the lymphocytes in the FSC/SSC, allowing separated analysis of changes in fluorescence of each population (Fig. 3A). Different ratios of purified NK cells to parasites from 10:1, 5:1 down to

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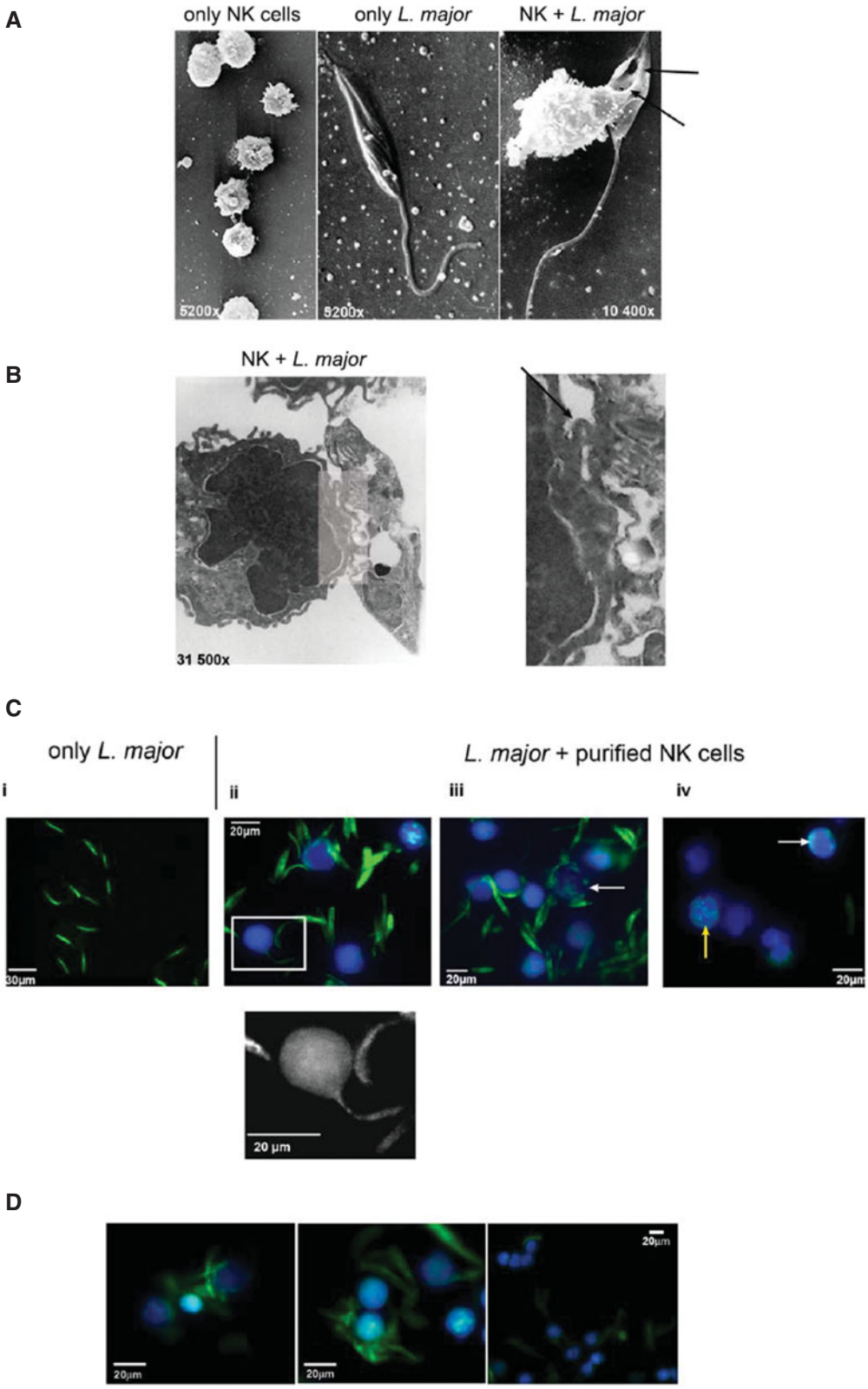

Fig. 1. Contacts between human NK cells and *Leishmania major* promastigotes. Promastigotes of *L. major* and *L. aethiopica* were incubated with purified NK cells at a ratio of 1:1 for 4 h and analysed either by electron (A and B) or fluorescence (C and D) microscopy. Electron microscopy revealed tight contacts between NK cells and cell bodies of promastigotes of *L. major* (A) that seemed to be associated with fusion of the membranes (B, arrow). Contacts were confirmed in fluorescence microscopy using CMFDA-labelled *L. major*. CMFDA generated a homogenous fluorescence of the whole cell body (C i). Incubation of promastigotes with NK cells (C ii–iv) caused fuzzy clouds of fluorescence around NK cells (white arrows) or very distinct spots (yellow arrow). These interactions are reminiscent of fusion of the cell membranes of lymphocytes and promastigotes (B, left picture and C ii, enlargement of framed area; to enhance the contrast the magnification is shown as a black-white picture). Contacts of NK cells with *Leishmania* promastigotes were not restricted to *L. major* species but were also detectable using promastigotes of *L. aethiopica* with comparable consequences as for *L. major* (D). Data presented as representative pictures of 4 independent experiments.

