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NK1.1 cells are required to control T cell hyperactivity during *Trypanosoma cruzi* infection

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: This study evaluated the regulatory function of NK1.1$^+$ cells during *Trypanosoma cruzi* infection.

Matreial/Methods: Both thymectomized (Tx C57Bl/6) and euthymic C57Bl/6 mice (C57Bl/6) were infected intraperitoneally with the Tulahuen strain. NK1.1$^+$ cells were depleted *in vivo* by anti-NK1.1 mAb. Spleen cells were analyzed by flow cytometry for the expression of CD44 and CD69 on T cells. Supernatants from splenocytes were used to measure nitrite concentration (quantified by Griess reagent). Interleukin 2 and IFN-gamma levels were determined by ELISA. The protocols used herein were approved by the Institutional Committee for Ethics. Student's t or Kruskal-Wallis tests were applied, as indicated.

Results: The number of T cells expressing CD69 increased progressively during *T. cruzi* infection in NK1.1 cell-depleted C57Bl/6 mice. In spite of an increased early T cell activation during infection, the percentage of CD4$^+$ CD44^{high} T cells did not augment in NK1.1 cell-depleted C57Bl/6 mice compared with untreated C57Bl/6 controls. Serum levels of IFN-gamma in anti-NK1.1-treated mice were higher than in non-depleted animals. Con-A-stimulated spleen cell supernatants from NK1.1 cell-depleted animals contained increased levels of IL-2 and nitric oxide (NO) during early infection.

Conclusions: After the first week of infection, NO overproduction and high levels of IFN-gamma in anti-NK1.1-treated C57Bl/6 mice appeared to be related to susceptibility and hyperactivation of peripheral T cells. Finally, this study suggests a novel regulatory function of NK1.1$^+$ cells during *T. cruzi* infection. Without NK1.1 cells, T lymphocytes are hyperactivated but do not differentiate to effector/memory T cells in infected C57Bl/6 mice.

key words: ***T. cruzi*** **• NK1.1 T cells • regulatory cells • IFN-gamma • nitric oxide • Interleukin-2**

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BACKGROUND

Trypanosoma cruzi is the primary agent of Chagas' disease, which affects several million people in Central and South America [1]. This protozoan parasite has a complex life cycle and is found in at least three morphologically distinct forms: infective for mammalian cells (metacyclic or blood trypomastigotes), insect replicative epimastigotes, and intracellular replicative amastigotes. Mammalian cell invasion by *T. cruzi* and its intracellular replication are essential for the completion of the parasite's life cycle. The parasite grows and replicates in a variety of host mammalian cells, especially macrophages and muscle cells [2–3]. Macrophages, NK cells, and T cells play an important role in the control of infection [4–13].

T. cruzi multiplies in resident macrophages, but is usually killed by these activated cells. Several reports suggest that the activation of the trypanocidal activity of macrophages is due to cytokines released by activated T [12] and NK [6] cells, which enhance the killing of intracellular amastigotes (reviewed in [3]). IFN-gamma, in particular, is widely recognized as one of the major priming mediators of macrophage activation and has been implicated as a key activating factor for the killing of *T. cruzi* [4,5,14], being mainly produced by NK1.1$^+$ cells [6]. The depletion of NK1.1$^+$ cells causes high mortality and increases parasitemia in young euthymic mice, but produces no such effects in thymectomized young C57Bl/6 mice infected with *T. cruzi* [15]. In addition, we have previously described that treatment of euthymic mice by anti-NK1.1 mAb did not upregulate the percentage of CD4$^+$ CD45RB^{low} or CD8$^+$ CD45RB^{low} memory/effector T cells as it did in untreated control mice, during infection. Also, it was shown that resistance to *T. cruzi* infection has been related to high numbers of memory/effector T cells prior to infection in thymectomized or aged animals [15]. These results suggest that NK cells are involved in the generation of the effector memory T cell pool during the acute phase of *T. cruzi* infection and prompted us to investigate early steps of T cell activation in NK-depleted mice as well as the formation of central memory T cells, defined by a high expression of the CD44 molecule.

In this study we show that the number of CD4$^+$ and CD8$^+$ T cells expressing the early activation marker CD69 increased progressively during infection in susceptible mice groups (euthymic and euthymic NK cell-depleted) compared with resistant groups (thymectomized and NK cell-depleted thymectomized). Also, NK cell-depleted euthymic mice presented lower numbers of CD4$^+$ CD44^{high} memory T cells than untreated euthymic controls at the end of the acute phase of the infection. In addition, splenocytes from euthymic mice depleted of NK1.1$^+$ cells produced high levels of IL-2 and nitric oxide (NO) in supernatants of Con A-stimulated spleen cells, and the mice had high levels of IFN-gamma in serum, showing a state of T cell hyperactivation. Finally, this study suggests that without NK1.1 cells there is a naïve T cell accumulation and early T cell activation on peripheral lymphocytes in infected euthymic mice.

MATERIAL AND METHODS

Animals

C57Bl/6 mice (1–2 month old) were obtained from the Institute of Biomedical Sciences (Department of Immunology) of the University of São Paulo. C57Bl/6 mice (two months old, euthymic or thymectomized) were infected as described below. The animals were kept under conventional conditions and were manipulated according to institutional guidelines. All the protocols used in this study were approved by the Committee for Ethics in Animal Experimentation of the University of São Paulo. All protocols involved in this study were committed to promoting and ensuring the well-being of animals.

