NK Cells Contribute to the Control of *Trypanosoma cruzi* Infection by Killing Free Parasites by Perforin-Independent Mechanisms

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The protozoan parasite *Trypanosoma cruzi* circulates in the blood as trypomastigotes and invades a variety of cells to multiply intracellularly as amastigotes. The acute phase leads to an immune response that restricts the proliferation of the parasite. However, parasites are able to persist in different tissues, which causes the pathology of Chagas’ disease. Natural killer (NK) cells play an important role in innate resistance to a variety of pathogens. In the present study we analyzed whether NK cells participated in the control of experimental *T. cruzi* infection. NK cells were depleted from C57BL/6 mice by antiasialo antibodies. This treatment caused an increased parasitemia during the acute phase, but tissue parasite burdens were not significantly altered according to quantitative real-time PCR. Our results demonstrated that NK cells were activated during the initial phase of a *T. cruzi* infection and exhibited a contact-dependent antiparasitic activity against extracellular parasites that was independent from perforin. Thus, NK cells limit the propagation of the parasite by acting on circulating *T. cruzi* trypomastigotes.

The protozoan parasite *Trypanosoma cruzi* infects a number of mammalian species and is the etiologic agent of Chagas’ disease of humans. Although it is a major health problem in Latin America—18 million people are chronically infected and 50,000 people die annually from Chagas’ disease—no convincing therapy is available. The parasites infect a variety of host cells, where they replicate intracellularly as amastigotes. After rupture the parasites are released as trypomastigotes that spread via the bloodstream to infect other host cells. The acute phase is characterized by high parasitemia and is followed by a chronic phase. Although during this phase almost no parasites are found in the blood, *T. cruzi* can persist life-long in various tissues (22). The pathology of Chagas’ disease is associated with the chronic phase. Although different mechanisms have been proposed to be responsible for the observed pathology, there is a growing body of evidence that parasite persistence is the primary course of Chagas’ disease (16, 29, 30, 31). Moreover, using sensitive molecular detection methods, it was demonstrated that the parasitic load is involved in the exacerbation of disease (33).

In *T. cruzi*-infected mice, protective immunity depends critically on the production of gamma interferon (IFN-γ) (32). It was shown that the induction of a Th1 immune response is capable of restricting the growth of *T. cruzi* (1, 14, 18, 21). The protective capacity of CD4⁺ T cells relies on IFN-γ-mediated activation of macrophages (10), whereas cytotoxic cells, i.e., cytotoxic T lymphocytes and natural killer (NK) cells, can directly lyse infected host cells by employing a concerted action of perforin, granzymes, and Fas ligand. Although T cells are absolutely required for the clearance of the pathogen, the innate immune response plays a pivotal role in the early phase. Thus, it was shown that depletion of NK cells results in increased parasitemia and mortality during the acute phase (6, 26). This has stimulated the interest in mechanisms of both innate and adaptive immunity that contribute to parasite clearance.

In the present study, we therefore analyzed further the contribution of NK cells to the immune effector mechanisms against *T. cruzi*. We demonstrated that depletion of NK cells prior to infection resulted in an increased parasitemia, whereas tissue burden was not influenced. Our data suggest that NK cells develop contact-dependent effector mechanisms against free parasites during infection.

### MATERIALS AND METHODS

**Parasites and mice.** *T. cruzi* strain Tulahuen (WHO reference strain M/HOM/CH/00/Tulahuen C2) is maintained by serial passage in BALB/c mice. In vitro culture was carried out by weekly inoculation of semiconfluent L929 cells with trypomastigotes drawn from the supernatants of previously infected cells. SCID mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Wild-type C57BL/6 mice and interleukin-12-deficient (IL-12⁻/⁻) mice were bred in the institute’s animal facility. Perforin⁻/⁻ mice on a C57BL/6 background were obtained from the MPI of Immunology (Freiburg, Germany). Male mice aged 6 to 8 weeks were experimentally infected with *T. cruzi* by inoculation of blood-form trypomastigotes into the footpad. Parasitemia was determined in 5 μl of blood that was obtained by tail vein puncture and lysed in 45 μl of NH₄Cl (0.87% [wt/vol]). Viable parasites were counted in a Neubauer chamber. Epimastigotes of the Tulahuen strain were obtained from cultures with LIT medium as described elsewhere (17).