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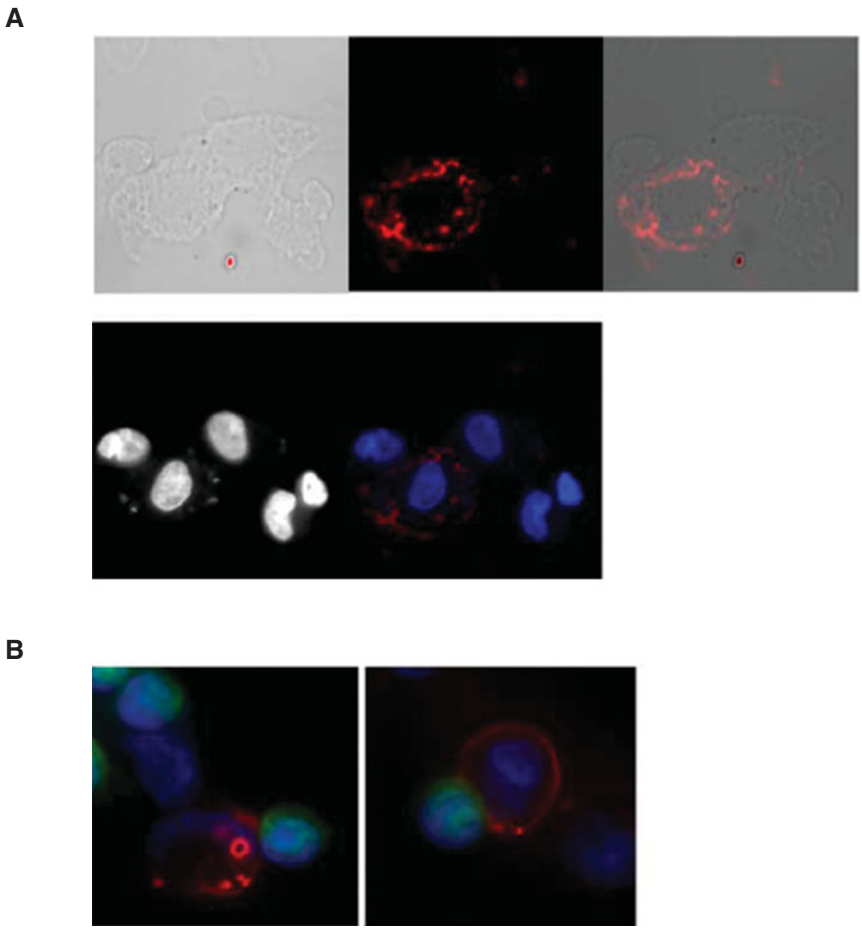

Fig. 2. Expression of *Leishmania*-derived molecules on the surface of infected monocytes enables contacts between infected cells and NK cells. Purified $CD14^+$ monocytes were infected with metacyclic promastigotes of *L. major* for 2 days and then incubated with purified CMFDA-labelled NK cells (green) for 4 h. Cells were fixed and stained for LPG (red) and nuclear HOECHST 33342 staining (blue). Infection of monocytes led to well-detectable intracellular LPG expression that was partly associated with amastigotes but also with LPG expression on the surface of monocytes (A). Incubation of monocytes with NK cells revealed tight interactions between NK cells and infected monocytes (B). Data presented as representative pictures of 4 independent experiments.

1:1 were assessed. As shown in Fig. 3A, Gate 1, not only the number of $CMFDA^+$ parasites decreased at an E:T ratio of 10:1 but also the total number of parasites, indicating an efficient killing and complete disruption of *L. aethiopica* when co-cultured with NK cells. Killing of *L. aethiopica* at an E:T ratio of 5:1 was evident already after 1 h of incubation (over 30% loss of fluorescence of the promastigotes) and further increased with time (up to 60% after 4 h). However, almost no killing was detected at a 1:1 PBMC to promastigote ratio (Fig. 3B).

In addition, according to the observed adopted fluorescence of NK cells, a shift in the population of NK cells was measurable after incubation with CMFDA-labelled parasites in flow cytometry (Fig. 3A, Gate 2). This phenomenon was more pronounced at an E:T ratio 1:1 compared to 10:1. Of note is the finding that exposure of NK cells to promastigotes 1:1 also caused an obvious reduction of NK cell number since the cell counts in Gate 2 were remarkably decreased. We took this as an indication of increased mortality of NK cells after contact with *Leishmania* and this path was then followed using microscopic methods.

Indications of NK cell death following co-culture with Leishmania *promastigotes*

Investigation of interactions between live NK cells and promastigotes by light microscopy showed cellular contacts that left the NK cells with multiple vesicle-like structures resembling necrotic cells (Fig. 4A). Counting the total numbers of viable NK cells after incubation with promastigotes of both *L. aethiopica* and *L. major*, revealed a significant decrease of viable NK cells (Table 1). Interestingly, this occurs in the absence of detectable Trypan blue-positive (dead) cells. In addition, only a small increase in dead NK cells was observed after 12 h in culture as assessed by the proportion of propidium iodide-positive (PI^+) cells using flow cytometry