Parasites

Mice were infected intraperitoneally with 1000 blood-form trypomastigotes of the Tulahuen strain of *T. cruzi* [15]. For infection, blood from an acutely infected mouse was mixed with heparin and diluted in balanced salt solution. An aliquot of 0.2 ml of this mixture was injected into normal mice. Control mice received the same volume of similarly diluted blood obtained from an uninfected mouse. The levels of parasites were evaluated in 5 µl of blood.

Thymectomy

Mice were thymectomized as previously described [16]. Survival after surgery was 60–80%. Thymectomized C57Bl/6 mice were infected one month thereafter. The completeness of thymectomy was confirmed by autopsy and results from any mice with thymic remnants were excluded from this study.

Treatment *in vivo* with monoclonal antibody (mAb)

Mice were depleted of NK1.1$^+$ cells by *i.p.* injection of anti-NK1.1 mAb (800 µg/mouse one day before infection and 400 µg/mouse/day every two days after infection). The NK1.1 mAb (PK136, American Type Culture Collection, Rockville, MD), obtained from ascitic fluid, was used after ammonium sulfate precipitation. Control ascitic fluid was obtained by injecting BALB/c mice with complete Freund's adjuvant (CFA) *i.p.* and then semi-purified by ammonium sulfate precipitation. Control mice received the same amount of protein from control ascitic fluid for the same length of time as the mice treated with mAb. For experiments *in vitro* and *in vivo*, we also used mouse immunoglobulin G, which produced results similar to those obtained in untreated mice, as described elsewhere [6]. The depletion of NK1.1$^+$ cells was monitored by antibody staining of spleen cells and flow cytometry (as described below). Depletion of NK1.1$^+$ CD3$^-$ cells was invariably greater than 95% after one week of treatment. Splenocytes (10^6) were stained at 4°C with optimal dilutions of PE- or FITC-conjugated monoclonal antibodies. Viable cells were identified by their ability to exclude propidium iodide and were analyzed after gating for lymphocytes using forward and orthogonal light scatter characteristics. Depletion of NK1.1$^+$ (alpha beta TCR$^+$) T cells was greater than 85% after one week of treatment.

Staining of spleen cells and flow cytometry

The animals were analyzed from day 0 to day 22 after infection. Spleen cells were isolated as described [7] [8] and placed in ice-cold PBS supplemented with 5% Fetal Calf Serum and 0.1% azide. Staining was done as previously described [17] using the antibody-fluorochrome conjugates listed be-

Table 1. Parasitemic peak of C57B1/6 mice infected with *T. cruzi*.

Groups of mice	Mean number of trypomastigotes/ml $\times 10^{-3}$
Tx C57Bl/6	167.40 (30**)
Tx C57Bl/6 PK-treated	139.50 (25**)
C57Bl/6 PK-treated	4464* (800**)
C57Bl/6	1953* (350**)

Mice were infected intraperitoneally with 10^3 bloodstream forms of *T. cruzi* (Tulahuen strain). Each mean corresponds to five mice. Parasitemia was evaluated in 5 µl of blood.
* $P<0.01$ compared with Tx C57Bl/6 and Tx C57Bl/6 PK-treated mice. (Mann-Whitney U-Wilcoxon test);
** indicates the parasite numbers found in each 100 microscopic fields

low. The fluorochrome-conjugated monoclonal antibodies used (purchased from Pharmingen, San Diego, CA) were: phycoerythrin-(PE)-anti-NK1.1 (PK136), PE-rat anti-mouse CD4, PE-rat anti-mouse CD8, FITC-rat anti-mouse CD44 (Pgp-1), and FITC-rat anti-mouse CD69. After staining, the cells were fixed with 1% paraformaldehyde in PBS and analyzed using a FACScan (Becton and Dickinson). Ten thousand events were recorded per sample.

In vitro cell culturing

Spleen cells were resuspended in complete medium (RPMI 1640 supplemented with 10% FCS, 5×10^{-5} M 2-mercaptoethanol, sodium pyruvate, glutamine, non-essential amino acids, and 10 mM Hepes) at a concentration of 5×10^6 cells/ml. All cultures were done in triplicate in flat-bottomed microplates and maintained in a humidified atmosphere with 5% CO_2 at 37°C. For the measurement of IFN-gamma and NO production in supernatants, 5×10^6 spleen cells from the different experimental groups were cultured either in complete medium alone or with 10 µg of Con A per ml, for 24 h, in a total volume of 1 ml. As stated in Results, supernatants were collected after 24 h, centrifuged, filtered (0.22 µm), and stored at –86°C before assaying as described below.

IFN-gamma serum levels

The serum levels of IFN-gamma were quantified [18] using a two-site sandwich ELISA with purified XMG1.2 and AN18 mAbs (obtained from Pharmingen, San Diego, CA). The lower limit of detection for IFN-gamma in this test was 0.05 ng/ml.

Interleukin-2 and NO measurements

Interleukin-2 (IL-2) production was evaluated using a two-site sandwich ELISA [18] with purified rat anti-mouse IL-2 (G281-2626 and biotinylated JES6-SH4) purified mAbs (Pharmingen, San Diego, CA). For NO determinations, blood samples from infected mice were collected from the tail vein several days after infection. Nitrate was reduced to nitrite in serum samples with nitrate reductase as previously described [19]. The nitrite concentration in serum and in spleen cell supernatants (after lysing red cells by osmotic shock) was assayed by mixing the sample with Griess reagent [20]. The A550 nm was read 10 min later, and the NO_2^- concentration was determined by reference to a standard curve of $NaNO_2$ (1-100 µM). The lower limit of detection for NO_3 in this test was 5 µM. A group of mice was not infected and was used as normal controls. NO measurement was below 5 µM in this group.

