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Leishmania-Infected Macrophages Are Targets of NK Cell-Derived Cytokines but Not of NK Cell Cytotoxicity▽‡

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Natural killer (NK) cells are important components of a protective immune response against intracellular pathogens such as *Leishmania* parasites, which reside within myeloid cells. Previous *in vivo* studies in murine cutaneous or visceral leishmaniasis showed that NK cells are activated by conventional dendritic cells in a Toll-like receptor 9-, interleukin-12 (IL-12)-, and IL-18-dependent manner during the early phase of infection and help to restrict the tissue parasite burden by unknown mechanisms. Here, we tested whether NK cells contribute to the control of *Leishmania* infections by lysing or by activating infected host cells. Coculture experiments revealed that activated NK cells from poly(I:C)-treated mice readily killed tumor target cells, whereas *Leishmania infantum*- or *L. major*-infected macrophages or dendritic cells remained viable. Infection with *Leishmania* did not significantly alter the expression of NK cell-activating molecules (retinoic acid early transcript alpha [Rae-1α], mouse UL16-binding protein-like transcript 1 [MULT-1], CD48) or inhibitory molecules (major histocompatibility complex [MHC] class I, nonclassical MHC class 1b molecule Qa-1) on the surface of myeloid cells, which offers an explanation for their protection from NK cell cytotoxicity. Consistent with these *in vitro* data, *in vivo* cytotoxicity assays revealed poor cytolytic activity of NK cells against adoptively transferred infected wild-type macrophages, whereas MHC class I-deficient macrophages were efficiently eliminated. NK cells activated by IL-12 and IL-18 stimulated macrophages to kill intracellular *Leishmania* in a cell contact-independent but gamma interferon-, tumor necrosis factor-, and inducible nitric oxide synthase-dependent manner. We conclude that *Leishmania* parasites, unlike viruses, do not render infected myeloid cells susceptible to the cytotoxicity of NK cells. Instead, soluble products of NK cells trigger the leishmanicidal activity of macrophages.

Natural killer (NK) cells participate not only in the control of tumors and the rejection of transplants but also in the development of protective immunity against intracellular pathogens (31, 51, 77). One of their central effects is the lysis of infected host cells. This has been best demonstrated for infections with viruses that up- or downregulate activating or inhibitory NK cell receptors or encode NK cell-activating ligands in their genome (2, 37, 38, 40, 71). Several studies suggested that NK cells also exert cytolytic effects against host cells infected with nonviral pathogens (1, 47, 53, 55, 74). On the other hand, both viral and nonviral infections exist in which host cells acquire resistance to NK cell lysis (10, 20, 21, 40). However, even without host cell lysis, NK cells might still operate as cytotoxic effectors, either by direct killing of extracellular microbes or by inducing the death of intracellular pathogens via the transfer of perforin or granulysin (1, 25, 42, 45, 52, 68, 75).

In addition to their cytotoxic activity, NK cells fulfill several regulatory functions, which also contribute to the control of infectious pathogens. NK cell-mediated host cell lysis itself facilitates T cell responses (30). Further enhancement results from NK cell-derived cytokines. Gamma interferon (IFN-γ) and tumor necrosis factor (TNF) pave the way for development of Th1 cells (32) and activate macrophages for the expression of antimicrobial effector mechanisms, such as inducible nitric oxide synthase (iNOS) (5, 6). Recently, human NK cells were found to produce IL-22, which promoted phagolysosomal fusion and impaired the growth of mycobacteria within macrophages (16). Finally, during viral infections that trigger a strong expansion of NK cells or during later phases of infections, NK cells might also acquire memory-like protective functions (70) or negative regulatory activities such as those seen, for example, in murine visceral leishmaniasis (44).

Infections with different species of the protozoan parasite *Leishmania* lead to cutaneous (e.g., *Leishmania major*) or visceral (e.g., *L. infantum*) disease. A series of findings argues for a protective role of NK cells in murine leishmaniasis, at least during the early phase of infection. These include (i) the aggravated course of *Leishmania* infections in NK cell-deficient *bg*/*bg* (beige) mice (28) or in NK cell-depleted mice (34, 36, 57), (ii) the disease-ameliorating effect of the activation or transfer of NK cells in *Leishmania*-infected mice (34, 43, 56), (iii) the expression of IFN-γ by NK cells early after infection *in vivo* (3, 17, 24, 39, 57, 58, 61), and (iv) the involvement of NK cells in the protection elicited by dendritic cell (DC)-based vaccines (54). However, it should be noted that the NK cell depletions were performed only transiently prior to and early

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during infection using reagents (e.g., anti-NK1.1 antibody) that were not entirely selective for NK cells.

Various cytokines and cell types participate in NK cell activation in cutaneous or visceral leishmaniasis. The key players are (i) interleukin-12 (IL-12), which is essential for NK cell activation (58) and released by myeloid DCs in response to *Leishmania* in a Toll-like receptor 9 (TLR9)- and vascular cell adhesion molecule-1/very late antigen-4-dependent manner *in vitro* and *in vivo* (22, 39, 61, 65); (ii) IFN-α/β, which accounts for the early expression of iNOS and thereby facilitates IL-12 responsiveness in *L. major*-infected mice (17, 18); (iii) IL-18, which supports NK cell activation in *L. infantum*-infected mice (24); and (iv) antigen-primed $CD4^+$ T cells that activate NK cells via IL-2 in the *L. major* mouse model (4, 57). *In situ*, NK cells form part of the early inflammatory infiltrate after *Leishmania* infection at the site of infection and colocalize with myeloid cells *in vivo* (3, 72).

To date, we have only a partial understanding of the mechanisms by which NK cells might contribute to the control of *Leishmania* parasites *in vivo*. During *L. major* infection, NK cell-derived IFN-γ was shown to drive the Th1 differentiation of $CD4^+$ T cells (57) and to restrict early parasite dissemination (17, 33). On the basis of *in vitro* experiments, NK cells might also lyse infected macrophages (1, 55). There are conflicting data whether NK cells directly kill extracellular *Leishmania* promastigotes (1, 43), an activity which could result from their constitutive expression of leishmanicidal granulysin or related molecules (68). More popular is the thought that NK cell-derived IFN-γ activates macrophages for the expression of iNOS (17); however, until now there has been no direct *in vitro* or *in vivo* evidence for this notion.

In the present study, we tested NK cells for their ability to lyse or activate *Leishmania*-infected macrophages. Unexpectedly, *Leishmania*-infected macrophages were entirely resistant to the cytotoxic activity of NK cells *in vitro* and *in vivo*. Instead, activated NK cells efficiently stimulated macrophages for the destruction of intracellular *Leishmania* in an IFN-γ-, TNF-, and iNOS-dependent manner.