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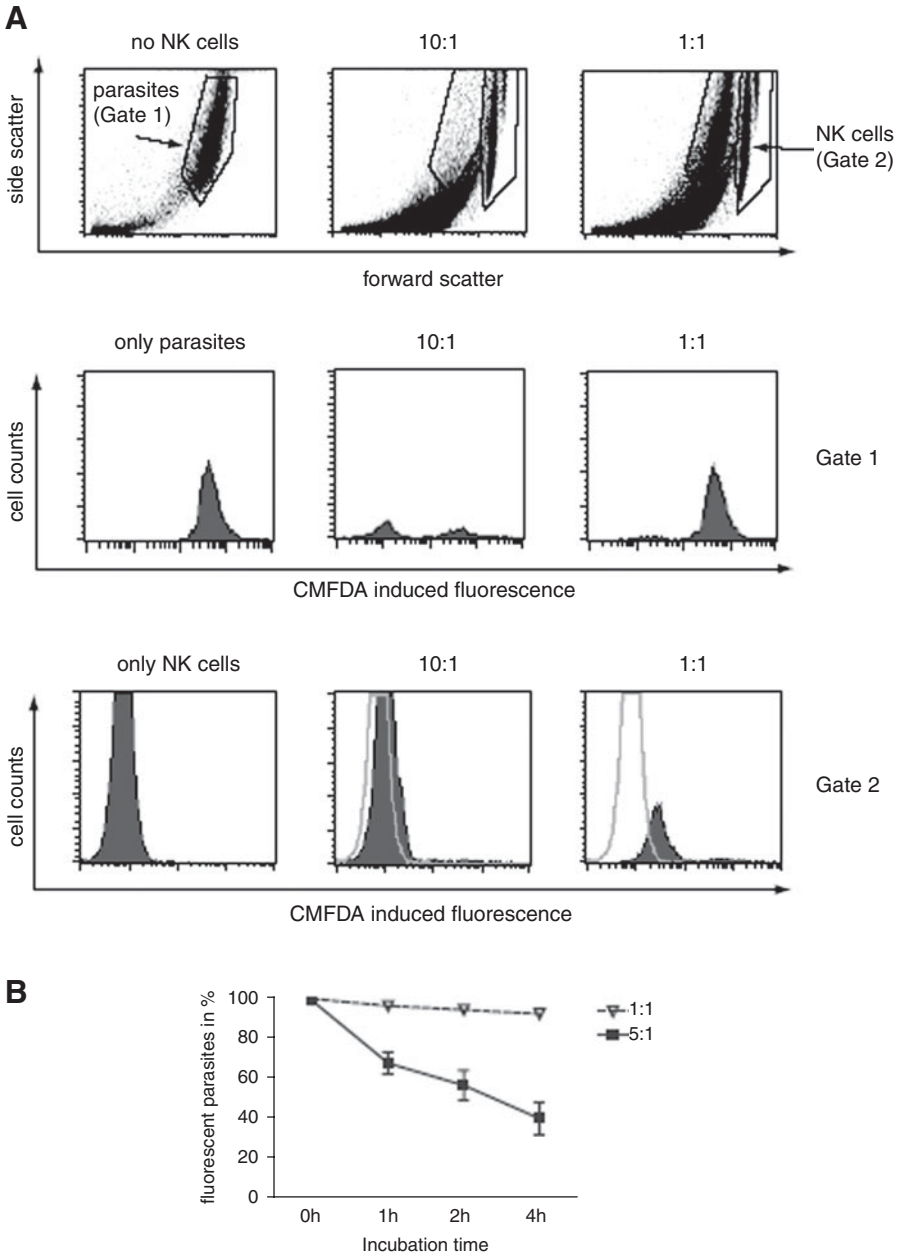

Fig. 3. Direct lysis of *Leishmania aethiopica* promastigotes by human NK cells. Purified NK cells were incubated with CMFDA-labelled promastigotes of *L. aethiopica* and cytotoxicity was analysed by flow cytometry. A population of *L. aethiopica* promastigotes (Gate 1) could be distinguished from the population of lymphocytes (Gate 2) in forward-side-scatter diagram (A) allowing assessment of changes in fluorescence in the separated populations indicating lysis of parasites. Of note: the forward scatter is on a log scale whereas the side scatter is on a linear scale. Promastigotes and lymphocytes were incubated at different ratios for 4 h at 26 °C in the dark. The histogram represents the gated parasites (Gate 1) or gated NK cells (Gate 2). Incubation of NK cells with parasites decreased not only fluorescence of promastigotes but reduced drastically the number of promastigotes in their specific area (evident at a ratio of 10:1 in Gate 1) indicating complete destruction of body structure. A ratio of 1:1 NK cells to promastigotes seemed to have almost no effect on *Leishmania* viability. In contrast, equal numbers of NK cells and promastigotes led to significantly reduced numbers of NK cells in gate 2 and the remaining NK cells showed increased levels of fluorescence which was not that prominent at an E:T ratio of 10:1. The time-course of lytic activity of NK cells against *Leishmania* revealed resistance of promastigotes at a ratio of 1:1 but fast initiation of lysis with an E:T of 5:1 after 1 h leading to a decreased percentage of viable *Leishmania* which was further increased after 4 h (B). Representative histogram and scatter-gram or summary with standard deviation (S.D.) of 3 independent experiments are shown.

analysis, suggesting total NK cell damage. In this respect it is notable that NK cells showed no signs of apoptosis after 4 and 24 h of incubation with *Leishmania*, respectively (data not shown).