Statistical analysis

The results are presented as means ±SEM. The significance of differences was determined by the Student's t test to evaluate differences between the experimental and control groups, unless indicated otherwise (as Kruskal-Wallis nonparametrical test used in NO, IL-2 and IFN-gamma determinations). P-values below 0.05 were considered significant.

RESULTS

The relative number of splenic $CD4^+$ and $CD8^+$ T cells bearing the CD69 marker increases during infection in susceptible mice

Thymectomized and NK1.1 cell-depleted thymectomized mice (Tx C57Bl/6 or Tx C57Bl/6 PK-treated) are resistant to infection (as described in [15]). These two groups of mice were both infected and used as resistant controls of euthymic C57Bl/6. At day 23 after infection, the parasitemic peak of C57Bl/6 or C57Bl/6 PK-treated was 1953 and 4464×10^3 trypomastigote forms/ml in blood, respectively (Table 1). Tx C57Bl/6 or Tx C57Bl/6 PK-treated (resistant controls) had 167.4 and 139.5×10^3 trypomatigotes/ml the same day after infection. Since T cells acquire an activated/memory phenotype during aging or after thymectomy, and resistance to *T. cruzi* infection correlates with the relative number of activated/memory $CD45^-$ CD4 T cells [15], we evaluated another activation marker such as very early antigen expression (VEA expression), CD69 [21]. Figure 1 shows the gated percentage (of the total CD4 or CD8 T cell subpopulations) of splenic $CD4^+$ $CD69^+$ (Figure 1A) and $CD8^+$ $CD69^+$ (Figure 1B) T cells in uninfected control mice (day 0 post-infection) and in *T. cruzi* infected mice. The number of $CD4^+$ $CD69^+$ T cells (Figure 1A) was sustained on day 6 post-infection (p.i.), but that of $CD8^+$ $CD69^+$ T cells (Figure 1B) is highly increased on day 6 p.i. in C57Bl/6 (treated or not with anti-NK1.1, compared with Tx controls). In addition, the percentage of T cells expressing CD69 increased progressively up to day 22 post-infection in C57Bl/6 mice (treated or not with PK136 mAb) on both CD4 (Figure 1A) and CD8 (Figure 1B) T cell populations. The treatment of the mice with anti-NK1.1 antibodies (C57Bl/6 PK-treated mice) did not affect the percentage of $CD69^+$ $CD4^+$ or $CD8^+$ cells on any of the time points studied (Figure 1).

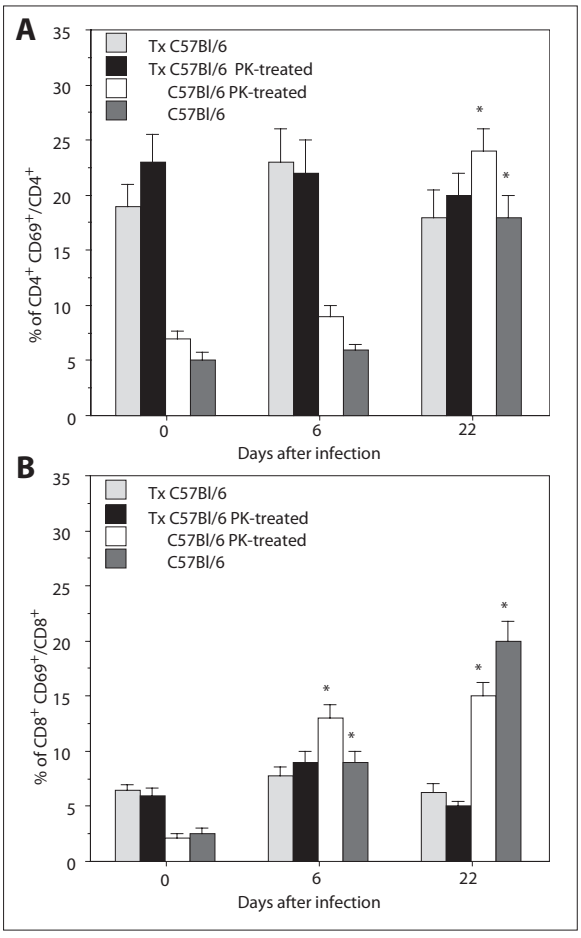

Figure 1. Increase in early activation marker expression in peripheral T cell subpopulations in infected C57Bl/6 mice as determined by CD69 expression. Splenocytes from infected mice or normal C57Bl/6 mice were stained with anti-CD4 or anti-CD8 plus anti-CD69 antibodies, as a marker for recent T cell activation, before and two times after infection. Gated $CD4^+$ (**A**) or $CD8^+$ (**B**) T cell subpopulations were analyzed by FACS. Infected mice were either untreated (C57Bl/6 and Tx C57Bl/6) or treated with anti-NK1.1 antibodies (PK-treated). The results are expressed as the mean ±S.E.M. of cells from five mice. *P<0.01 for C57Bl/6 susceptible euthymic mice (treated or not with mAb) compared with Tx (treated or not with mAb) resistant mice, on day 0 (Figure 1A) and day 0 plus day 6 (Figure 1B) after infection.