MATERIALS AND METHODS

Mice. Female or male C57BL/6 (B6) mice were purchased from Charles River, Sulzfeld, Germany. Breeding pairs of B6 protein tyrosine phosphatase (PTPRC; $CD45.1^+$) congenic mice and of mice with a disrupted iNOS gene (35) ($iNOS^{-/-}$; 11th generation backcross to B6), IFN-γ gene (14) ($IFN\text{-}\gamma^{-/-}$; 10th generation backcross to B6), or IFN-γ receptor ($IFN\text{-}\gamma R^{-/-}$; 10th generation backcross to B6) were from the Jackson Laboratory (Bar Harbor, ME). Bone marrow from β_2-microglobulin-deficient ($\beta_2m^{-/-}$) mice (29) (11th generation backcross to B6; Jackson) was a kind gift of D. Vöhringer (Institute for Immunology, LMU Munich, Germany). Breeding pairs of recombination activating gene 2 (RAG2)/common γ-chain double mutant ($RAG2/\gamma c^{-/-}$) mice, which were generated by intercrossing $RAG2^{-/-}$ mice (62) (10th generation backcross to B6) with $\gamma c^{-/-}$ mice (19) (12th generation backcross to B6), were obtained from J. Kirberg (MPI, Freiburg, Germany). Breeding pairs of $RAG1^{-/-}$ mice (48) (Jackson; 10th generation backcross to B6) were provided by T. Winkler (Department of Genetics, FAU Erlangen, Erlangen, Germany).

All mice were bred and kept under specific-pathogen-free conditions at the Franz Penzoldt Center for Animal Research, Universitätsklinikum Erlangen, and at the animal facility of the institute following the animal welfare protocol approved by the government of Middle Franconia (registration no. 54-2532.1-24/08). For experiments, sex-matched mice at the age of 6 to 12 weeks were used.

Parasites and infection of mice. The origin, propagation, and preparation of *L. infantum* promastigotes (strain MHOM/00/98/LUB1) (8) and of *L. major* amastigotes (strain MHOM/IL/81/FEBNI) (64) were described before (61, 69). For infection of mice with *L. infantum*, 10^7 promastigotes were injected intravenously (i.v.) via the tail vein or intraperitoneally (i.p.).

Myeloid cells and *in vitro* infection. Bone marrow-derived macrophages (BM-Mϕ) were generated by culturing 6×10^6 nucleated bone marrow cells in 50 ml conditioned Dulbecco modified Eagle medium (supplemented with 10% [vol/vol] fetal calf serum [FCS], 50 μM 2-mercaptoethanol, 1% [vol/vol] 100× nonessential amino acids, 5% [vol/vol] horse serum, and 15% [vol/vol] supernatant [SN] from L929 fibroblast cultures [ATCC clone CCL-1] as a source of macrophage colony-stimulating factor) in Teflon bags for 8 days (59). Peritoneal exudate cells (PECs) were harvested from the peritoneal cavities of mice 4 days after i.p. injection of 3 ml of 4% (wt/vol) Brewer's thioglycolate broth (Difco, Detroit, MI) and contained >90% $CD11b^+$ $F4/80^+$ peritoneal exudate macrophages (PE-Mϕ) (60). Bone marrow-derived dendritic cells (BM-DCs) were differentiated by culturing 6×10^6 bone marrow cells in RPMI 1640 medium (catalog no. 21875; Invitrogen, Karlsruhe, Germany) supplemented with 10% (vol/vol) FCS, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (termed "complete RPMI 1640 medium"), to which 10% (vol/vol) SN from the recombinant mouse-granulocyte macrophage colony-stimulating factor-transfected Ag8653 myeloma cell line (78) was added. The cells were cultured for 8 days in 60-cm^2 culture dishes (Nalge Nunc International, Rochester, NY) with 10 ml of medium initially, before fresh medium was added on days 3 and 5 (41).

For infection, BM-Mϕ or PE-Mϕ were cocultured with *L. infantum* promastigotes or skin lesion-derived *L. major* amastigotes at ratios from 1:4 to 1:10 (see figure legends) for 14 to 16 h or 4 h, respectively, in Teflon bags using complete RPMI 1640 medium. Infection of BM-DCs was carried out similarly, except for using petri dishes. After infection, the cells were harvested and washed three times with complete RPMI 1640 medium to remove free parasites. The percentage of infected macrophages (60 to 90%) was determined by Diff-Quik (Medion Diagnostics AG, Switzerland) staining.

In some experiments involving the transfer of infected macrophages *in vivo*, the parasites used for infection were labeled with the cell proliferation dye eFluor 670 (eBioscience, Frankfurt, Germany). Briefly, *L. infantum* promastigotes were resuspended in 5 ml of phosphate-buffered saline (PBS) to which 1 μl of a 5 mM dye solution was added, and mixture was vortexed and incubated at 37°C for 10 min. To stop the reaction, ice-cold complete RPMI 1640 medium was added, followed by 5 min incubation on ice. Additional washes with ice-cold PBS were performed to remove excess dye. All cell culture reagents and media contained less than 10 pg/ml endotoxin as determined by a colorimetric *Limulus* amebocyte lysate assay (QCL-1000; Lonza, Cologne, Germany).

Antibodies and flow cytometry. For surface phenotyping and cell sorting, fluorochrome (fluorescein isothiocyanate, phycoerythrin, or allophycocyanin [APC])-labeled or biotinylated monoclonal antibodies against the following mouse antigens were used: NK1.1 (PK136), CD3ε (145-2C11), CD11b (M1/70), CD11c (HL3), CD48 (HM48-1), and mouse UL-16-binding protein-like transcript 1 (MULT-1; 5D10) (all from eBioscience); Qa-1 (6A8.6F10.1A6) and $H\text{-}2k^b$ (AF6-88.5; BD Biosciences, Heidelberg, Germany); F4/80 (CI:A3-1; AbD Serotec, Düsseldorf, Germany); and pan-retinoic acid early transcript 1 (pan-Rae-1) (186107; R&D Systems, Wiesbaden, Germany). The specificity of the stainings was verified by the use of appropriate isotype control antibodies. Propidium iodide (PI) was included at 1 μg/ml in the final wash to detect dead cells.

Purification of NK cells. Spleens were aseptically harvested from B6 wild-type (WT) or $IFN\text{-}\gamma^{-/-}$ mice, and single-cell suspensions were prepared in complete RPMI 1640 medium. After passage through a 100-μm-mesh-size cell strainer (BD Falcon), red blood cells were lysed by NH_4Cl treatment. NK cells were purified by MACS technology using anti-DX5 MicroBeads (Miltenyi, Bergisch-Gladbach, Germany) and by subsequent MoFlo sorting gating on $NK1.1^+$ $CD3^-$ cells (purity > 99%).

In vitro NK cell cytotoxicity assay. After determination of the percentage of $NK1.1^+$ $CD3^-$ NK cells within the splenocyte population by fluorescence-activated cell sorter (FACS) analysis, splenocytes were added to target cells at different NK cell/target cell ratios and incubated for a period of 4 h (YAC-1 cells) or 4 to 12 h (BM-Mϕ, PE-Mϕ, DCs) in complete RPMI 1640 medium at 37°C in 5% CO_2, 95% humidified air. The target cells were previously labeled with ~150 μCi ^{51}Cr (specific activity, 400 to 1,200 Ci/g; Perkin-Elmer, Rodgau, Germany) for 90 min; and the spontaneous (target cells alone), maximal (^{51}Cr-labeled cells directly added to LUMA plate), and coculture-elicited ^{51}Cr release into the SNs was measured using a TopCount NXT microplate gamma counter (Perkin-Elmer).