To further explore the promastigote-NK cell lytic interaction, *Leishmania* promastigotes were labelled with monoclonal antibody against LPG. Not all promastigotes were recognized by this specific

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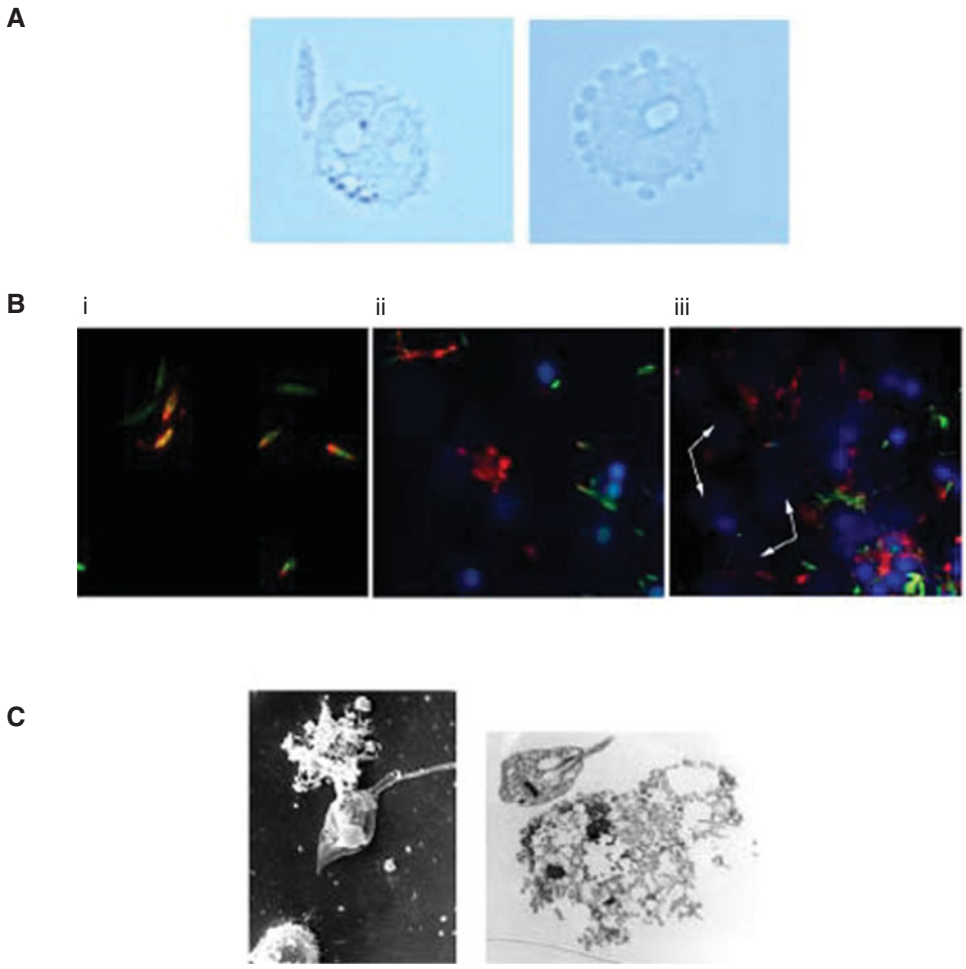

Fig. 4. Cytotoxic activity against promastigotes of NK cells leads to destruction of NK cells. Unlabelled purified NK cells were incubated with CMFDA-labelled (green), LPG-stained (red) *Leishmania major* promastigotes at a ratio of 1:1 for 4 h. Cells were observed in a viable state in bright light microscopy or fixed and nuclear staining using HOECHST 33342 was performed. In bright light microscopy numerous contacts between NK cells and promastigotes could be detected. This affected predominantly NK cells as it could be detected in flow cytometry at this ratio. NK cells revealed numerous vesicle and bubble-like structures within and on the surface of the cells resembling necrotic cells (A). Using fluorescence microscopy, the control cultures where parasites were incubated without NK cells, not all CMFDA-labelled promastigotes (green) were marked with the LPG-antibody (B, i). However, in the presence of NK cells (blue) spots of LPG revealed complete destruction of parasite bodies (B, ii and iii). In addition large amounts of diffuse DNA were detectable (iii; arrows). SEM as well as TEM confirmed that as well as promastigote destruction, NK cells suffer after exposure to *L. major* (C). Data presented as representative pictures of 4 independent experiments.

monoclonal antibody; however, using this anti-LPG antibody, intact promastigotes could be distinguished from disrupted ones in which the destroyed cell membrane appeared as LPG-labelled red spots while the cell surface of live promastigotes revealed a constant expression of LPG. The monoclonal antibody revealed significant destruction of the promastigote membrane when cultured with NK cells (Fig. 4B).

Furthermore, co-incubation of NK cells with *Leishmania* showed, in addition to disruption of promastigotes, diffuse DNA by HOECHST 33342 staining, reminiscent of non-viable cells (Fig. 4B arrows). No such staining was observed in control NK cell cultures incubated for the same time-period with medium alone (data not shown). It could not be ascertained using fluorescent microscopy whether this DNA originated from lysed parasites or dead NK cells. On the other hand there were some indications of death and complete disruption of NK cells as well (Fig. 4C).

Co-culture of NK cells with Leishmania *promastigotes causes general decreased cytotoxicity*

Fluorescence microscopy revealed cellular contacts with fluorescent transfer and indication of cell death for both NK cells and parasites. However, numerous NK cells appeared normal in microscopy and thus the question arose as to whether only those NK cells which got into contact with the promastigotes underwent necrosis and all the other NK cells

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Table 1. Percentages of dead/viable purified NK cells after exposure to *Leishmania* promastigotes

(5×10^5 NK cells were incubated with 5×10^5 *Leishmania* promastigotes for 4 h and subsequently stained with propidium iodide for 10 min on ice and analysed in flow-cytometry or counted in light microscopy. There were no Trypan blue-positive cells, there were simply less cells to count. Results are summarized as mean ± S.D. of 6 independent experiments.)