The number of splenic $CD44^-$ cells increased progressively in susceptible mice during acute *T. cruzi* infection

Figure 2 shows the relative numbers of $CD4^+$ and $CD8^+$ T cells that do not express the CD44 molecule (naïve T cells) in the different experimental groups, as defined by $CD4^+$ $Pgp\text{-}1^-$ (gated on total CD4 or CD8 T cells). The percentage of $CD4^+$ $Pgp\text{-}1^-$ cells is decreased in uninfected C57Bl/6 control mice (day 0, Figure 2A) compared with infected mice (day 22, Figure 2B), and accounted for approximately 23% and 27% of the entire splenic $CD4^+$ T cell population on day 22 after infection in untreated and anti-NK1.1-treated mice, respectively. Conversely, in mice resistant to infection as Tx C57Bl/6 (PK-treated or not), this phenotype represented about 5–7.2% of the total $CD4^+$ T cell population on day 22 post-infection (Figure 2B). $CD8^+$ $Pgp\text{-}1^-$ T cells were also analyzed, and Figure 2C shows that the percentage of this phenotype is increased in uninfected Tx C57Bl/6 and Tx C57Bl/6 PK-treated mice, compared with infected mice (Figure 2D). These last two groups of mice have this cell population greatly decreased after infection (Figure 2D). However, $CD8^+$ $Pgp\text{-}1^-$ cells decreased only slightly in C57Bl/6 mice and not at all in C57Bl/6 PK-treated mice after infection.

Overall decrease in T cells expressing high levels of CD44 molecules in *T. cruzi* infected mice depleted of $NK1.1^+$ cells

The percentage of $CD44^{high}$ T cell expression was also analyzed in spleen cells during infection (Figure 3). $CD4^+$ $CD44^{high}$ decreased in C57Bl/6 PK-treated mice compared with the untreated control mice, which accounted from 25.9% to 19.1%. Tx PK-treated decreased to 14.2% compared with Tx-untreated mice (29.7%). $CD8^+$ $CD44^{high}$ T cells remained practically unchanged in Tx PK-treated (14.1%) compared with Tx-untreated mice (16.3%). This same cell population amounted to 28.9% in C57Bl/6 PK-treated mice and 27% in controls, in clear contrast to $CD8^{low}$ $CD44^{mid}$ T cells. T lymphocytes that were $CD8^{low}$ $CD44^{mid}$ were decreased in C57Bl/6 PK-treated mice (Figure 2) compared with Tx C57Bl/6 PK-treated and C57Bl/6 controls, 3% compared with 7% and 4.2%, respectively (Figure 3).

Regulation of IFN-gamma production by NK1.1 cells *in vivo*

The levels of IFN-gamma were measured in serum from infected mice. Figure 4 shows that IFN-gamma was elevated in all infected mice after the first week of infection. Eight days after infection, C57Bl/6 PK-treated and Tx C57Bl/6 mice treated with mAb or not had higher serum IFN-gamma levels than C57Bl/6 untreated controls, which produced intermediate amounts of this cytokine. On day 16 post-infection, the levels of serum IFN-gamma decreased in all groups compared with eight days after infection. C57Bl/6 mice had very low levels of serum IFN-gamma on day 16 after infection. C57Bl/6 PK-treated mice still produced more of this cytokine than untreated controls during this phase of infection. By day 22 after infection, IFN-gamma was barely detectable in the sera of infected mice (not shown).

$NK1.1^+$ cells regulate the production of NO

To determine whether the increased IFN-gamma production in PK-treated mice could induce cell activation and NO production, the levels of NO_2 production by cultured, Con A-stimulated spleen cells from the different experimental groups were assessed after a 48-hour incubation using cells obtained four days post-infection. At this point, the NO_2 production *in vitro* was much higher in C57Bl/6 PK-treated mice than in the other experimental groups (Figure 5). To evaluate the production of NO during infection, spleen-cell supernatants were also analyzed on different days after infection. Spleen cells from C57Bl/6 PK-treated mice or control mice were cultured with Con A and the supernatants were collected after 24 hours of incubation (Figure 5 – insert).