***In vivo* NK cell cytotoxicity assay.** WT or major histocompatibility complex (MHC) class I-deficient ($\beta_2m^{-/-}$) BM-Mϕ were infected with *L. infantum* promastigotes at a parasite/macrophage ratio of 7:1 and incubated in Teflon bags for 18 h. Splenocytes (from uninfected B6 $CD45.1^+$ mice) or BM-Mϕ (from uninfected or infected B6 $CD45.1^+$ or $\beta_2m^{-/-}$ $CD45.1^-$ mice) were resuspended in

10 ml of PBS containing 0.1% bovine serum albumin and were labeled with 0.5 or 5 μM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen), respectively, for 10 min at 37°C. Labeling of cells was stopped by the addition of ice-cold complete RPMI 1640 medium and by two washes with ice-cold PBS. After the cells were counted, WT BM-Mϕ ($CD45.1^+$, $CFSE^{hi}$), $\beta_2m^{-/-}$ BM-Mϕ ($CD45.1^-$, $CFSE^{hi}$), and WT splenocytes ($CD45.1^+$, $CFSE^{low}$) were mixed in equal ratios and injected i.p. into WT B6 recipient mice (2.5×10^5 cells per population in a total volume of 0.5 ml PBS/mouse) that had been i.p. treated with PBS, poly(I:C) (50 μg), or *L. infantum* promastigotes (10^7) 18 h prior to cell transfer. At 16 h after cell transfer, the mice were killed, the peritoneal exudates were collected, and the percentage of CFSE-labeled cells recovered from the peritoneal cavity was determined by FACS analysis. The cytotoxicity for each mouse group was measured using the following formula (12): percent specific lysis = $[1 - (r^{naïve}/r^{primed})] \times 100$, where r is the number of $CD45.1^+$ $CFSE^{lo}$ splenocytes/number of $CFSE^{hi}$ Mϕ ($CD45.1^+$ WT or $CD45.1^-$ $\beta_2m^{-/-}$) from naïve (i.e., PBS-treated) or primed [i.e., poly(I:C)-treated or *L. infantum*-infected] mice. In the case of PBS-treated mice, the calculated cytotoxicity value was 0% lysis, as r^{primed} equals $r^{naïve}$. Any alteration in r^{primed} ($CD45.1^+$ $CFSE^{lo}$ splenocytes/$CFSE^{hi}$ Mϕ) observed in poly(I:C)- or *L. infantum*-treated mice was regarded as specific cytotoxicity.

Intracellular cytokine staining of NK cells. Splenocytes from naïve B6 mice were cultured for 8 or 24 h with medium alone, with recombinant mouse IL-12 (rmIL-12) plus rmIL-18 (10 ng/ml each; R&D Systems), or with 50 ng/ml phorbol myristate acetate (Sigma-Aldrich) plus 750 ng/ml ionomycin (Sigma-Aldrich) in the presence of 10 μg/ml brefeldin A. After the cells were stained for NK cell surface markers ($CD3^-$ $NK1.1^+$ or $CD3^-$ $DX5^+$), the cells were fixed with Cytoperm Cytofix fixative (BD Biosciences) for 20 min and incubated with APC-conjugated rat anti-mouse IFN-γ (XMG1.2; eBioscience) or rat anti-mouse TNF (MP6-XT22; BD Biosciences) (61).

Coculture of NK cells and macrophages. BM-Mϕ generated from B6 WT, IFN-$\gamma R^{-/-}$, or $iNOS^{-/-}$ mice were plated at 1.5×10^5/400 μl in Lab-Tek eight-well Permanox chamber slides (catalog no. 177445; Nalge Nunc International) using complete RPMI 1640 medium and were allowed to adhere for 2 h in air with 5% CO_2 and 95% humidity. After removal of nonadherent cells by three washes with PBS, macrophage monolayers were infected for 18 h with stationary-phase *L. infantum* promastigotes in a 10:1 parasite/cell ratio (1.5×10^6/400 μl), which led to an infection rate of 40 to 80% (Diff-Quik staining). Cells were washed three times with PBS to remove extracellular parasites and were cocultured with purified NK cells at a NK cell/macrophage ratio of 6:1 in the presence or absence of cytokines (rmIL-12, 10 ng/ml; rmIL-18, 10 ng/ml; R&D Systems). In some experiments the macrophages were treated with rmIFN-γ (20 ng/ml; kindly provided by G. Adolf, Boehringer Ingelheim, Vienna, Austria) and/or rmTNF (10 ng/ml; R&D Systems), and in others they were treated with a rat anti-TNF neutralizing antibody (clone MP6-XT22; R&D Systems) or a control rat IgG (Jackson ImmunoResearch, Dianova, Hamburg) at a final concentration of 5 μg/ml. The final culture volume was adjusted to 200 μl per well. Cells received 50 μl fresh medium (containing the respective stimuli and reagents) every 24 h. After 72 h, SNs were collected for cytokine and NO_2^- determinations and the monolayers were washed three times with PBS and stained with Diff-Quik for analysis of intracellular *Leishmania*. The parasite load in macrophages was calculated by determining the mean number of parasites per 100 macrophages of the culture (percent infection rate × average number of parasites per infected cell) in five randomly selected microscopic fields at ×1,000 magnification. The number of parasites in the nonstimulated macrophages (medium only) was set equal to 100%.

For transwell experiments, macrophages were plated at 0.5×10^6/500 μl on 12-mm-diameter round coverslips (catalog no. CB00120RA1; Thermo Scientific) placed in a 24-well cell culture dish and infected with *L. infantum* promastigotes in a 5:1 parasite/cell ratio. Cytokines were added as described above, and NK cells were seeded in a NK cell/macrophage ratio of 2:1 either directly to the macrophage monolayer or in Costar transwell inserts (0.4-μm pore size; Corning Life Sciences, Wiesbaden, Germany) placed above the coverslip. The final volume in the macrophage compartment was adjusted to 1 ml, and that in the insert was adjusted to 150 μl. After 72 h of coculture, the intracellular parasite load was determined as described above.

Assays for cytokine and nitrite determination. TNF and IFN-γ in SNs of coculture experiments were determined using commercial enzyme-linked immunosorbent assay systems from eBioscience and BD Biosciences, respectively. The NO_2^- content was measured with the Griess assay using 50 μl of culture SN and an equal volume of Griess reagent (9).

Statistical analysis. Standard errors of the mean (SEMs) were determined, and statistical significance was analyzed using either the nonparametric Mann-Whitney test or the unpaired Student t test. A P value of <0.05 was considered significant.