	NK cells viability	
Leishmania	Propidium iodide$^+$ dead cells	% Live cells
Medium	1·1 ± 1·3	100
L. aethiopica	5·2 ± 2·0	53 ± 23
L. major	5·2 ± 7·7	37 ± 16

remained viable and active, or whether all NK cells suffered from the exposure to promastigotes but only some died. We assessed the general state of NK cells by a rADDC using Fc expressing murine P815 coupled with anti-CD16 or anti-NKG2D. As reported earlier incubation of NK cells with promastigotes of *Leishmania* had drastic impact on the expression of certain NK receptors whereas others remained unaffected (Lieke *et al*. 2008). This refers to CD16 for which expression was significantly lowered after exposure to promastigotes and NKG2D that was not affected by *Leishmania* (Fig. 5A). However, both receptors are involved in cytotoxicity of NK cells. Fig. 5B shows that co-incubation of NK cells with *L. major* reduced the ability of NK cells to kill the MHC class I-deficient cell line K562. While there was some background killing of uncoated P815 cells by non-exposed NK cells, NK cells exposed to promastigotes showed no killing of uncoated P815 cells (Fig. 5C). Furthermore, CD16 (Fig. 5D) and also NKG2D (Fig. 5E) triggered killing was reduced in NK cells exposed to *Leishmania*. Overall, NK cells co-cultured with *Leishmania* promastigotes exhibited a decreased general cytolytic activity compared to non-exposed NK cells which was not dependent on loss of CD16 expression.

DISCUSSION

In this study we present data indicating that recognition of *Leishmania*-derived molecules by NK cells leads to lysis of promastigotes, generally reduced NK activity and increased cell death. Furthermore, we have shown contact between NK cells and *Leishmania*-infected monocytes expressing molecules of the promastigotes on their surface.

The development of *Leishmania* spp. in the mammalian host starts with the bite of sand flies releasing promastigotes into the wound in the skin through which they might enter the blood stream or infect cells nearby. Promastigotes are protected from complement lysis by generation of a surface coat covering the whole cell membrane including the flagellum (Ilgoutz and McConville, 2001). Furthermore, proteins of the surface coat bind to molecules on macrophages allowing the entrance to the final host cell (Russell, 1987; Brittingham *et al*. 1995). These surface coat-derived molecules are expressed independently of MHC molecules shortly after infection on the surface of infected cells (Descoteaux and Turco, 2002).

Therefore, it is unlikely that NK cells get into extensive contact with promastigotes *in vivo,* however, using promastigotes for *in vitro* studies as the wearer of the surface coat is a valid model to investigate consequences of NK recognition of the corresponding molecules. The greater relevance is if the detected recognition of *Leishmania*-derived molecules by NK cells provokes comparable activity, particularly the lytic activity, against infected cells as it does against extracellular promastigotes. Our results show complete disruption of extracellular promastigotes, indicating engagement of receptors eliciting cytotoxicity. In addition, we confirmed expression of a major *Leishmania* surface coat molecule: LPG on infected monocytes. However, it cannot be expected that infected monocytes are lysed by autologous NK cells even if they express NK cell activating *Leishmania*-derived molecules since the expression of self-MHC protects monocytes from NK activity. Another possibility of interactions of infected monocytes and NK cells is the more likely impairment of NK cell activity after binding the *Leishmania* molecules. It needs to be elucidated whether the cell death and general NK cell inhibition is a result of lytic activity or is mediated alone by interaction with the *Leishmania*-derived molecules. We made several attempts to determine whether the contacts that are detectable between NK cells and infected LPG-expressing monocytes induced the same outcome as when using promastigotes. However, we were unable to confirm this due to limitations in the *in vitro* methods at this point, in part due to the low infection rate of monocytes *in vitro*.

In this study the two species *L. major* and *L. aethiopica*, both agents of cutaneous leishmaniasis, were used. While the knowledge of the immune response against *L. major* is based on multiple studies in mouse models and *in vitro* infections, *L. aethiopica* lacks an adequate mouse model (Akuffo *et al*. 1990). However, *ex vivo* data of patients with acute CL gave strong indications of impaired NK cell activity with both agents: *L. major* infection reduced the IFN-γ response of NK cells (Lieke *et al*. 2008) and acute *L. aethiopica* infection decreased NK cell numbers in the blood of patients (Maasho *et al*. 1998). The possibility of direct inhibition of NK cells by *Leishmania* and *Leishmania*-derived molecules is underlined by direct binding of *Leishmania* gp63 leading to blockage of NK cell proliferation. Thus,