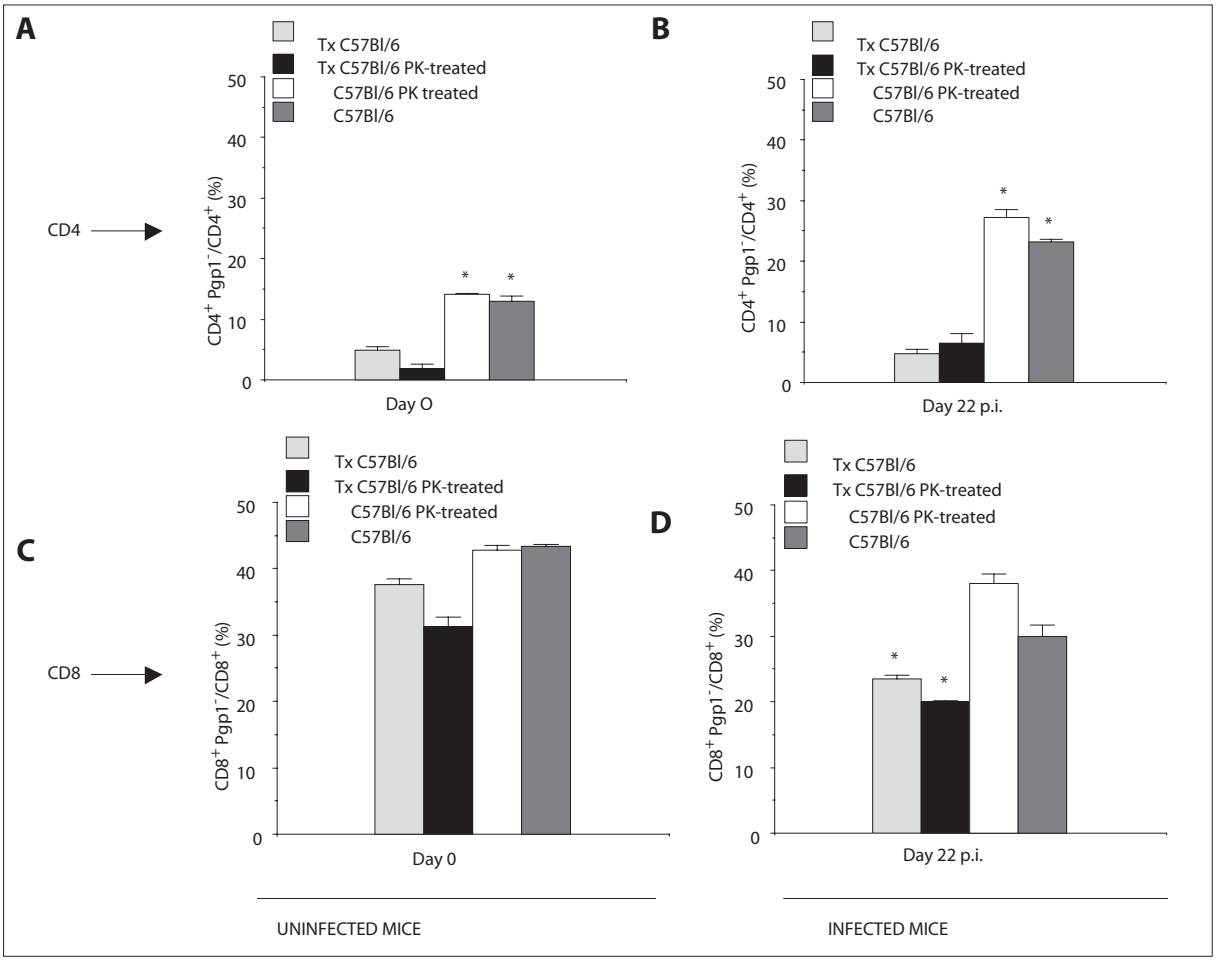

Figure 2. CD44-negative T cells in spleen cells from normal or infected C57Bl/6 mice. The percentages of gated $CD4^+$ (**A** and **B**) and $CD8^+$ (**C** and **D**) T cells that were negative for CD44 staining ($Pgp\text{-}1^-$) are shown. Infected C57Bl/6 and Tx C57Bl/6 were treated or not with anti NK1.1. The columns (mean ±S.E.M.) represent the percentage of spleen cells CD44 negative in the gated CD4 or CD8 cell populations from five mice. Stainings were performed individually, as described in Methods. *P<0.05 compared with the same uninfected groups or to Tx control mice.

In Con A-stimulated spleen cells from C57Bl/6 PK-treated mice, NO_2 production increased four and eight days after infection. By day 16 after infection, NO_2 production by Con A-stimulated cells from C57Bl/6 PK-treated mice was similar to that of cells from untreated infected mice. From the 16th to the 22nd day after infection, infected C57Bl/6 PK-treated euthymic mice had higher serum levels of NO_2/NO_3 than the other groups of mice (data not shown).

Increased production of IL-2 by spleen cells from euthymic mice depleted of NK1.1 cells, during early infection

As showed above, anti-NK1.1-treated mice were highly susceptible and produced high levels of IFN-gamma in sera and NO in supernatants of cultured spleen cells. Because IL-2 production is usually increased on naïve T cells [22–23], this interleukin was also evaluated in spleen cell supernatants. Spleen cells from C57BL/6 or Tx C57BL/6 (both treated or not with anti-NK1.1) were cultured with Con A and the supernatants were collected after 24 (Figure 6A) or 48 hours of incubation (Figure 6B). In Con A-stimulated spleen cells from C57Bl/6 PK-treated or C57Bl/6 mice, IL-2 production increased compared with both Tx groups of mice after 24 hours of incubation (Figure 6A), amounting to 2.7 and 1.9 ng/ml, respectively, in C57Bl/6 PK-treated and C57Bl/6 mice. Decreased IL-2 production was observed in the other groups of mice and amounted to 1.5 in the Tx C57BL/6 and 0.7 ng/ml in the Tx C57BL/6 PK-treated mice. After 48 hours (Figure 6B), IL-2 recovered from supernatants was sustained in Tx C57BL/6 PK-treated, but decreased in the other groups of mice (Tx C57Bl/6, Tx C57Bl/6 PK-treated and C57Bl/6), denoting high IL-2 consumption.