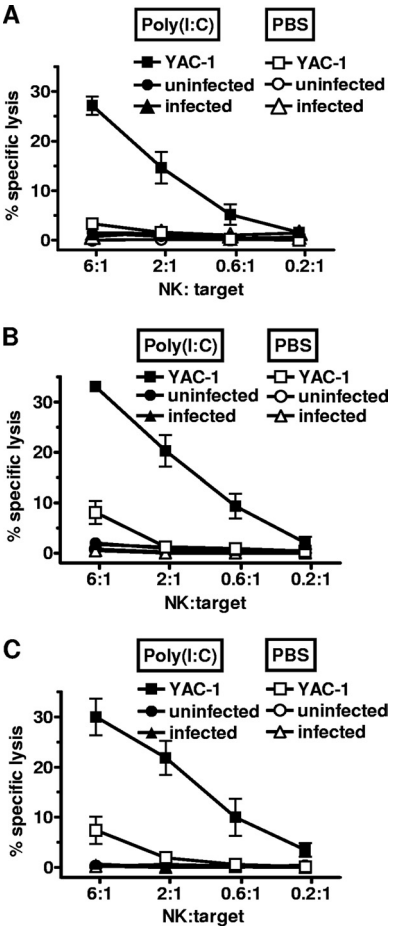

FIG. 1. *In vivo*-activated NK cells do not lyse *Leishmania*-infected host cells *in vitro*. B6 $RAG1^{-/-}$ mice (2 to 3 mice per group) were treated i.p. with 50 μg of poly(I:C) or with PBS. Splenocytes from these mice were incubated with ^{51}Cr-labeled BM-Mϕ (A), PE-Mϕ (B), or BM-DCs (C) at the indicated NK/target cell ratio for 12 h. The myeloid target cells were either uninfected or infected with *L. major* amastigotes (parasite/cell ratio = 4:1). Splenocytes incubated with YAC-1 tumor cells were used as a positive control for NK cell cytotoxicity. Results shown represent the mean (±SEM) of 2 to 3 independent experiments.

RESULTS

Activated NK cells exhibit cytolytic properties against tumor targets but do not lyse *Leishmania*-infected myeloid cells *in vitro*. We investigated whether activated NK cells exerted cytotoxic effects against infected host cells *in vitro*. To this end, different types of myeloid cells, all of which function as host cells for *Leishmania*, were cocultured with splenocytes isolated from PBS or poly(I:C)-treated mice, and chromium (^{51}Cr) release assays were performed. As expected, *in vivo*-activated NK cells efficiently lysed YAC-1 tumor targets, which lack expression of MHC class I, one of the strongest NK cell-inhibitory molecules, on the cell surface. In contrast, BM-Mϕ, PE-Mϕ, and BM-DCs were resistant to NK cell cytotoxicity *in vitro*, regardless of whether they were uninfected or infected with *L. infantum* promastigotes or *L. major* amastigotes and whether the coculture period lasted 4 or 12 h (Fig. 1 and data not shown). Importantly, infection with *Leishmania* did not increase the susceptibility of myeloid cells to NK cell-mediated

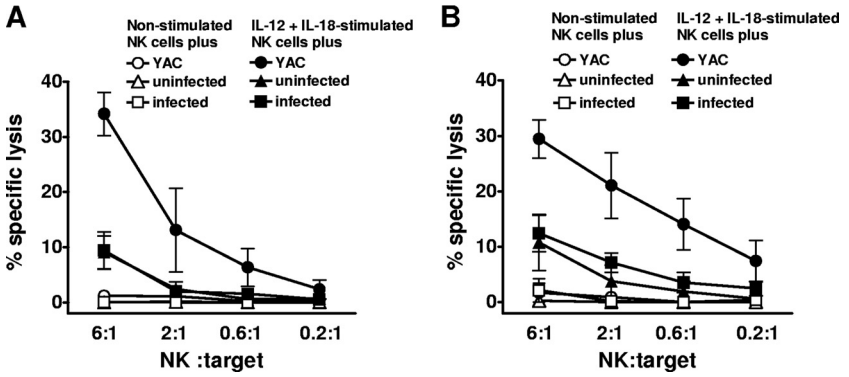

FIG. 2. *In vitro*-activated NK cells readily kill YAC-1 tumor targets but are not cytotoxic against uninfected or infected B6 BM-Mϕ. NK cells from the spleens of RAG1$^{-/-}$ mice were stimulated for 18 h with rmIL-12 plus rmIL-18 and used as effector cells in 4-h (A) and 18-h (B) chromium-51 release assays. The mean (±SEM) of three independent experiments is shown as percent specific lysis against uninfected and *L. infantum*-infected BM-Mϕ (parasite/cell ratio = 10:1) or YAC-1 tumor cells.

lysis. The same observation was made when we used NK cells that were purified from naïve RAG1$^{-/-}$ mice and activated *in vitro* by IL-12 and IL-18, two key stimulators for the activation of NK cells during *L. major* and *L. infantum* infection (Fig. 2). Thus, neither *in vivo*- nor *in vitro*-activated NK cells show the capacity to specifically lyse *Leishmania*-infected myeloid cells.

***Leishmania* infection does not modulate the expression of ligands for activating or inhibitory NK cell receptors on the surface of myeloid cells.** The cytotoxic activity of NK cells is controlled by a set of activating and inhibitory receptors on their surface. NK cells recognize cells that overexpress ligands for activating receptors or underexpress ligands for inhibitory receptors, or both. A shift in the balance from inhibitory to activating signals will result in target cell lysis by NK cells (31). We therefore tested CD11b$^+$ F4/80$^+$ BM-Mϕ, CD11b$^+$ F4/80$^+$ PE-Mϕ, and CD11b$^+$ CD11c$^+$ BM-DCs for the surface expression of (i) MHC class I antigens (the ligands for the inhibitory members of the Ly49 NK cell receptor family) and Qa-1^b (a nonclassical MHC-like molecule that primarily functions as a high-affinity ligand of the inhibitory NK cell receptor NKG2A [13]); (ii) CD48, which interacts with the 2B4 receptor on NK cells and transmits inhibitory or activating signals, depending on the degree of receptor expression and cross-linking (11, 27); and (iii) Rae-1α and MULT-1, which are ligands of the activating NK cell receptor NKG2D (31). In general, infection of the three different myeloid cell populations with *L. infantum* promastigotes did not alter the expression of these ligands. In two of four experiments, we measured a slight upregulation of MHC class I and Qa-1^b selectively in infected BM-Mϕ and a downregulation of Rae-1α only in infected PE-Mϕ and not in the other two cell populations (Fig. 3). Importantly, in none of the four experiments did we observe an NK cell ligand expression pattern that would promote the activation of NK cells. Comparable results were obtained when the infections were performed in the presence of naïve or activated NK cells or when *L. major* amastigotes freshly isolated from skin lesions were used (data not shown). From these data we conclude that the resistance of myeloid cells to NK cell lysis *in vitro* might result from maintaining the balance between activating versus inhibitory NK receptor ligands, despite infection with *Leishmania*.

