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Re-circulation of lymphocytes mediated by sphingosine-1-phosphate receptor-1 contributes to resistance against experimental infection with the protozoan parasite *Trypanosoma cruzi*

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ABSTRACT

T-cell mediated immune responses are critical for acquired immunity against infection by the intracellular protozoan parasite *Trypanosoma cruzi*. Despite its importance, it is currently unknown where protective T cells are primed and whether they need to re-circulate in order to exert their anti-parasitic effector functions. Here, we show that after subcutaneous challenge, $CD11c^+$-dependent specific $CD8^+$ T-cell immune response to immunodominant parasite epitopes arises almost simultaneously in the draining lymph node (LN) and the spleen. However, until day 10 after infection, we observed a clear upregulation of activation markers only on the surface of $CD11C^+PDCA1^+$ cells present in the LN and not in the spleen. Therefore, we hypothesized that $CD8^+$ T cells re-circulated rapidly from the LN to the spleen. We investigated this phenomenon by administering FTY720 to *T. cruzi*-infected mice to prevent egress of T cells from the LN by interfering specifically with signalling through sphingosine-1-phosphate receptor-1. In *T. cruzi*-infected mice receiving FTY720, CD8 T-cell immune responses were higher in the draining LN and significantly reduced in their spleen. Most importantly, FTY720 increased susceptibility to infection, as indicated by elevated parasitemia and accelerated mortality. Similarly, administration of FTY720 to mice genetically vaccinated with an immunodominant parasite antigen significantly reduced their protective immunity, as observed by the parasitemia and survival of vaccinated mice.

We concluded that re-circulation of lymphocytes mediated by sphingosine-1-phosphate receptor-1 greatly contributes to acquired and vaccine-induced protective immunity against experimental infection with a human protozoan parasite.

1. Introduction

T cells are important mediators of the adaptive immune response against infections caused by intracellular microorganisms, including the digenetic intracellular protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas disease (American trypanosomiasis). Genetic deficiency or specific treatments leading to the depletion of $CD4^+$ or $CD8^+$ T cells critically impairs the acquired immunity observed during experimental mouse infection [1–4]. Although, the anti-parasitic effect exerted by the T cells is largely mediated by IFN-γ, other mediators may also participate in the efficient elimination of parasites from the host [1–4].

In inbred mouse strains or humans, MHC class II-restricted $CD4^+$ T cells recognize multiple antigens from *T. cruzi* [5–9], whereas MHC class Ia-restricted $CD8^+$ T cells are primarily specific for immunodominant epitopes that are expressed by surface antigens members of a large family of *T. cruzi* proteins named *trans*-sialidases (TS) [1,4,9–18]. T cells are not only critical for acquired immunity, but they are also important mediators of protective immunity in response to vaccination with recombinant proteins, plasmid DNA, and bacteria- and virus-based vaccine constructs against *T. cruzi* [19–25]. Additionally, as in the case of immunity acquired during infection, IFN-γ is a key mediator of protective immunity [25]. Despite the important role of T-cell mediated immune responses, it is currently unknown where protective T cells are primed and whether they need to re-circulate in order to exert their anti-parasitic effector functions during acquired immune responses.

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With this aim, we first evaluated the kinetics of $CD8^+$ T-cell activation in the LN and spleen following a subcutaneous parasite challenge. Although the kinetics of activation in both locations were very similar, we detected the presence of clearly activated $CD11C^+$ Plasmacytoid Dendritic Cells 1^+ (PDCA-1) cells only in the LN. $CD11C^+$ PDCA-1^+ are known for their capacity to secrete large amounts of type I IFN upon activation. But most important for our purposes, very recently, they have been implicated in the priming of $CD8^+$ T cells [26]. Based on that, we hypothesized that $CD8^+$ T cells were activated at the LNs and re-circulated rapidly to the spleen.