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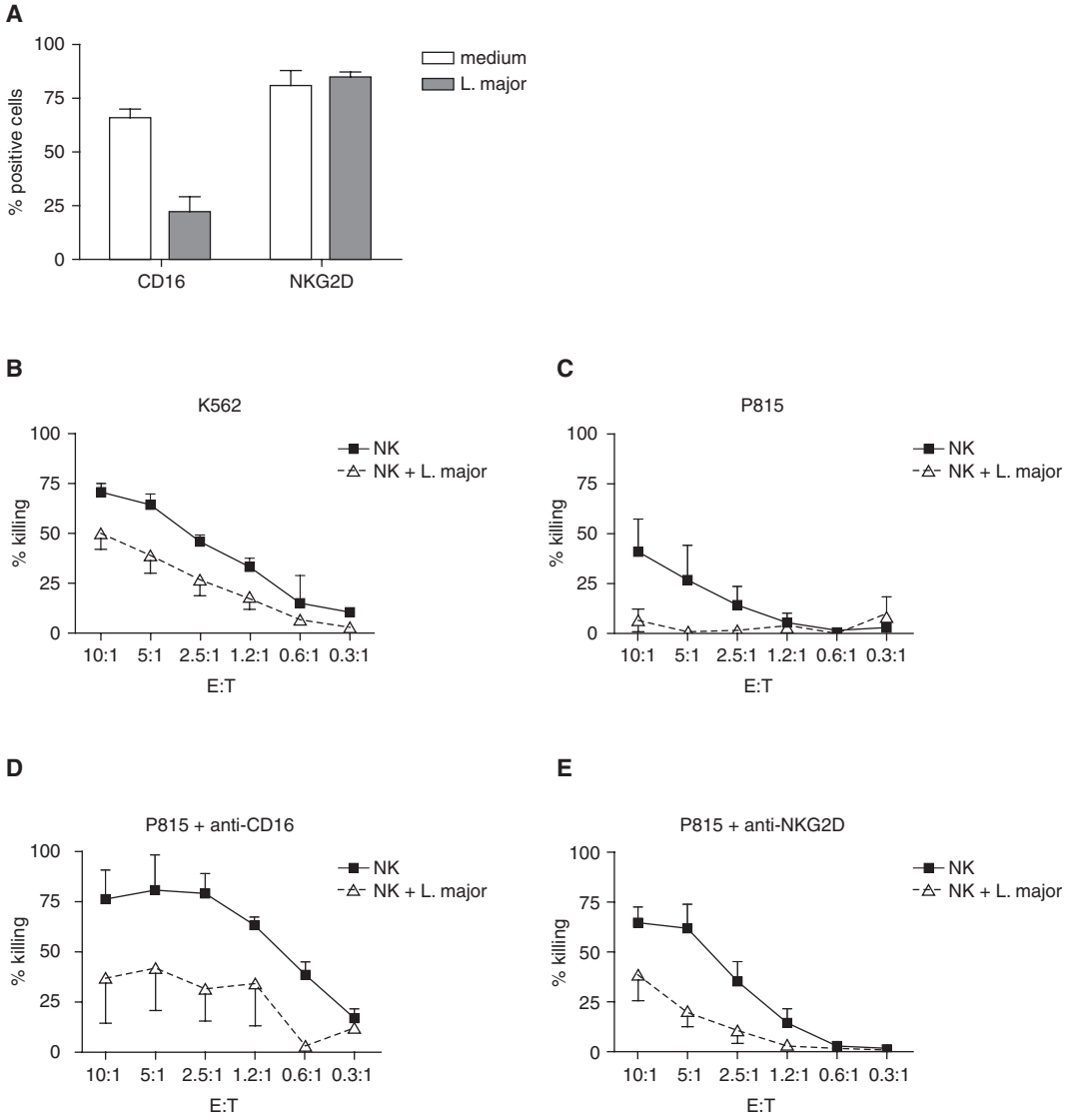

Fig. 5. NK cells exhibit general signs of exhaustion after incubation with *Leishmania*. Purified NK cells were incubated in medium alone or with *L. major* promastigotes for 24 h and then stained for the expression of CD16 and NKG2D (A). After exposure to *L. major* or incubation only in medium, NK cells were incubated in variable effector:target (E:T) ratios with ^{51}Cr-labelled regular target K562 (B) and murine P815 cells for 4 h, respectively. P815 cells were either untreated as non-target cells (C) or coated with anti-human CD16 (D) or anti-human NKG2D (both 5 μg/ml) (E). Diagrams show mean ± S.D. of 3 independent experiments.

our artificial setting might give a reasonable explanation of effects that occur during acute CL in human, highlighting once more the importance of NK cell participation in protozoan infection and the efforts parasites make to avoid NK activity.

This hypothesis is in line with findings that *Leishmania* established several mechanisms to escape immunity of mammalian hosts avoiding recognition and/or clearance (reviewed by (Zambrano-Villa *et al*. 2002)). Once promastigotes enter the macrophages, inflammatory responses in the monocytes are silenced, especially the secretion of IL-12 (Carrera *et al*. 1996) which has a strong potential for activation of NK cells (Newman and Riley, 2007). Despite all efforts, *Leishmania* failed to prevent NK cell activity (Scharton and Scott, 1993). It would only be a logical consequence if the parasites try to suppress NK cell activity for as long as possible to get a head start in the fight for survival.