NK cells do not kill *Leishmania*-infected macrophages *in vivo*. The failure of NK cells to recognize infected macrophages as targets *in vitro* might reflect the lack of additional NK cell-activating signals such as those delivered by myeloid DCs. Hence, we sought to study the cytotoxicity of NK cells against infected macrophages *in vivo*. For this purpose, a mixture of CFSE-labeled WT BM-Mϕ (uninfected or infected with *L. infantum* promastigotes), β_2m$^{-/-}$ BM-Mϕ (uninfected or infected; these served as a positive control due to their MHC class I deficiency), and WT splenocytes (used as a reference population for the transferred cells) were injected into the peritoneal cavity of mice whose NK cells had already been activated by the treatment with poly(I:C) or the infection with *L. infantum* 18 h prior to the cell transfer. At 16 h after cell transfer the mice were killed and peritoneal exudates were collected and analyzed by flow cytometry. The transferred cells were distinguished from each other and the immune cells of the recipient by their congenic marker (CD45.1$^+$) and by the labeling with different concentrations of CFSE. The infection status of the transferred macrophages was ascertained by using fluorescently labeled parasites (data not shown). The percent decrease in the number of CFSE^{hi} macrophages relative to the number of CFSE^{lo} splenocytes was used as a measure of cytotoxicity (see Materials and Methods). As expected, β_2m$^{-/-}$ BM-Mϕ (CFSE^{hi} CD45.1$^-$) were readily eliminated from the peritoneal cavity of poly(I:C)-treated or *L. infantum*-infected mice. In contrast, only minimal cytotoxicity was observed against WT macrophages in the very same mice. However, this minimal cytotoxicity was not specific for infected macrophages, as it was also observed with uninfected target cells (Fig. 4). The ultimate proof that both poly(I:C) and *L. infantum* led to proper activation of NK cells in the peritoneal cavity and that the observed lysis of β_2m$^{-/-}$ BM-Mϕ resulted from NK cell activity was obtained by comparison of the cytotoxicity against β_2m$^{-/-}$ BM-Mϕ in WT versus RAG1$^{-/-}$ or RAG2/γc$^{-/-}$ recipient mice. Whereas the cytotoxicity observed in RAG1$^{-/-}$ mice, which contain NK cells but lack T and B cells, was comparable to that in WT mice,

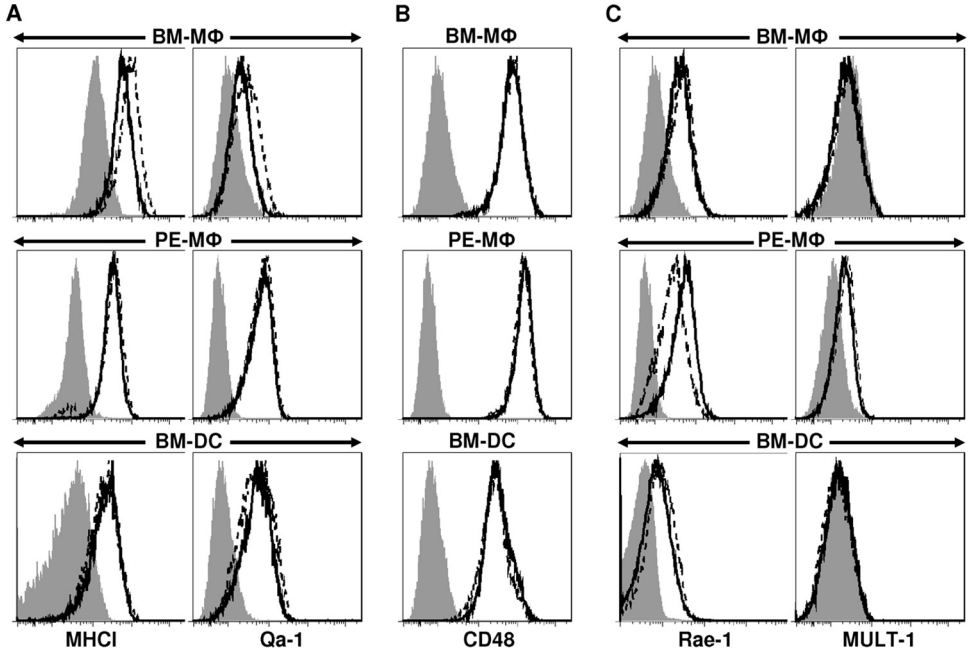

FIG. 3. *Leishmania* infection does not alter the levels of NK cell regulatory molecules on the surface of macrophages or DCs. BM-Mϕ, PE-Mϕ, or BM-DCs were infected with *L. infantum* promastigotes at a parasite/cell ratio of 7:1 for 14 h. $CD11b^+$ $F4/80^+$ Mϕ and $CD11c^+$ $CD11b^+$ DCs were stained for the expression of the NK cell-receptor ligands MHC class I and Qa-1^b (A; inhibitory), CD48 (B; inhibitory or activating), or Rae-1α and MULT-1 (C; activating). The histograms represent the expression levels of these molecules on the surface of uninfected (solid lines) or infected (dashed lines) cells. Isotype controls (shaded) were included for each staining. The data are representative of 4 independent experiments.

there was no target cell lysis detectable in RAG2/$\gamma c^{-/-}$ mice, which are devoid of all lymphocytes, including NK cells (Fig. 5). Together, these results demonstrate that, albeit activated NK cells have the potential to eliminate MHC class I-deficient macrophages, *Leishmania*-infected macrophages are spared from their cytotoxicity.

Activated NK cells stimulate BM-Mϕ for the kill of intracellular *Leishmania* in a cell contact-independent but IFN-γ-, TNF-, and iNOS-dependent manner. Having excluded target cell lysis as a mechanism by which NK cells confer protection in leishmaniasis, we investigated their ability to trigger leishmanicidal activities within macrophages in a nonlytic fashion.

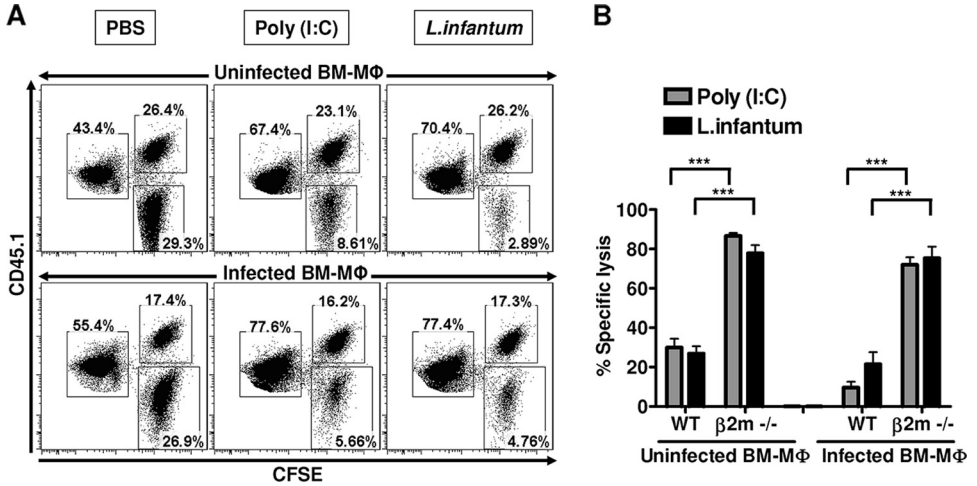

FIG. 4. *Leishmania*-infected macrophages are resistant to NK cell lysis *in vivo*. WT B6 mice ($CD45.1^-$) were treated i.p. with PBS, poly(I:C) (50 μg), or *L. infantum* promastigotes (10^7 parasites). At 18 to 20 h posttreatment or postinfection, 2.5×10^5 CFSE-labeled uninfected or infected BM-Mϕ ($CFSE^{hi}$ $CD45.1^+$, *L. infantum* promastigote/cell ratio for infection = 7:1) from congenic B6 PTPRC mice were injected i.p. along with an equal number of uninfected or infected MHC class I-deficient ($\beta_2m^{-/-}$) BM-Mϕ ($CFSE^{hi}$ $CD45.1^-$, parasite/cell ratio for infection = 7:1) and uninfected WT splenocytes ($CFSE^{lo}$ $CD45.1^+$). (A) The percentage of CFSE-labeled cells recovered from the peritoneal cavity at 14 to 16 h posttransfer was analyzed by flow cytometry. After gating on CFSE-positive cells, the transferred populations were distinguished on the basis of their differential intensity of CFSE fluorescence (high or low) and expression of CD45.1 (positive or negative). (B) The bar graph represents the mean (±SEM) of percent specific lysis obtained from four independent experiments with four mice per group as described in Materials and Methods. ***, $P < 0.0001$ (Mann-Whitney test).