**Flow cytometry.** To check NK cell depletion by antiasialo treatment, blood of infected mice was taken 12 days postinfection (p.i.). Erythrocytes were lysed by addition of 2 ml of Aqua dest., and thereafter 2 ml of double-concentrated phosphate-buffered saline (PBS) was added to reach physiological salt concentrations. The remaining cells were washed twice with PBS supplemented with 1% fetal calf serum (FCS) and were subsequently stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse CD49b/pan-NK cell (DX5) monoclonal antibody and Cy-chrome-conjugated rat anti-mouse CD3 monoclonal antibody, respectively. After incubation on ice for 30 min, cells were washed three times with PBS supplemented with 1% FCS. Stained cells were fixed by addition of 2%...
parafomaldehyde and examined with a FACScan using the CellQuest software (Becton Dickinson, Mountain View, Calif.).

**Parasitemia and survival.** NK cells were depleted in vivo with a rabbit anti-mouse asialo GM1 polyclonal antiserum (Cedarlane). Fifty microliters of antialosialo antiserum was injected intraperitoneally (i.p.) on days −1, +3, and +7 and with the infection with T. cruzi. Control mice were treated with 50 μl of PBS i.p. at the same time points. Mice were infected with either \(10^4\) or 250 T. cruzi into the footpad suspended in PBS, respectively. Parasitemia was determined starting at day 7 p.i. every 3 to 4 days. For NK cell depletion with monoclonal anti-NK.1 antibodies (PK136; American Type Culture Collection), mice were injected with 100 μg i.p. on days −1, 0, +2, +6, and +10 after the infection.

**Cytotoxicity assay.** A total of 10⁵ spleen cells from C57BL/6 mice were incubated overnight in vitro either with 200 μg of polyclonal (P1-C [polyinosinic-polycytidil]; Sigma, Taufkirchen, Germany) or with 10⁶ viable T. cruzi trypomastigotes obtained from in vitro culture. As a control, spleen cells were incubated with medium alone. To analyze NK cell activation, spleen cells were incubated with YAC-1 cells that were previously labeled with radioactive ⁵¹Cr (Amersham Bioscience, Freiburg, Germany) for 90 min at 37°C and then washed with warm medium. Labeled target cells were incubated with the respective spleen cells at the indicated ratio at 37°C. After 4 h the supernatant was harvested and analyzed for radioactivity using a gamma counter.

**ELISPOT assay.** For ELISPOT analysis, a matched pair of anti-IFN-γ antibodies was used (Becton Dickinson). Spleen cells from SCID mice (2 × 10⁵) were incubated either with medium, with 2 × 10⁶ live T. cruzi trypomastigotes, or with the same number of dead parasites (heat inactivated [95°C for 15 min]) for 24 h in anti-IFN-γ-coated ELISPOT plates (MultiScreen HA; Millipore, Bedford, Mass.). Analysis was performed as described by the manufacturer. Cytokine-producing cells were detected using biotinylated anti-IFN-γ followed by avidin-conjugated peroxidase. Spots were developed with substrate buffer (100 mM Tris [pH 7.5], 800 μg of dianaminobenzidin/ml, and 400 μg of NiCl₂/ml analyzed with an ELISPOT reader (Bioreader 2000). Data were displayed as the number of cytokine-producing cells per 10⁵ cells (spot-forming units).