To evaluate this possibility, we administered an immunosuppressive drug, FTY720, to interfere with T-cell signalling *via* the sphingosine-1-phosphate receptor-1 (S1P1). This receptor is expressed on T cells that respond to S1P1 by emigrating out of the thymus, LN, and bone marrow [27–29]. Following T-cell activation, S1P1 is transiently downmodulated, resulting in prolonged residence of T cells within lymphoid tissues and improved priming efficacy. FTY720 interferers with this process, since upon application, it becomes rapidly phosphorylated to FTY720-P, thus behaving as a strong S1P1 agonist. This results in sustained inhibition of S1P1 signalling, effectively trapping naive and recently activated T cells within the secondary lymphoid. Although FTY720 allows T-cell priming, it efficiently blocks migration of activated T cells from the LNs to the peripheral tissues and thereby precludes peripheral T-cell responses [27–29].

Essentially, we observed that administration of FTY720 after challenge with *T. cruzi* in mice that normally survive acute infection (C57Bl/6) or susceptible vaccinated A/Sn mice led to a significant increase in the susceptibility to infection, as indicated by elevated parasitemia and accelerated mortality. Together, these results corroborate the hypothesis that re-circulation of T lymphocytes mediated by S1P1 plays an important role during acquired or vaccine-induced protective immune responses to *T. cruzi* infection.

2. Methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (http://www.cobea.org.br/). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institutional Animal Care and Use Committee at the Federal University of Sao Paulo (Id # CEP 0426/09).

2.2. Mice and parasites

Female 8-week-old mice (C57BL/6 and A/Sn) were purchased from CEDEME (Federal University of São Paulo). Transgenic mice expressing the diphtheria toxin receptor (DTR) under control of the CD11c promoter (CD11c-DTR) on a C57BL/6 background were derived as described and were maintained in our colony as heterozygotes [30]. Blood-derived trypomastigotes of the Y strain of *T. cruzi* were obtained from A/Sn mice infected 7–8 days earlier. Each C57BL/6 or A/Sn mouse was challenged sub-cutaneously (s.c.) at the base of the tail with a final dose containing 10^4–10^5 or 150 parasites, respectively, in a final volume of 0.1 mL. Parasite development was monitored by counting the number of blood-derived trypomastigotes in 5 μL of fresh blood collected from the tail vein [10].

2.3. Administration of DT or FTY720

Wild type (WT) and CD11c-DTR mice were treated i.p. with 2 doses of 50 ng diphtheria toxin from *Corynebacterium diphteriae* (DT, Sigma), 48 h before and on the same day of challenge. In addition, infected WT mice were treated every other day, beginning on the same day of infection, with doses of 20 μg FTY720 (Cayman Chemical, Ann Arbor, MI) per mouse (1 mg/kg) in a final volume of 0.2 mL. The control mice were injected with the diluent only.

2.4. Peptides

Peptides were purchased from Genscript (Piscataway, NJ). Purity was as follows: VNHRFTLV, 97.2% and TsKb-20 (ANYKFTLV), 99.7%.

2.5. Recombinant plasmids and adenoviruses

Plasmid pIgSPCl.9 and the human replication-defective adenovirus type 5 containing the *asp-2* gene were described previously [22,24,25,31]. Heterologous prime-boost immunization involved priming i.m. with 100 μg of plasmid DNA followed by a dose of viral suspension containing 2×10^8 plaque-forming units (pfu) of adenovirus 21 days later in the same locations. Immunological assays or challenges were performed 14 days after viral inoculation (boost).

2.6. Phenotypic cell analyses by flow cytometry

The panel of conjugated antibodies used for FACS analyses were CD11c-FITC (clone HL3), CD19-PECy7 (clone 1D3), CD8α-PerCP (clone 53-6.7), CD86-APC (clone GL1), CD80-APC (clone 16-10A1), CD40-APC (clone 3/23) all from BD; PDCA-1-PE (clone JF05-1C2.4.1) from Miltenyi Biotec. Single-cell suspensions from Inguinal lymph nodes or spleen were stained for surface markers on ice for 20 min, and then washed twice in buffer containing PBS, 0.5% BSA, and 2 mM EDTA fixed in 4% PBS-paraformaldehyde solution for 10 min. At least 300,000 events were acquired on a BD FACSCanto II flow cytometer and then analyzed with FlowJo (Tree Star, Ashland, OR).