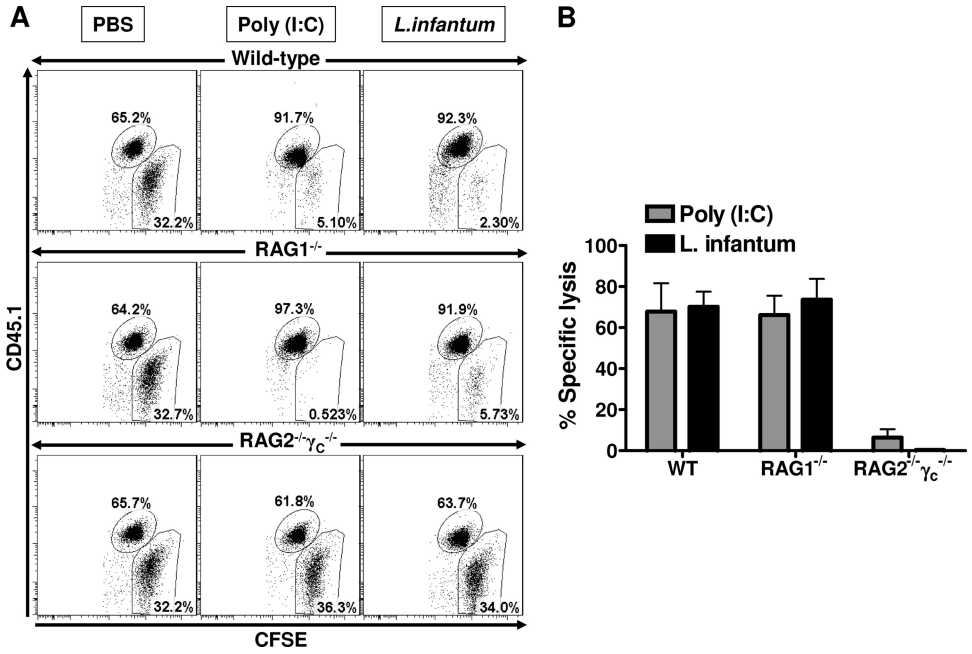

FIG. 5. NK cells account for the cytotoxic activity against myeloid cells detected in the peritoneal cavity of infected or poly(I:C)-treated mice. B6 WT, RAG1$^{-/-}$, and RAG2/γc$^{-/-}$ mice were treated i.p. with PBS, poly(I:C) (50 μg), or *L. infantum* promastigotes (10^7 parasites). At 18 to 20 h posttreatment or postinfection, CFSE-labeled uninfected MHC class I-deficient ($\beta_2m^{-/-}$) BM-Mϕ (CFSE^{hi} CD45.1$^-$) and WT splenocytes (CFSE^{low} CD45.1$^+$) were injected into the peritoneal cavity. (A) The percentage of CFSE-labeled cells recovered from the peritoneal cavity at 14 to 16 h posttransfer was analyzed by flow cytometry and used to calculate the percent specific lysis. (B) The bar graph depicts the mean (±SEM) of percent specific lysis of WT, RAG1$^{-/-}$, or RAG2/γc$^{-/-}$ peritoneal cells against uninfected $\beta_2m^{-/-}$ BM-Mϕ targets in two independent experiments with three mice per group.

We therefore cocultured *L. infantum*-infected BM-Mϕ with highly purified NK cells from naïve mice in the presence of IL-12 and IL-18, which are required for optimal NK cell activation in mouse cutaneous and visceral leishmaniasis (24, 39, 61). After 72 h of coculture, the total parasite load decreased by 76% to 97% (Fig. 6A, C, and D). In the nonstimulated 72-h control cultures (Mϕ only), the macrophage infection rate varied between 38% and 74% and the absolute number of parasites per 100 Mϕ was between 64 and 344, whereas in the presence of NK cells plus IL-12 and IL-18, the infection rate went down to 4% to 24% and the parasite numbers per 100 Mϕ ranged from 2 to 43 in this series of experiments. Importantly, there was no correlation between the reduction of the macrophage parasite load induced by activated NK cells and the absolute parasite numbers in the control cultures (data not shown). Thus, NK cell-mediated activation of infected macrophages is not influenced by the parasite burden within the macrophages.

To further characterize the ability of activated NK cells to trigger antiparasitic effects in macrophages, we titrated the NK cell/BM-Mϕ ratio (6:1, 3:1, 1:1, 0.1:1, to 0.01:1) in two independent experiments. Comparable reductions in the parasitic load were observed with NK cell/BM-Mϕ ratios from 6:1 down to 0.1:1, indicating that less than one NK cell per macrophage is sufficient to elicit parasite killing by the host cell (data not shown).

The macrophage-activating effect of NK cells was independent of physical interactions between both cell populations, as it was also observed under transwell conditions (Fig. 6B). Furthermore, it was mediated by IFN-γ and TNF and the expression of iNOS. This was revealed by (i) the analysis of IFN-γR$^{-/-}$ BM-Mϕ (Fig. 6C, left panel), IFN-γ$^{-/-}$ NK cells (Fig. 6C, right panel), anti-TNF antibodies (Fig. 6D), and iNOS$^{-/-}$ BM-Mϕ (Fig. 6C, left panel); (ii) the determination of cytokine and NO_2^- levels in the respective culture SNs (Table 1); and (iii) the detection of cell-associated IFN-γ, but not of TNF, in activated NK cells using intracellular cytokine staining (data not shown). As controls, BM-Mϕ were stimulated with IFN-γ plus TNF, which led to the expected, almost complete elimination of intracellular parasites (7, 69) (Fig. 6A to D), whereas the use of IFN-γ or TNF alone (Fig. 6A) or of IL-12 plus IL-18 in the absence of NK cells (Fig. 6A, C, and D) was largely ineffective. Likewise, NK cells added to the infected BM-Mϕ monolayers in the absence of IL-12 plus IL-18 caused no or only very limited (4.1% to 35.7%) killing of intracellular *Leishmania*, which was not observed when the two cell populations were separated from each other (Fig. 6B, direct contact versus transwell).