**Quantitative real-time PCR of T. cruzi DNA.** Quantification of tissue parasite burdens was performed as described previously (11). Specimens of about 20 mg were separately analyzed. A 121-bp sequence of the 140/116-kDa antigen gene of T. cruzi (accession no. U15616) was amplified with forward primer GGCTGCA GAGTGCAGGTTGT, reverse primer GCATATCGCCGAAAACAGCA, and an internal probe, carboxyfluorescein-TAGGCTTCCATGATGCAAAAAGAAAC AAAAAGAAA-tetramethyl carbonylhydroxamidine-TA. Reaction mixtures of 0.05 ml contained a 200 nM concentration of each primer, a 100 nM concentration of probe, a 0.2 nM concentration of each deoxynucleoside triphosphate, 2 mM MgCl₂, 1 U of AmpliTaq Gold, 50 mM KCl, 0.01 mM EDTA, 10 mM Tris-HCl (pH 8.3), and 0.005 ml of template DNA. PCR reagents were obtained from Applied Biosystems (Weiterstadt, Germany). Thermal cycling comprised an initial denaturation step of 15 min at 95°C followed by 45 cycles of 20 s at 95°C and 40 s at 58°C on an ABI Prism 7700 SDS instrument (Applied Biosystems). A 347-bp stretch of the murine β-actin gene was used for quantification of host DNA contamination. Amplification was carried out essentially as described elsewhere (25). Both T. cruzi and β-actin sequences were quantified individually for each DNA sample according to the methods of Bustin et al. (4). The quantity of parasite DNA in a specimen was expressed in relation to that of its content of β-actin DNA.

**Purification or depletion of NK cells from spleen cell cultures.** A total of 10⁵ spleen cells from C57BL/6 mice were washed three times in PBS supplemented with 1% FCS and stained with FITC-labeled anti-mouse CD49b/pan-NK cell (DX5) monoclonal antibody. Cells were washed three times and were resuspended in PBS supplemented with 0.1% bovine serum albumin and 2 mM EDTA. Magnetic-activated cell sorter anti-FITC microbeads (Miltenyi Biotec) were incubated overnight with T. cruzi trypomastigotes. Activation of NK cells was analyzed by using chromium-labeled YAC cells. These cells do not express major histocompatibility complex class I molecules and therefore serve as targets for activated NK cells. Infection of spleen cells led to an increased lysis of YAC cells in comparison to untreated control cells (Fig. 1A). To further investigate if only cytolytic pathways of NK cells were activated or if cytokine expression was also induced, trypomastigotes were incubated with spleen cells from SCID mice that lack B and T cells. Viable parasites led to a strong induction of IFN-γ-producing cells in comparison to medium control or heat-inactivated trypomastigotes (Fig. 1B). In addition, we found that DX5-positive NK cells from in vitro-infected spleen cells as well as those isolated from lymph nodes of infected mice produced IFN-γ, as analyzed by intracellular fluorescence-activated cell sorter staining (data not shown). These data show that infection with T. cruzi in vitro and in vivo activates cytokine secretion as well as lytic pathways of NK cells.

**Depletion of NK cells leads to an increased parasitemia.** In order to confirm that NK cells contribute to parasite clearance in vivo, C57BL/6 mice were treated with antisialo antibodies on days −1, 0, 3, and 7. The efficiency of NK cell depletion was determined on day 12 in peripheral blood by flow cytometry (Fig. 2). Treatment of mice with antisialo antibodies resulted in an almost complete removal of DX5-positive NK cells from the blood during the acute phase of infection, although we could not exclude that some NK cells remained in different tissue. It is interesting that NK cell-depleted mice displayed for each data point. For quantification of membrane leakage, 10⁵ culture-derived trypomastigotes of T. cruzi were washed in serum-free medium and labeled with 4 μM CellTracker Green (5-chloromethylfluorescin diacetate [CMFDA]; Molecular Probes, Eugene, Ore.) for 30 min at 37°C. Cells were washed in warm medium containing 5% FCS and were further incubated for 30 min at 37°C. Trypomastigotes were washed again, and 4 × 10⁵ cells were incubated with 4 × 10⁵ C57BL/6 spleen cells, which were either untreated or prestimulated with 0.2 mg of poly(I-C)per ml overnight, for 4 h at 37°C. Cells were washed in PBS supplemented with 1% FCS, fixed with 2% paraformaldehyde, and analyzed with a FACScan (Becton Dickinson, Mountain View, Calif.). In some experiments spleen cells were incubated overnight with 25 mM strontium chloride (Sigma, Taufkirchen, Germany) to induce degranulation. Cells were subsequently washed and used as described above.