2.7. Sorting of cells by flow cytometry

PDCA-1^+ cells were isolated from LN collected from C57BL/6 mice infected 5 days earlier s.c. with 10^4 *T. cruzi* parasites. As controls, we used PDCA-1^+ cells isolated from LN of naïve C57BL/6 mice ($n = 15$). Inguinal lymph nodes were removed, collagenase-treated, and the single cell suspension was stained with the following antibodies: CD3 Pacific Blue (500A2), IA^b FITC (25-9-17), CD11c APCCy7 (HL3) all from BD, and PDCA-1 PE (JF05-1C2.4.1) from Miltenyi Biotec. $CD3^-$ IA^{b+} $CD11c^+$ PDCA-1^+ cells were then sorted in a BD FACSAria III cell sorter.

$CD8^+$ cells were obtained from C57BL/6 mice ($n = 2$) s.c. infected with 10^4 *T. cruzi* parasites. Spleens were removed 15 days after infection. Following red blood cell lysis, a single cell suspension was stained with CD8 PE (53-6.7) from BD and positive cells were subjected to sorting in a BD FACSAria III cell sorter. As determined by FACS analysis, the purity of the $CD8^+$ was 98%.

2.8. Immunological T-cell assays

Ex vivo ELISPOT (IFN-γ) or *in vivo* cytotoxicity assays were performed exactly as described previously [13,25]. Briefly, the *in vivo* cytotoxicity assays, C57BL/6 splenocytes were divided into two populations and labeled with the fluorogenic dye carboxyfluorescein diacetate succinimidyl diester (CFSE Molecular Probes, Eugene, Oregon, USA) at a final concentration of 10 μM (CFSEhigh) or 1 μM (CFSElow). $CFSE^{high}$ cells were pulsed for 40 min at 37 °C with 1 μM of the H-2 K^b ASP-2 peptide (VNHRFTLV) or TsKb-20. $CFSE^{low}$ cells remained unpulsed. Subsequently, $CFSE^{high}$ cells were washed and mixed with equal numbers of $CFSE^{low}$ cells before injecting intravenously (i.v.) 30×10^6 total cells per mouse. Recipient animals were mice that had been infected or not with *T. cruzi*. Spleen cells or