From these data we conclude that IL-12/IL-18-stimulated NK cells activate *Leishmania*-infected macrophages in a cell contact-independent but IFN-γ- and TNF-dependent manner for the expression of iNOS, which is the key effector mechanism in murine leishmaniasis (17, 50, 67).

DISCUSSION

NK cell cytotoxicity against host cells infected with nonviral pathogens. Host cell lysis is considered to be a rapidly available and highly efficient effector mechanism of NK cells during infections with intracellular pathogens. Its protective role in

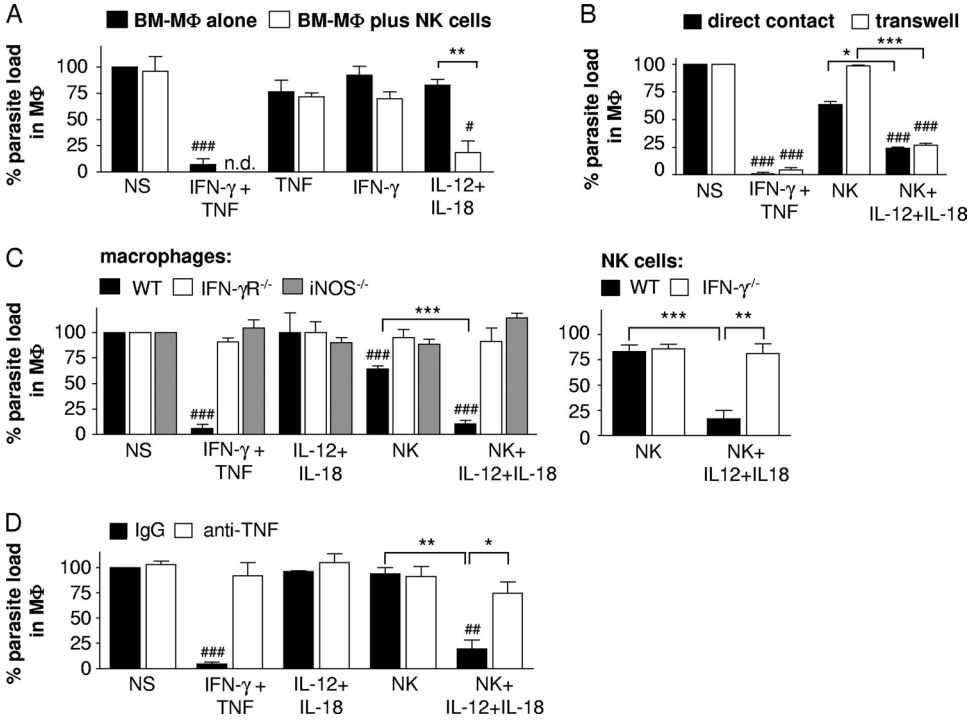

FIG. 6. Activated NK cells stimulate infected macrophages to kill intracellular *Leishmania* in a cell contact-independent but IFN-γ-, TNF-, and iNOS-dependent manner. BM-Mϕ were infected with *L. infantum* promastigotes for 18 h in 8-well chamber slides (A, C, and D; parasite/cell ratio = 10:1) or 24-well plates (B; parasite/cell ratio = 5:1) and were subsequently cocultured with the indicated cytokines and/or purified splenic NK cells from naïve mice. After 72 h the intracellular parasite load was determined (mean [±SEM] of 3 to 4 independent experiments). Rhombus-shaped symbols denote significance compared to unstimulated cells (NS). * or #, $P < 0.05$; ** or ##, $P < 0.01$; *** or ###, $P < 0.001$ (Student's *t* test); n.d., not detectable. (A) BM-Mϕ monolayer alone versus NK cells cocultured with BM-Mϕ (ratio, 6:1). (B) Coculture of NK cells with BM-Mϕ (ratio, 6:1). The NK cells were added directly to the BM-Mϕ plated on coverslips in a 24-well plate (direct contact) or seeded into culture inserts placed above the infected BM-Mϕ monolayer (transwell). (C) (Left panel) B6 WT NK cells were cocultured with B6 WT, IFN-γR$^{-/-}$, or iNOS$^{-/-}$ BM-Mϕ (ratio, 6:1); (right panel) B6 WT or IFN-γ$^{-/-}$ NK cells were cocultured with B6 WT BM-Mϕ (ratio, 6:1). (D) NK cells and BM-Mϕ from B6 WT mice were cocultured (ratio, 6:1) with the addition of anti-TNF neutralizing antibody or normal IgG (5 μg/ml).

viral diseases is well documented and highly plausible, because viruses require host cells for replication. Very little is known whether NK cell-mediated lysis of host cells also contributes to the control of nonviral pathogens. In the present study, we tested the hypothesis that myeloid cells infected with the facultative intracellular parasite *Leishmania* also become a target of NK cell cytotoxicity *in vitro* and *in vivo*. This hypothesis was based on several considerations. First, *Leishmania* released from lysed myeloid cells is thought to be highly susceptible to humoral defense mechanisms of the host (e.g., complement), so that target cell lysis even without simultaneous parasite destruction could be beneficial to the host organism. Second, NK cells and myeloid cells frequently colocalize during cutaneous or visceral leishmaniasis (3, 72), raising the possibility of functional interactions. Third, previous experiments involving activation, depletion, or transfer of NK cells provided evidence for a protective role of NK cells in cutaneous and visceral leishmaniasis (3, 17, 24, 28, 34, 36, 39, 43, 56–58, 61).

To our knowledge, the present study is the first to address the issue of NK cell-mediated target cell lysis in a nonviral infection model *in vivo*. The data clearly show that upon infection with *Leishmania* parasites WT macrophages do not become susceptible to cytolysis by activated NK cells either *in vitro* or *in vivo*. Resistance to NK cell lysis was paralleled by an unaltered expression of activating or inhibitory NK cell receptors on the surface of the infected host cells. For the *in vivo* analysis, we had to resort to a newly established *L. infantum* peritoneal infection model, because infected macrophages that were transferred i.v. into previously i.v. infected mice became trapped in the lung and did not home to the sites of NK cell activation in the spleen or liver (data not shown). The validity of the peritoneal model was ascertained by verifying the presence of activated NK cells in the peritoneal cavity after i.p. infection, which was unequivocally demonstrated by the expression of IFN-γ in peritoneal NK1.1$^+$ CD3$^-$ cells (data not shown) and the prominent lysis of susceptible β_2m$^{-/-}$ macrophage targets by these NK cells (Fig. 4).