Human NK cells obviously express receptors which are as yet not identified in detail but are known to recognize prominent molecules on the surface of infectious promastigotes. The interplay of NK cells with those molecules provokes opposed reactivity of NK cells. We reported earlier induction of IFN-γ secretion by human NK cells after incubation with live but not heat-killed promastigotes of *L. aethiopica* (Nylen *et al*. 2003), suggesting that not only the recognition of *Leishmania*-derived molecules by NK receptors leads to activation but the density of those molecules is critical, indicating specific receptor-ligand interactions. On the other

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hand, we found signs of suppression of NK cell proliferation and decreased expression of several characteristic NK receptors after exposure to *Leishmania* promastigotes caused by gp63 (Lieke *et al*. 2008). We now show data supporting direct activation of NK cytotoxicity. Direct activation of NK cells in protozoan infection is controversially discussed since other reports claim that the activation of NK cells depends on the presence of IL-12 thus needing the help of accessory cells which can be infected macrophages or even dendritic cells (reviewed by Newman and Riley, 2007). Furthermore, Schleicher *et al*. (2007) excluded any activation of NK cells by extracellular parasites. This is rebutted not only by our own results in this and a previous study with *Leishmania* parasites but also by studies using other protozoan parasites (Hatcher and Kuhn, 1982; Artavanis-Tsakonas *et al*. 2003; Lieke *et al*. 2004).

Nevertheless, all observed interactions were contact dependent. Thus, we visualized the contacts and to our surprise found that the formation of contacts resulted in fusion of cell membranes. A comparable phenomenon has been described by Stinchcombe *et al*. (2001) who found membrane bridges between cytotoxic T cells and target cells with a central diameter between 50 and 95 nm. However, no transfer of cytoplasm was described. Using CMFDA, we found several signs of exchange of cytosol between *Leishmania* promastigotes and NK cells. The most striking characteristic of CMFDA is the hydrophilic outcome of the fluorescent form of the molecule whereby no interactions with membranes withhold the dye in the labelled cells. Membrane leakage and flow of cytosol allows free mobility of the dye.

In addition, we checked whether the increased fluorescence of NK cells is caused by internalization of CMFDA-labelled cytoplasm or by attached fluorescent vesicles on the cell membrane or unspecific uptake of parasite-derived cytosol from the culture medium. Therefore, NK cells were incubated with lysates of CMFDA-labelled promastigotes, but under these conditions no comparable shift in flow cytometry was detectable (data not shown).

Exchange of cytoplasmic components with participation of lymphocytes has been described for regulatory T cells and activated $CD4^+$ T cells, leading to transfer of cAMP from the Treg to the activated T cells via gap junctions resulting in suppression of T cell proliferation (Bopp *et al*. 2007). However, electron microscopy gave no indication of gap junction connection in our experiments. To our knowledge this is the first report of cytoplasmic exchange between a cytotoxic lymphocyte and a protozoan parasite. The biological significance, however, remains to be clarified.

In summary, our results currently reported and previous results demonstrate the existence of as yet unspecified NK receptors that interfere directly with *Leishmania*-derived molecules which build the surface coat of promastigotes but are also expressed on infected monocytes in the earliest phase after invasion of host cells. The binding of surface molecules elicits cytokine release (Nylen *et al*. 2003) and cytotoxicity but also blocks NK proliferation (Lieke *et al*. 2008) and, as a final consequence, induces destruction of NK cells. Although we failed to confirm these results with infected monocytes the results provide insight into the possible interaction of *Leishmania* and the immune response at a very essential time-point for establishment of infection.

REFERENCES

Akuffo, H., Maasho, K., Blostedt, M., Hojeberg, B., Britton, S. and Bakhiet, M. (1997). Leishmania aethiopica derived from diffuse leishmaniasis patients preferentially induce mRNA for interleukin-10 while those from localized leishmaniasis patients induce interferon-gamma. *Journal of Infectious Diseases* **175**, 737–741.

Akuffo, H. O., Walford, C. and Nilsen, R. (1990). The pathogenesis of *Leishmania aethiopica* infection in BALB/c mice. *Scandinavian Journal of Immunology* **32**, 103–110.

Artavanis-Tsakonas, K., Eleme, K., McQueen, K. L., Cheng, N. W., Parham, P., Davis, D. M. and Riley, E. M. (2003). Activation of a subset of human NK cells upon contact with Plasmodium falciparum-infected erythrocytes. *Journal of Immunology* **171**, 5396–5405.

Becker, I., Salaiza, N., Aguirre, M., Delgado, J., Carrillo-Carrasco, N., Kobeh, L. G., Ruiz, A., Cervantes, R., Torres, A. P., Cabrera, N., Gonzalez, A., Maldonado, C. and Isibasi, A. (2003). *Leishmania* lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. *Molecular and Biochemical Parasitology* **130**, 65–74.

Belosevic, M., Finbloom, D. S., Van Der Meide, P. H., Slayter, M. V. and Nacy, C. A. (1989). Administration of monoclonal anti-IFN-gamma antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with *Leishmania major*. *Journal of Immunology* **143**, 266–274.

Bopp, T., Becker, C., Klein, M., Klein-Hessling, S., Palmetshofer, A., Serfling, E., Heib, V., Becker, M., Kubach, J., Schmitt, S., Stoll, S., Schild, H., Staege, M. S., Stassen, M., Jonuleit, H. and Schmitt, E. (2007). Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *Journal of Experimental Medicine* **204**, 1303–1310.

Boyum, A. (1968). Separation of leukocytes from blood and bone marrow. Introduction. *Scandinavian Journal of Clinical and Laboratory Investigation Supplement* **97**, 7–106.

Brittingham, A., Morrison, C. J., McMaster, W. R., McGwire, B. S., Chang, K. P. and Mosser, D. M. (1995). Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. *Journal of Immunology* **155**, 3102–3111.

Bryceson, A. D. (1969). Diffuse cutaneous leishmaniasis in Ethiopia. I. The clinical and histological features of the disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **63**, 708–737.