**Scanning electron microscopy.** Either 10⁶ NK cells or T cells were purified from poly(I-C)-stimulated spleen cells as described above and were incubated with 10⁶ epimastigotes. Cells were subsequently fixed with 2% glutaraldehyde in sodium cacodylate buffer and applied to poly-(l-lysine)-coated cover slides (Cell Locate; Eppendorf). After 2 h slides were washed and fixed again with 1% osmium oxide for 30 min at 4°C. After repeated washing, cells were dehydrated with increasing ethanol concentrations and subjected to critical point drying. Samples were spotted with gold and analyzed in a scanning electron microscope (PSEM 500; Philips).

**Statistical analysis.** Results are presented as the means plus standard deviations. The number of individual experiments is indicated in each figure legend. Statistical analysis was generally performed with the unpaired Student’s t test by using the Prism software (GraphPad Software, San Diego, Calif.). The level of significance was set at a P value of <0.05.

**RESULTS**

**Activation of NK cells by T. cruzi.** Several studies have shown that infection with T. cruzi in vitro and in vivo leads to a strong induction of IL-12 and IL-18, which are known to stimulate NK cells as well as T cells (9, 20). To determine the contribution of NK cells to the very early immune response against trypanosomes, spleen cells from C57BL/6 mice were incubated overnight with T. cruzi trypomastigotes. Activation of NK cells was analyzed by using chromium-labeled YAC cells. These cells do not express major histocompatibility complex class I molecules and therefore serve as targets for activated NK cells. Infection of spleen cells led to an increased lysis of YAC cells in comparison to untreated control cells (Fig. 1A). To further investigate if only cytolytic pathways of NK cells were activated or if cytokine expression was also induced, trypomastigotes were incubated with spleen cells from SCID mice that lack B and T cells. Viable parasites led to a strong induction of IFN-γ-producing cells in comparison to medium control or heat-inactivated trypomastigotes (Fig. 1B). In addition, we found that DX5-positive NK cells from in vitro-infected spleen cells as well as those isolated from lymph nodes of infected mice produced IFN-γ, as analyzed by intracellular fluorescence-activated cell sorter staining (data not shown). These data show that infection with T. cruzi in vitro and in vivo activates cytokine secretion as well as lytic pathways of NK cells.

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levels of IFN-\(\gamma\) in the serum similar to those found in untreated mice upon infection, which indicated that also in the absence of NK cells a robust Th1 response was mounted (data not shown). Infection with a sublethal dose of \(10^4\) \(T.\) cruzi resulted in a comparable survival rate of NK cell-depleted and control mice (Fig. 3A). However, mice lacking NK cells suffered from a significantly increased parasitemia in comparison to control mice when infected with either a sublethal dose of \(10^4\) (Fig. 3B) or with a nonlethal dose of \(0.25 \times 10^3\) \(T.\) cruzi trypomastigotes (Fig. 3C). The observed effects were not restricted to NK depletion by antiasialo antibodies, since we depleted NK cells in some experiments by using anti-NK1.1. This led to a similar increase in parasitemia but did not influence survival significantly (data not shown). Since the expression of this marker is not only restricted to NK cells, we used antiasialo-mediated depletion of NK cells for further experiments.

**Tissue burdens from NK cell-depleted mice are comparable to those from untreated mice.** To analyze the influence of NK cell depletion on parasite burden during a \(T.\) cruzi infection, we employed quantitative PCR to measure the relative \(T.\) cruzi DNA concentrations in tissues. During the acute phase (day 12 p.i.), lymph nodes, heart, and skeletal muscle from both groups of mice were analyzed for the quantity of \(T.\) cruzi DNA in relation to their content of murine \(\beta\)-actin DNA (Fig. 4A). In contrast to parasitemia, the amount of parasitic DNA in the different tissues obtained from both groups was not significantly different. The highest tissue load was found in specimens from muscle and fivefold less in lymph nodes, whereas the tissue load from the heart was lowest. It is interesting that, in contrast to what was expected, all samples from NK cell-depleted mice exhibited a slightly decreased parasitic load in comparison to untreated mice, although the difference was not found to be statistically significant.