Discussion

In a normal immune system, most peripheral T cells are resting cells [24]. However, a small percentage of peripheral T cells already express markers that characterize an activated/memory phenotype. This may reflect encounter with environmental antigens, with self-antigens, or with both, as previously suggested [25–26]. In addition, during an immune response, resting T cells are activated, start to proliferate, and develop effector functions [22]. These T cells are called activated/memory T cells. Activated/memory T cells may migrate to inflammatory

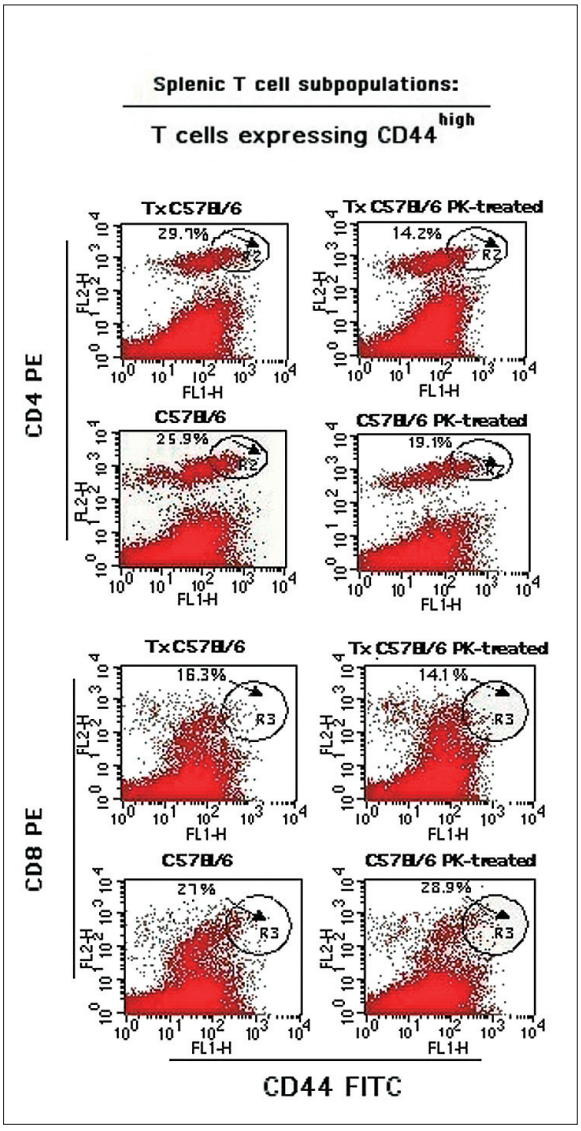

Figure 3. CD44-high T cells in spleen cells from C57Bl/6 mice. Infected C57Bl/6 and Tx C57Bl/6 were treated or not with anti-NK1.1. Dots are representative of two independent experiments. Regions show the percentage of spleen cells CD44 -high in the gated CD4 or CD8 T cell populations (total CD4 or CD8 T cells), from five mice. Stainings were performed individually, as described in Methods.

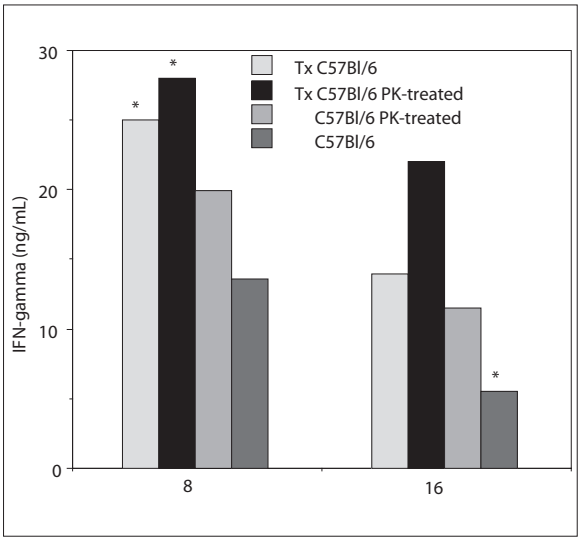

Figure 4. Serum IFN-gamma levels in infected C57BL/6 mice with or without anti-NK1.1 mAb treatment. IFN-gamma levels were measured by ELISA. Infected mice were either euthymic or Tx C57Bl/6 mice. The mice were treated (or not) with anti-NK1.1 antibodies (PK-treated). The columns are the means of triplicate values for five mice per group, and are representative of two different experiments (n=5). *P<0.01 compared with C57Bl/6 PK-treated (Kruskal-Wallis test).

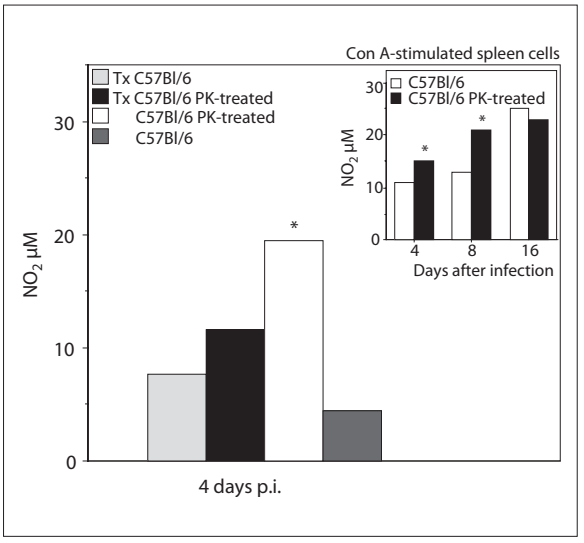

Figure 5. Effect of anti-NK1.1 mAb administration on the levels of NO in stimulated spleen cell supernatants from *T. cruzi* (Tulahuen)-infected mice. Mice were treated with PK136 mAb (PK-treated) from one day before infection onwards. The levels of NO_2 in supernatants from Con A-stimulated spleen cells were measured using cells obtained four days after infection (4 days p.i.), for C57Bl/6 or Tx C57Bl/6 groups of mice or 4, 8, and 16 days after infection for euthymic PK-treated or untreated controls (insert). Infected mice were either untreated (C57Bl/6) and thymectomized (Tx) and/or treated with anti NK1.1 antibodies (PK-treated). All supernatants were collected after 24 h and the levels of NO_2 were measured as described in Methods. The columns represent the mean of the results found, n=4–5 mice. *P<0.01 compared with the controls (Kruskal-Wallis test).

sites and, therefore, are of fundamental importance in the defense against pathogens that grow in other tissues than in the lymphoid tissue [22,27]. For instance, a parasite-specific immunity mediated by effector cells is critical for the control of *T. cruzi* infection, especially by strains such as the Colombian and Tulahuen, which grow in muscle cells [2,15].