Several previous studies suggested that NK cells exert cytotoxic effects on host cells infected with various nonviral intracellular pathogens. These include human monocytes harboring *Mycobacterium tuberculosis* (74), human erythrocytes carrying *Plasmodium falciparum* schizonts (53), and possibly also human neutrophils after phagocytosis of *Haemophilus influenzae* (47). There are also two reports that claimed *in vitro* NK cell cytotoxicity against BALB/c macrophages infected with promastigotes of *L. major* or *L. amazonensis*, respectively (1, 55). However, there are several problems with the experimental design of both studies. These include (i) the application of IL-2-activated killer (LAK) cells without further phenotypic characterization (55) or with proven contamination by NKT

TABLE 1. Cytokine and NO_2^- levels in SNs of BM-Mϕ cultures and BM-Mϕ/NK cocultures[a]

Culture no.	BM-Mϕ	Cultured with:			Measured concn (±SEM)[b]		
		NK	Stimulus	Blocking agent	IFN-γ (ng/ml)	TNF (pg/ml)	NO_2^- (μM)
1	WT				0 (±0)	6 (±3)	0 (±0)
2	WT		IFN-γ + TNF		12 (±4)	1,750 (± 512)	56 (±3)
3	WT		IL-12 + IL-18		0 (±0)	18 (±3)	3 (±3)
4	WT	WT			0 (±0)	69 (±29)	0 (±0)
5	WT	WT	IL-12 + IL-18		144 (±43)	274 (±67)	50 (±4)
6	IFN-γR$^{-/-}$				0 (±0)	4 (±1)	0 (±0)
7	IFN-γR$^{-/-}$		IFN-γ + TNF		22 (±4)	652 (±131)	0 (±0)
8	IFN-γR$^{-/-}$		IL-12 + IL-18		0 (±0)	24 (±18)	0 (±0)
9	IFN-γR$^{-/-}$	WT			0 (±0)	76 (±51)	0 (±0)
10	IFN-γR$^{-/-}$	WT	IL-12 + IL-18		276 (±43)	241 (±62)	0 (±0)
11	WT			IgG	0 (±0)	5 (±4)	0 (±0)
12	WT			Anti-TNF	0 (±0)	1 (±0)	0 (±0)
13	WT		IFN-γ + TNF	IgG	13 (±3)	801 (±356)	48 (±3)
14	WT		IFN-γ + TNF	Anti-TNF	6 (±3)	221 (±140)	4 (±2)
15	WT		IL-12 + IL-18	IgG	0 (±0)	13 (±4)	0 (±0)
16	WT		IL-12 + IL-18	Anti-TNF	0 (±0)	8 (±6)	0 (±0)
17	WT	WT		IgG	0 (±0)	110 (±89)	0 (±0)
18	WT	WT		Anti-TNF	0 (±0)	50 (±37)	0 (±0)
19	WT	WT	IL-12 + IL-18	IgG	106 (±75)	168 (±81)	52 (±5)
20	WT	WT	IL-12 + IL-18	Anti-TNF	116 (±72)	67 (±35)	49 (±13)

[a] BM-Mϕ from B6 or IFN-γR$^{-/-}$ mice were infected for 18 h with a 10-fold excess of *L. infantum* promastigotes and subsequently cultured for 72 h with different cytokines with or without addition of splenic NK cells from B6 mice. Under some conditions, anti-TNF blocking antibody or a control IgG was added. SNs were tested for cytokine and NO_2^- content.

[b] Data are expressed as mean (±SEM) of 3 to 6 independent experiments.

and $CD8^+$ T cells (1), which are known to lyse and activate *Leishmania*-infected myeloid cells (10, 66), (ii) the use of extremely high LAK cell-Mϕ ratios (≥10:1) and *Leishmania*-Mϕ ratios (≥40:1) (55), and (iii) the nonquantitative analysis of cytotoxicity without including uninfected macrophages as controls (1).

Thus, on the basis of our own *in vitro* and *in vivo* results, we argue that highly purified NK cells do not exert a cytolytic effect against infected macrophages *in vitro*, except for the small degree of lysis already seen with uninfected cells. This notion is further supported by the observation that human immature DCs infected with *L. infantum* were resistant to NK cell-mediated lysis due to the upregulation of HLA-E (a ligand for the inhibitory NK cell receptor CD94/NKG2A) (10) and that LAK cells also failed to lyse mouse macrophages infected with the related pathogen *Trypanosoma cruzi* (79).

NK cells and cytokine-mediated macrophage activation. IFN-γ and TNF are known activators of macrophages, and IFN-γ is the key cytokine for the induction of iNOS in mice. *In vitro* IFN-γ-mediated induction of iNOS requires endogenous TNF (for a review, see reference 6). As NK cells are an early source of IFN-γ in *Leishmania* infections (3, 17, 39, 57, 61), it might appear to be trivial to postulate NK cell cytokine-mediated macrophage activation. However, whether this actually occurs upon contact of activated NK cells with infected macrophages has never been tested. In fact, in response to *Leishmania*, macrophages can release IL-10 (49), which is one of the cytokines that is able to antagonize NK cell functions and to suppress the release of NK cell-activating cytokines (15, 73). In addition, NK cells themselves produce IL-10 (44, 46), which then might block macrophage activation, including the expression of iNOS (5, 6). The results reported in the present study convincingly show that in an NK cell/macrophage coculture system, IL-12 and IL-18 drive the production of sufficiently high levels of IFN-γ and TNF for the subsequent induction of macrophage antileishmanial activities. In accordance with our previous analyses on the origin of IFN-γ in macrophage cultures (60), the comparative measurement of IFN-γ in macrophage cultures versus NK cell/macrophage cocultures and intracellular cytokine staining identified NK cells as the primary source of IFN-γ (Table 1, cultures 19 versus 15, and data not shown). With respect to TNF, macrophages rather than NK cells appear to be the producers, because NK cells were negative for intracellular TNF (data not shown) and IFN-γ is known to activate *Leishmania*-infected macrophages for the expression of endogenous TNF (23).

Although IFN-γ is an essential component of the macrophage-activating effect of IL-12/IL-18-stimulated NK cells (Fig. 6C), it is possible that, in addition to IFN-γ, other soluble factors secreted by NK cells are involved. This is suggested by the observation that the decrease of the parasite burden in the macrophage-NK cell coculture system was considerably smaller when rmIL-12 plus rmIL-18 was replaced by rmIFN-γ (Fig. 6A). It is also worth noting that the role of TNF is not necessarily restricted to the synergistic induction of iNOS together with IFN-γ, as iNOS-independent antileishmanial effects of TNF have been repeatedly reported (63, 76). This possibility is supported by our observation that anti-TNF treatment of IL-12/IL-18-stimulated BM-Mϕ/NK cell cocultures largely blocked the killing of *Leishmania* parasites, without causing a significant reduction of nitrite accumulation (Fig. 6D; Table 1, cultures 20 versus 19).

In some of our experiments on the interaction of NK cells with infected macrophages, we noticed a small decrease of the

macrophage parasite burden even in the presence of nonactivated NK cells (Fig. 6B and C). The effect was IFN-γ and iNOS dependent (Fig. 6C), which argues against the possibility that naïve NK cells act by the release of perforin and other cytotoxins that are taken up by the neighboring infected macrophage and mediate *Leishmania* killing, as suggested for other pathogens in an NK cell-free *in vitro* system (75).

In conclusion, despite being potent cytotoxic effectors, NK cells activated by *Leishmania* infection fail to recognize infected macrophages as targets, most likely due to the lack of proper NK cell stimulatory signals. This finding provides a perfect example of host-pathogen interaction where *Leishmania* use macrophages as a safe niche for their initial survival, while the host avoids unwanted killing of infected cells, which otherwise could lead to the release and dissemination of viable parasites. However, due to their capacity to respond to IL-12 and IL-18 by releasing IFN-γ, NK cells efficiently assist macrophages in the control of *Leishmania* parasites.

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