To investigate whether NK cell depletion had an influence on tissue burden during the chronic phase, NK cell-depleted and untreated control mice were infected with a nonlethal dose of 250 trypanosomes. As shown before, NK cell-depleted mice exhibited an increased parasitemia during the acute phase in comparison to control mice (Fig. 3C). All mice survived, and after day 30 parasites were undetectable in the blood. To analyze the tissue burden during the chronic phase, the parasitic loads of different organs of the respective groups were quantified either 30 days or 1 year after infection by quantitative PCR (Fig. 4B and C). Whereas the parasitic burdens of both heart and lymph node were low, the highest amount of parasitic DNA was found in skeletal muscle tissue. As for the acute phase, there were no differences in tissue burdens in the chronic phase between both groups of mice. A direct comparison of \(T.\) cruzi DNA derived from skeletal muscle from both the acute and chronic phases indicated that tissue burden increased with time, indicating that this tissue is most susceptible for parasite persistence. In contrast, the parasitic burdens of heart tissue and lymphatic tissue remained constant with time.

**NK cells exhibit antiparasitic mechanisms against extracellular trypanosomes.** The increase of parasitemia in the blood of NK-depleted mice indicated that NK cells contribute to the killing of extracellular parasites. To further characterize these NK cell-mediated mechanisms that contribute to the control of extracellular trypanosomes, an in vitro system using either free epimastigotes or trypomastigotes as target cells and spleen cells from different mice as a source of NK cells was established. Naive spleen cells were able to kill free parasites after coincubation, as indicated by a rapid loss of motility observed with light microscopy (Table 1). Since the observed killing was very rapid, a contribution of a specific T-cell response is very unlikely and NK cells were thought to be the most promising candidate of effector cells. This was further corroborated by...
the finding that stimulation of spleen cells with poly(I:C), which is known to activate NK cells (7), led to an increased activity against trypanosomes. Even spleen cells from SCID mice, which lack B and T cells, exhibited similar activity against trypanosomes in comparison to spleen cells from wild-type mice. To ascertain that NK cells were involved, isolated DX5-positive NK cells were incubated with parasites. In contrast to unstimulated NK cells, NK cells that were purified from poly(I:C)-stimulated spleen cells were highly active against trypanosomes. To exclude that metabolically active cells might influence the survival of trypanosomes, they were incubated with the B-cell hybridoma A20, which had no influence on the parasites.

In order to analyze the molecular mechanisms that lead to the killing of free trypanosomes by NK cells, parasites were stained with the fluorescent dye CMFDA and were incubated with spleen cells from either wild-type or SCID mice. This assay directly quantifies cytolytic reactions, since membrane leakage can be analyzed by measuring the amount of fluorescent parasites by using flow cytometry. Incubation of trypanosomes with naive spleen cells resulted in rapid loss of the CMFDA from parasites, which was dependent on the effector cell-to-parasite ratio (Fig. 5). To validate that NK cells are responsible for the observed lysis of parasites, NK cells were depleted from spleen cells of C57BL/6 mice by magnetic cell sorting. This treatment led to a decreased lytic activity (Fig. 6A). Furthermore, spleen cells from SCID mice, which lack B and T cells, were capable of lysing parasites. These data indicate that NK cells are responsible for the major activity against free parasites. The lytic activity was not further decreased by depletion of either CD4+ or CD8+ T cells, which indicated that T cells were not responsible for the observed trypanocidal activity that remained after depletion of NK cells. In order to further evaluate which mechanisms were employed to lyse free trypanosomes, NK cells were preincubated with strontium chloride, which induces degranulation of cytotoxic cells (28). This treatment significantly decreased the antiparasitic effect, suggesting that cytotoxic granules are involved in parasite lysis. Although in this assay membrane leakage was directly quantified, spleen cells from perforin-deficient mice were as active as those from wild-type mice (Fig. 6B). Interestingly, NK cells purified from C57BL/6 mice after stimulation with poly(I:C) were as active as spleen cells. In contrast, unstimulated NK cells exhibited no lytic activity (Fig. 6C). To further analyze the requirements for NK cell activation by trypanosomes, we incubated spleen cells from IL-12−/− mice with parasites. These spleen cells were not able to lyse parasites, even when they were stimulated by poly(I:C). This indicates that IL-12 produced by spleen cells after incubation with T. cruzi is necessary for activation of NK cells and therefore explains why unstimulated and highly purified NK cells are not able to lyse parasites.