The generation of effector T cells restrains the growth of an intracellular infection and, at the same time, triggers regulatory mechanisms to ensure self-tolerance after the infection has been controlled [7,11]. Although the majority of effector T and B cells are generated in the peripheral lymphoid organs, some T cells may leave the thymus functionally active as effector cells. This seems to be the case for

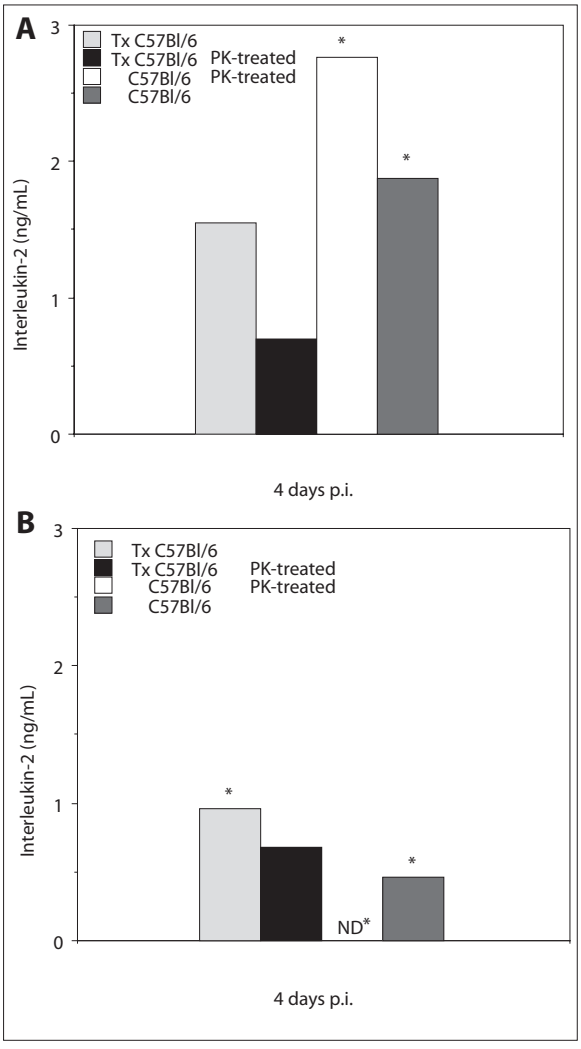

Figure 6. Interleukin-2 detection in supernatants from spleen cells of infected mice. The levels of IL-2 in supernatants from Con A-stimulated spleen cells were measured four days after infection of C57Bl/6 or Tx C57Bl/6 mice (PK-treated or untreated). Infected mice were either untreated (C57Bl/6 and Tx C57Bl/6) and/or treated with anti-NK1.1 antibodies (PK-treated). All supernatants were collected after 24 (**A**) or 48 (**B**) hours and the levels IL-2 were measured as described in Methods. Columns represent the mean of the results found to 4-5 mice. ND indicates Not Detected. *P<0.01 compared with control groups (Kruskal-Wallis test).

a subpopulation of NK1.1$^+$ and regulatory CD4$^+$ CD25$^+$ T cells [28]. In addition, the thymus is responsible for feeding peripheral lymphoid organs with T cells that go to the resting peripheral pool, becoming a reservoir of memory/activated precursor cells [29]. In addition, these resting peripheral T cells may produce the majority of IL-2 during the first steps of activation following antigen encounter [23]. Therefore, one would expect that the absence of the thymus or its hypofunction, with a reduced or absent output, would diminish, with time, the number of resting T cells in the peripheral lymphoid pool. Whether the reduction or expansion of the peripheral resting T cell pool by thymic activity can modify the outcome of an infection is not known. However, we have previously shown that the presence of a fully functional thymus is associated with higher parasitemia and mortality, and this is also seen in NK1.1$^+$ cell-depleted euthymic mice [15]. In addition, multiple injections of thymocytes applied in thymectomized or aged mice induced higher parasitemia and mortality during *T. cruzi* infection [8]. These observations suggest that increased thymic output may be related to susceptibility to *T. cruzi* infection, along with other mechanisms. Acute depletion of NK1.1 cells by a single injection of anti-NK1.1 mAb in normal euthymic C57Bl/6 mice induced an increase in the numbers of recent thymic emigrants (Nomizo, A. et al, manuscript in preparation). Therefore, it seems that NK1.1$^+$ cells may, somehow, control T cell thymic output, thus modifying the production of newly formed T cells.