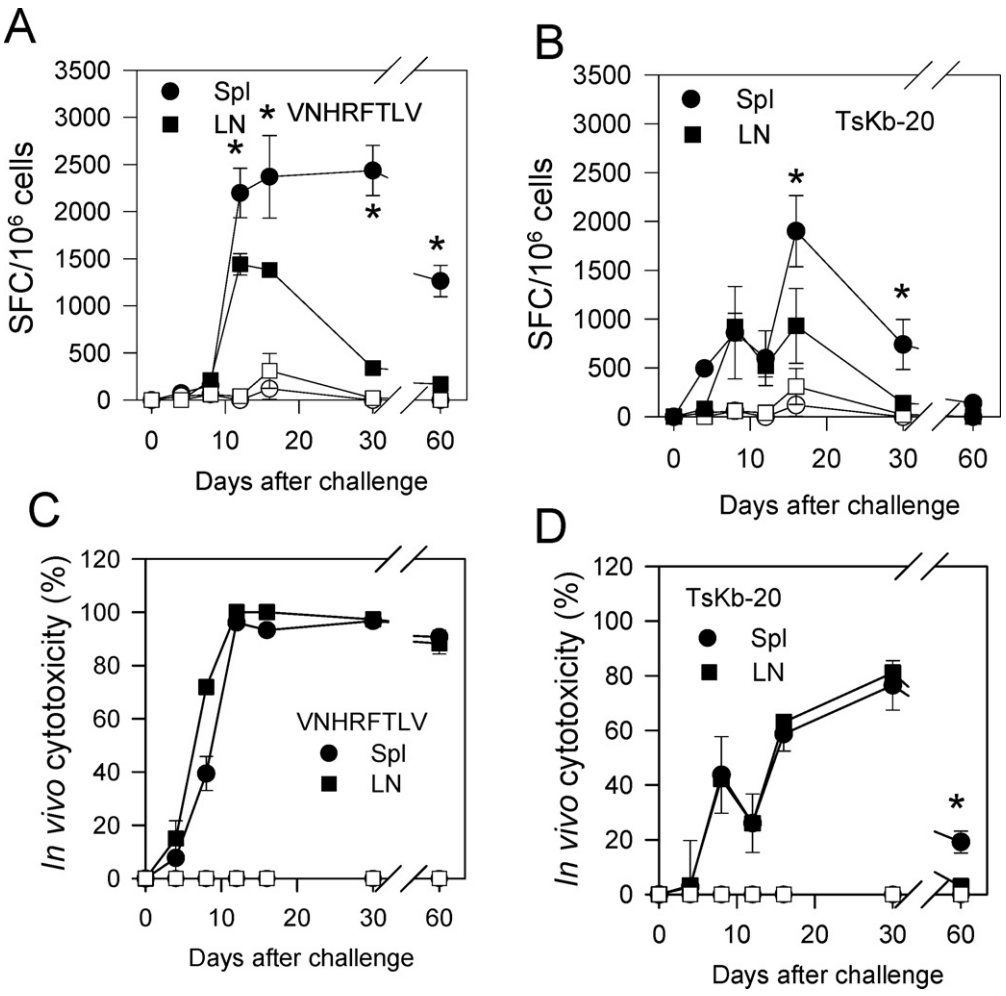

Fig. 1. Kinetics of the specific CD8⁺ T-cell-mediated immune responses in mice infected with *T. cruzi*
C57BL/6 mice were challenged s.c. with 10^4 blood-borne trypomastigotes of the Y strain of *T. cruzi*. At the indicated days, IFN-γ-producing cells were estimated *ex vivo* by using the ELISPOT assay in the presence of peptides VNHRFTLV (Panel A) or TsKb-20 (Panel B). The *in vivo* cytotoxic activities were estimated against target cells coated with peptides VNHRFTLV (Panel C) or TsKb-20 (D). The results are presented as the mean of the values of 4 mice ± SD per group, except for the *in vivo* cytotoxic activity of LN cells. In that case, we pooled cells from 4 mice for analysis. Asterisks denote the frequency of SFCs in the spleen of infected mice were significantly higher than the LN cells ($P < 0.05$).

lymph node cells of recipient mice were collected 20 h after transfer, fixed with 3.7% paraformaldehyde and analyzed by FACS as described above. The percentage of specific lysis was determined using the formula:

$$1 - \frac{\%\mathrm{CFSE}_{\mathrm{high}}\ \ \mathrm{infected}/\%\mathrm{CFSE}_{\mathrm{low}}\ \ \mathrm{infected}}{\%\mathrm{CFSE}_{\mathrm{high}}\ \ \mathrm{naive}/\%\mathrm{CFSE}_{\mathrm{low}}\ \ \mathrm{naive}} \times 100\%$$

The surface mobilization of CD107a and the intracellular expression of cytokines (IFN-γ and TNF-α) were evaluated after *in vitro* culture of splenocytes in the presence or absence of an antigenic stimulus. Cells were washed 3 times in plain RPMI and re-suspended in cell culture medium containing RPMI 1640 medium (pH 7.4), supplemented with 10 mM Hepes, 0.2% sodium bicarbonate, 59 mg/L penicillin, 133 mg/L streptomycin, and 10% Hyclone fetal bovine sera (Hyclone, Logan, Utah). The viability of cells was evaluated using 0.2% Trypan Blue exclusion dye to discriminate between live and dead cells. The cell concentration was adjusted to 5×10^6 cells/mL in a cell culture medium containing anti-CD28 (2 μg/mL, BD Pharmingen), brefeldin A (10 μg/mL, BD Pharmingen), monensin (5 μg/mL, Sigma, St. Louis, MO), and FITC-labeled anti-CD107a (Clone 1D4B, 2 μg/mL, BD Pharmingen). In half of the cultures, VNHRFTLV peptide was added at a final concentration of 10 μM. Cells were cultivated in V-bottom 96-well plates (Corning) in a final volume of 200 μL in duplicates, at 37 °C in a humid environment containing 5% CO_2. After no more than 12 h incubation, cells were harvested and stained for surface markers with Per-CP or PE-labeled anti-CD8 on ice for 20 min. To detect IFN-γ, or TNF-α by intracellular staining (ICS), cells were then washed twice in buffer containing PBS, 0.5% BSA, and 2 mM EDTA and then fixed and permeabilized for 20 min on ice with 100 μL Cytofix/Cytoperm (BD Pharmingen). After washing twice with 250 μL permwash buffer (BD Pharmingen), the cells were stained to detect intracellular markers using APC or PE-labeled anti-IFN-γ (clone XMG1.2) and PE-labeled anti-TNF-α (clone MP6-XT22). Finally, cells were washed twice and fixed in 1% PBS-paraformaldehyde. At least 300,000 events were acquired on a BD FACSCanto II flow cytometer and then analyzed with FlowJo (Tree Star, Ashland, OR).