These data suggest that on the one hand cytotoxic granules are involved in parasite killing but that on the other hand perforin is not solely responsible for lytic effector mechanisms. Using scanning electron microscopy, we found that preincubation of NK cells and free T. cruzi led to a rapid formation of intimate cell-cell contact (Fig. 7), which was not present when purified T cells were used (data not shown). In summary, coincubation of spleen cells from either C57BL/6 mice or from SCID mice with parasites led to a rapid loss of mobility (Table 1) and loss of membrane integrity (Fig. 5 and 6) of the parasites. This activity was found to be dependent on NK cells and was contact dependent. Indeed, neither conditioned medium from T. cruzi-infected spleen cells nor spleen cells from SCID mice that were separated from free trypanosomes in transwell experiments exhibited activity against free T. cruzi (data not shown).

DISCUSSION

In the mammalian host, T. cruzi cycles between two different developmental stages: extracellular trypomastigotes disseminate in the host, while intracellular amastigotes are forms of replication and persistence. However, most studies have analyzed the interactions of the immune system with intracellular forms. For this stage it is well established that IFN-γ plays a central role for the control of parasites, due to its capacity to activate inducible nitric oxide synthase-dependent microbicidal
activities in macrophages, which are known to be the first cells that are infected with *T. cruzi* (10, 13, 27, 33). Infected macrophages were shown to produce IL-12 and also IL-18, both of which are known to activate NK cells synergistically and to promote a Th1 immune response (1, 9, 20). The importance of IL-12 production was demonstrated by using IL-12-deficient mice, which were shown to be highly susceptible (1, 21). However, IL-18 deficiency did not interfere with resistance (11). Most probably, the IL-12 production which is induced by *T. cruzi* infection is sufficient to induce a robust Th1 response even in the absence of IL-18.

Several studies have shown that NK cells are activated during infection with *T. cruzi* trypomastigotes in vitro as well as in vivo (2, 34). Although immunity against *T. cruzi* infection depends critically on T cells, activated NK cells orchestrate the very early phase of the ongoing immune response in various ways: (i) NK cell-derived cytokines induce a Th1-biased T-cell response; (ii) they activate macrophages via secretion of IFN-γ; and (iii) they are capable of killing free pathogens directly. Consequently, it was shown that NK-depleted mice suffer from an increased parasitemia upon infection with *T. cruzi*, which was paralleled by a decreased IFN-γ level during the very early phase of infection (6, 26, 34). As already shown by others, we demonstrated that NK-depleted mice exhibit an increased peak parasitemia during the acute phase (6, 26). However, in contrast to other studies, we did not see a significant influence of NK cell depletion on the survival rate. This might be due to different experimental conditions, e.g., the route of infection, parasite number, and clonal variation of the pathogens, which might have major effects on the outcome of infection. These data imply that high levels of parasite tissue burden are more likely associated with death of the host than high levels of parasitemia. Indeed, using quantitative PCR we found that during peak parasitemia in the acute phase the parasitic load of various organs was comparable between NK-depleted and control mice. However, we cannot exclude that NK cells in some tissues are resistant to depletion by antiasialo antibodies that might account for similar parasitic loads in tissues between untreated and NK-depleted groups (8). However, these data exclude that the increased number of extracellular parasites in the blood of NK-depleted mice is due to an increased pool of intracellular parasites in infected tissue.