As demonstrated here, Tx and Tx anti-NK1.1-treated mice already had lower percentages of naïve CD4$^+$ T cells (before infection), as indicated by the decreased percentage of CD44$^-$ cells in these mice when compared with euthymic controls (Figure 2). In addition, the percentages of naïve CD8$^+$ CD44$^-$ T cells in Tx or Tx anti-NK1.1-treated mice decreased even further during infection (Figure 2D), suggesting that in our experimental model the remaining resting T cells were being activated during the infection. CD44^{high} CD45RB^{low} CD8$^+$ T cells accumulate in uninfected Tx mice (data not shown, see also ref. [15,30]). As suggested previously, CD44^{high} CD45RB^{low} CD8$^+$ T cell accumulation appears to dispense with the need for NK1.1$^+$ cells to control the infection [15]. However, the generation of activated/effector T cells is preceded by the activation of cells belonging to the resting T cell pool [31]. In accordance to this model, Wu and colleagues [32] recently showed that CD4 T cells could present a linear differentiation pathway, as defined by the transition from IFN-gamma-producing to resting persistent memory cells or to short lived IFN-gamma-producing cells. For this reason, we studied the pattern of T cell activation during *T. cruzi* infection, using markers such as the CD69 molecule, expressed during the early activation of T cells.

CD69 expression is down-regulated as T cells start to acquire effector activities, with these cells eventually becoming CD69 negative, CD45RB negative and/or CD44 high (reviewed in [21]). The percentage of splenic CD4$^+$ T cells bearing the CD69 molecule increased in C57Bl/6 or C57Bl/6 PK-treated mice 22 days after infection. In addition, on day 22 after infection the percentage of splenic CD8$^+$ CD69$^+$ T cell was still elevated in C57Bl/6 mice (with or without anti-NK1.1 mAb treatment) compared with Tx C57Bl/6 PK-treated mice, suggesting a reduction in the numbers of CD69$^+$ cell precursors after thymectomy. The increase in the percentage of early activated splenic CD8$^+$ T cells correlated with an increase in memory/activated CD8$^+$ T lymphocytes negative for CD45RB, but this correlation was lower in C57Bl/6 PK-treated mice [15]. Taken together, these results indicate that, in C57Bl/6 mice and in the absence of NK1.1$^+$ cells, naïve T cells accumulate in spleens and may be extensively activated in infected euthymic mice, in spite of a low rate of maturation and/or differentiation to activated/memory T cells in susceptible mice.

Since IFN-gamma, whose production is induced by IL-12, is one of the major cytokines associated with resistance [33], macrophage activation, and NO production (reviewed in [3]), we examined the levels of IFN-gamma in the sera of mice from

the different experimental groups at various time intervals. Consistent with the results cited above, anti-NK1.1 mAb-treated Tx mice showed higher amounts of IFN-gamma on day 8 after infection than did the untreated or PK-treated mice. In addition, C57Bl/6 PK-treated mice produced more IFN-gamma than the untreated controls after the first week of infection (Figure 4). On day 16 after infection, the overall production of IFN-gamma fell in all groups of mice, although the C57Bl/6 PK-treated mice still had increased amounts of this cytokine than their untreated controls, as did susceptible animals in another study [34]. Surprisingly, the higher production of NO and IFN-gamma by C57Bl/6 PK-treated mice seen here did not correlate with the higher mortality and higher levels of parasitemia found in this particular group [15]. As described in rats in another experimental model, this may indicate that, whatever is the source of the NO in those animals, this may not be involved in parasite control [35]. Alternatively, the increase in NO levels may constitute a partially effective attempt to control parasitemia in the absence of a putatively more efficient NO-unrelated T cell effector mechanism.

In another study, the depletion of NK1.1$^+$ cells led to a decreased production of IFN-gamma by spleen cells stimulated *in vitro* with live forms of *T. cruzi* [6]. This investigation was based on IFN-gamma found in supernatants of *T. cruzi* antigen-stimulated spleen cells, and this may account for the differences found between that and the present study.

We have proposed before that, in *T. cruzi*-infected young euthymic mice, NK1.1 cells contribute to the full differentiation of early activated T cells. NK T cells produce a large panel of cytokines, including IL-2, IL-4, IL-5, IL-10, IL-13, TNF-alpha, IFN-gamma, and GM-CSF [36–39], and this set of cytokines would make these cells important for conventional T cell differentiation. In fact, NK1.1 cells may be a candidate for inducing the differentiation of memory/activated conventional T cells, and for inducing immunoglobulin class-switching in *T. cruzi*-infected mice [15]. For instance, CD1d-restricted NK T cells limit parasitemia and augment the antibody response to a glycophosphoinositol-modified surface protein in mice infected with the CL strain of *T. cruzi* [40]. However, it was recently shown that CD1d-restricted NK T cells were not crucial for Ig class-switching in the antibody response during infection, when the Y strain of *T. cruzi* was used [41]. This apparent contradiction may be explained by the fact that the Tulahuen and CL strains of *T. cruzi* may generate a different immune response from that of the Y strain of *T. cruzi*. In fact, as the Y strain of *T. cruzi* grows preferentially in macrophages, an early T cell activation with high lymphoid production of IFN-γ and NO would be rather beneficial in this particular case. Finally, it has been described that WSX-1 (the receptor for IL-27) knockout mice present a state of T cell hyperactivation, with the production of high amounts of proinflammatory cytokines, including IFN-γ, in the acute phase of *T. gondii* or *T. cruzi* infection [42,43]. Therefore, the T cell hyperactivity found in NK-depleted mice could be similar to the situation found on WSX-1 knockout mice.

CONCLUSIONS

The results described herein suggest that susceptible mice appeared to be in a state of immune hyperactivation, producing high levels of IL-2, IFN-gamma, and NO and increased numbers of activated CD4$^+$ CD69$^+$ T cells. However, the latter do not appear to undergo full differentiation to effector memory T cells. Consequently, mice are unable to control *T. cruzi* tissue infection.

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