Carrera, L., Gazzinelli, R. T., Badolato, R., Hieny, S., Muller, W., Kuhn, R. and Sacks, D. L. (1996). *Leishmania* promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. *Journal of Experimental Medicine* **183**, 515–526.

Cerwenka, A. and Lanier, L. L. (2001). Ligands for natural killer cell receptors: redundancy or specificity. *Immunological Reviews* **181**, 158–169.

Convit, J. and Kerdel-Vegas, F. (1965). Disseminated cutaneous leishmaniasis; innoculation to laboratory animals, electron microscopy and fluorescent antibodies studies. *Archives of Dermatology* **91**, 439–447.

Cooper, M. A., Fehniger, T. A., Turner, S. C., Chen, K. S., Ghaheri, B. A., Ghayur, T., Carson, W. E. and Caligiuri, M. A. (2001). Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood* **97**, 3146–3151.

Descoteaux, A. and Turco, S. J. (2002). Functional aspects of the *Leishmania donovani* lipophosphoglycan during macrophage infection. *Microbes and Infection* **4**, 975–981.

Habibi, G. R., Khamesipour, A., McMaster, W. R. and Mahboudi, F. (2001). Cytokine gene expression in healing and non-healing cases of cutaneous leishmaniasis in response to in vitro stimulation with recombinant gp63 using semi-quantitative RT-PCR. *Scandinavian Journal of Immunology* **54**, 414–420.

https://doi.org/10.1017/S0031182011001363 Published online by Cambridge University Press

Hatcher, F. M. and Kuhn, R. E. (1982). Destruction of *Trypanosoma cruzi* by natural killer cells. *Science* **218**, 295–296.

Ilgoutz, S. C. and McConville, M. J. (2001). Function and assembly of the *Leishmania* surface coat. *International Journal for Parasitology* **31**, 899–908.

Karre, K. (2002). NK cells, MHC class I molecules and the missing self. *Scandinavian Journal of Immunology* **55**, 221–228.

Korbel, D. S., Finney, O. C. and Riley, E. M. (2004). Natural killer cells and innate immunity to protozoan pathogens. *International Journal for Parasitology* **34**, 1517–1528.

Laskay, T., Diefenbach, A., Rollinghoff, M. and Solbach, W. (1995). Early parasite containment is decisive for resistance to *Leishmania major* infection. *European Journal of Immunology* **25**, 2220–2227.

Lieke, T., Graefe, S. E., Klauenberg, U., Fleischer, B. and Jacobs, T. (2004). NK cells contribute to the control of *Trypanosoma cruzi* infection by killing free parasites by perforin-independent mechanisms. *Infection and Immunity* **72**, 6817–6825.

Lieke, T., Nylen, S., Eidsmo, L., McMaster, W. R., Mohammadi, A. M., Khamesipour, A., Berg, L. and Akuffo, H. (2008). *Leishmania* surface protein gp63 binds directly to human natural killer cells and inhibits proliferation. *Clinical and Experimental Immunology* **153**, 221–230.

Maasho, K. and Akuffo, H. O. (1992). Cells from healthy non-exposed individuals produce cytokines to selected fractions of *Leishmania* promastigotes. *Scandinavian Journal of Immunology Supplement* **11**, 179–184.

Maasho, K., Sanchez, F., Schurr, E., Hailu, A. and Akuffo, H. (1998). Indications of the protective role of natural killer cells in human cutaneous leishmaniasis in an area of endemicity. *Infection and Immunity* **66**, 2698–2704.

Newman, K. C. and Riley, E. M. (2007). Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. *Nature Reviews Immunology* **7**, 279–291.

Nylen, S., Maasho, K., Soderstrom, K., Ilg, T. and Akuffo, H. (2003). Live *Leishmania* promastigotes can directly activate primary human natural killer cells to produce interferon-gamma. *Clinical and Experimental Immunology* **131**, 457–467.

Nylen, S., Mortberg, U., Kovalenko, D., Satti, I., Engstrom, K., Bakhiet, M. and Akuffo, H. (2001). Differential induction of cellular responses by live and dead *Leishmania* promastigotes in healthy donors. *Clinical and Experimental Immunology* **124**, 43–53.

Russell, D. G. (1987). The macrophage-attachment glycoprotein gp63 is the predominant C3-acceptor site on *Leishmania mexicana* promastigotes. *European Journal of Biochemistry* **164**, 213–221.

Scharton, T. M. and Scott, P. (1993). Natural killer cells are a source of interferon gamma that drives differentiation of CD4+ T cell subsets and induces early resistance to *Leishmania major* in mice. *Journal of Experimental Medicine* **178**, 567–577.

Schleicher, U., Liese, J., Knippertz, I., Kurzmann, C., Hesse, A., Heit, A., Fischer, J. A., Weiss, S., Kalinke, U., Kunz, S. and Bogdan, C. (2007). NK cell activation in visceral leishmaniasis requires TLR9, myeloid DCs, and IL-12, but is independent of plasmacytoid DCs. *Journal of Experimental Medicine* **204**, 893–906.

Stinchcombe, J. C., Bossi, G., Booth, S. and Griffiths, G. M. (2001). The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity* **15**, 751–761.

Zambrano-Villa, S., Rosales-Borjas, D., Carrero, J. C. and Ortiz-Ortiz, L. (2002). How protozoan parasites evade the immune response. *Trends in Parasitology* **18**, 272–278.

https://doi.org/10.1017/S0031182011001363 Published online by Cambridge University Press