Interestingly, infected mice developed a strong Th1 response even in the absence of NK cells (data not shown). This suggests that the immune response against *T. cruzi* is strongly biased towards a proinflammatory reaction, so that an effective Th1 response was mounted even in the absence of NK cells, although they were shown to be a source of early IFN-γ (5, 34). This is in agreement with previous results that showed that IL-18-deficient mice produce significant lower amounts of IFN-γ upon infection with *T. cruzi* but are as well protected as wild-type mice (11). Most probably, C57BL/6 mice mount a Th1 response upon *T. cruzi* infection regardless of the early instructive cytokines produced by NK cells.

It was shown that appropriate vaccination methods have the potential to reduce the severity of *T. cruzi* infection (35). However, it was also shown that during *T. cruzi* infection immune-suppressive mechanisms favor persistence during both the acute as well as the chronic phase (19). Therefore, even a strong, Th1-biased immune response might not be able to clear...
all parasites from infected tissues. This suggests that the most effective time period to control parasite replication is the very early phase, in which the parasite is not protected intracellularly. To address this question, we infected control mice and NK-depleted mice with a low number of parasites, causing a chronic infection. As seen before, NK-depleted mice exhibited an increased parasitemia in comparison to control mice. Both groups of mice survived the acute phase and were analyzed for tissue parasite burden either 30 days or 1 year after infection. In NK-depleted and control mice, trypanosomal DNA in lymph nodes, spleen, and heart was low. In contrast, parasite persistence in the skeletal muscle was high, as already demonstrated for the acute phase, which was consistent with previous studies (11). This might reflect the tropism of the respective parasite and also the ineffectiveness of the immune system to control parasite growth in these tissues. Furthermore, it may be concluded from these results that even in the absence of NK cells, an effective adaptive immune response was mounted that controlled parasite replication in tissues other than skeletal muscle. In addition, these data imply that NK cells do not contribute to the control of intracellular replication but effectively control extracellular trypanosomes. The loss of viability of free trypanosomes after incubation with NK cells was already shown in vitro by Hatcher and Kuhn by light microscopy (12). We further analyzed the molecular mechanisms leading to parasite killing. Our data revealed that coincubation of activated NK cells and free trypanosomes is accompanied by a rapid contact formation between both that is followed by loss of mobility and membrane integrity of the parasitic target, as indicated by the loss of a fluorescent dye from the cytosol of the parasite. Neither contact formation with purified T cells was observed nor did the depletion of T cells lead to decreased parasite lysis, which indicates that killing of free trypanosomes

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**TABLE 1. Killing of *T. cruzi* in different preparations**

<table>
<thead>
<tr>
<th>Cells</th>
<th>% Dead parasites&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epimastigotes</td>
<td>Trypomastigotes</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>C57BL/6 spleen cells</td>
<td>39</td>
</tr>
<tr>
<td>C57BL/6 spleen cells + poly(I·C)</td>
<td>59</td>
</tr>
<tr>
<td>SCID spleen cells</td>
<td>31</td>
</tr>
<tr>
<td>SCID spleen cells + poly(I·C)</td>
<td>58</td>
</tr>
<tr>
<td>Isolated NK cells</td>
<td>0</td>
</tr>
<tr>
<td>Isolated NK cells + poly(I·C)</td>
<td>60</td>
</tr>
<tr>
<td>A20 (B-cell hybridoma)</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> The effect on *T. cruzi* was visualized by light microscopy. In each experiments the percentage of parasites that had lost their mobility was determined. Data were determined in three independent experiments, each of which was performed in triplicate.

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FIG. 4. Tissue parasite burdens from NK cell-depleted mice and control mice are comparable. *T. cruzi* DNA in lymph nodes (lymph n.), heart muscle (heart), and skeletal muscle (muscle) from mice infected with $10^4$ *T. cruzi* was analyzed at day 12 p.i. (A) or mice were infected with 250 *T. cruzi* and were analyzed 30 days p.i. (B) or 1 year p.i. (C).
is indeed mediated by NK cells. By using cytokine-deficient mice, we further demonstrated that trypanocidal effects of NK cells were dependent on IL-12, which was shown to be rapidly produced after *T. cruzi* infection (9). Preincubation of NK cells with strontium chloride, a substance known to induce degranulation of cytotoxic cells, inhibited parasite killing (28). These data suggest that cytotoxic granules are involved in the antiparasitic effects. However, it was shown that *T. cruzi* is resistant to the lytic action of purified perforin, a molecule that was the most likely candidate for this lytic activity (3). It remains pos-

FIG. 5. Spleen cells exhibit direct lytic effects against free trypanosomes. A total of 10^7 CMFDA-labeled trypomastigotes were left untreated (A) or were incubated with 5 × 10^5 (B), 1 × 10^6 (C), or 5 × 10^6 (D) spleen cells from C57BL/6 mice for 4 h at 37°C. Cells were then examined by flow cytometry. Using forward and side scatter parameters, a gate was confirmed that included only parasites and no spleen cells. This gate was subsequently analyzed for CMFDA intensity. This allowed the identification of parasites with low intensity (dead parasites, indicated by the bar) and high intensity (living parasites, right).

FIG. 6. Lysis of free trypanosomes was mediated by NK cells. (A) A total of 10^5 CMFDA-labeled trypomastigotes were incubated for 4 h at 37°C with 10^6 spleen cells from SCID or C57BL/6 mice that were depleted either of NK cells or of NK and T cells. (B) Effects of SrCl_2-treated spleen cells and spleen cells from perforin^−/−_mice. NK cells purified from poly(I·C)-stimulated spleen cells exhibited trypanolytic activity. (C) Stimulation of NK cells by trypanosomes is dependent on IL-12. Data were obtained by flow cytometry as described previously. Significant differences (P < 0.05) between experimental groups are indicated with asterisks. Each experiment was performed independently three to five times.

FIG. 7. Intimate interaction between NK cells and parasites. DX5-positive NK cells were purified by magnetic cell sorting. A total of 10^6 purified NK cells were incubated with 10^7 *T. cruzi* epimastigotes for 1 h at 37°C. Cells were subsequently fixed and analyzed by scanning electron microscopy. Representative areas are shown. No interaction was observed with purified T cells (data not shown).
sible that purified perforin in cell culture does not reach the critical concentration which is needed for polymorization, whereas in the contact zone between cytotoxic cells and their targets, a higher concentration would be achieved. Therefore, we used spleen cells from perforin-deficient mice. These cells lyse free parasites as actively as those from wild-type mice, suggesting that other factors are responsible for lysis of extracellular parasites. In contrast to our data with free parasites, it was demonstrated that perforin plays an important role for the control of intracellular parasites in vivo (23).

It was recently demonstrated that NK lysis, an antimicrobial factor from cytolytic cells of pigs, and peptides thereof are able to kill T. cruzi in vitro (15). Although a homologous factor in mice was not described yet, it may be speculated that other antimicrobial factors either alone or in synergism with perforin exhibit activity against T. cruzi. However, preliminary experiments revealed that a homogenate of NK cells was not directly lytic to T. cruzi, although trypanolytic fractions could be obtained by chromatography by using extracts from purified NK cells that were stimulated by poly(I·C) (data not shown). One explanation for this observation might be that a transfer of lytic granules to the parasite surface leads to a high local concentration of the respective factor(s), which then is accompanied by membrane leakage and subsequent parasite lysis. This concentration may not be reached when extracts of NK cells are used, or these factors might be neutralized by other molecules present.

It was very recently shown that highly purified human NK cells interact directly with free Leishmania parasites (24). Therefore, it is tempting to speculate that NK cells employ specific receptors not only for the recognition of infected cells but also for the recognition of foreign parasitic surface molecules.

The present study provides evidence that NK cells participate directly in the early immune response against T. cruzi by killing free parasites rather than in the control of the adaptive immune response. Furthermore, we demonstrated that lysis of the parasitic target appears to be mediated by contact-dependent exocytosis of lytic granules. However, lysis occurred independently from perforin. In addition, by using quantitative PCR we found that parasitemia is not causally linked to parasite tissue burden and that the latter is a more predictive value for mortality.

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