2.9. Statistical analysis

Values are expressed as means ± SD. These values were compared using Oneway ANOVA followed by Tukey's HSD tests (http://faculty.vassar.edu/lowry/VassarStats.html). The Logrank test was used to compare mouse survival rates after challenge with *T. cruzi* (http://bioinf.wehi.edu.au/software/russell/logrank/). The differences were considered significant when the *P* value was <0.05.

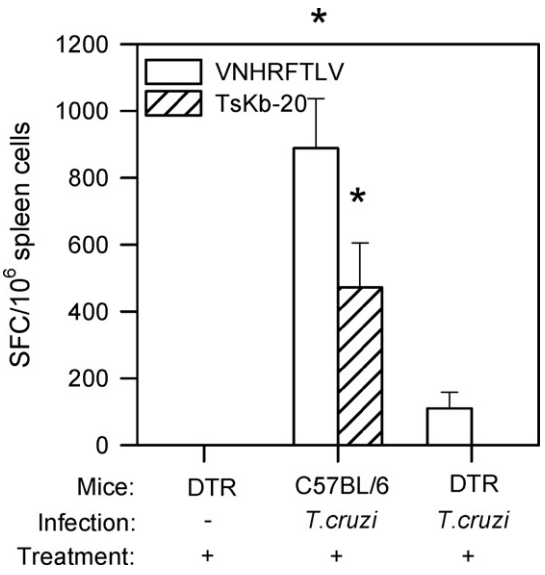

Fig. 2. CD11c$^+$ cells are required for the CD8 T-cell immune responses of mice infected with *T. cruzi*.
WT or CD11c-DTR mice were infected s.c. with 10^4 *T. cruzi* blood parasites. 48 h before and on the day of challenge, WT and CD11c-DTR mice were treated i.p. with 50 ng of DT. One week later, IFN-γ-producing spleen cells were estimated *ex vivo* by ELISPOT in the presence of peptides VNHRFTLV or TsKb-20. Results are expressed as means ± SD of 4 mice per group and are representative of experiments performed at twice with similar results. Asterisks denote that the number of spots forming cells (SFC) from infect WT mice were significantly higher when compared to naïve or infected CD11c-DTR mice ($P < 0.01$, one-way ANOVA).

3. Results

During experimental infection of H-2^b inbred mouse strains with parasites of the Y strain of *T. cruzi*, epitopes VNHRFTLV and TsKb-20 (ANYKFTLV) are recognized by H-2K^b-restricted CD8$^+$ cytotoxic T cells. In previous studies we have described that the first is the immunodominant epitope leading to a higher immune response and the second a sub-dominant epitope [10,12,13]. After s.c. challenge with infective trypomastigote forms of the parasite, detailed analyses of the kinetics of peptide-specific immune responses were determined *ex vivo* by ELISPOT and *in vivo* by cytotoxicity assays. At the indicated time points, spleen or LN cells were incubated *in vitro* with medium (control) or peptides (VNHRFTLV or TsKb-20). The number of peptide-specific IFN-γ secreting cells was determined by ELISPOT assay (13). Alternatively, at the indicated time points, target cells were labeled with CFSE and coated with peptides VNHRFTLV or TsKb-20 as described in Section 2. These cells were transferred to infected or naïve mice. Twenty hours later, spleen or LN cells were collected and the *in vivo* cytotoxicity estimated.

The results showed that the effector peptide-specific immune cells developed at a similar rate in both the draining LN and the spleen (Fig. 1A–D). The main transition occurred from days 4 to12 in both organs, for both peptides.

To determine the role of CD11c$^+$ cells during the expansion/maturation phase of the adaptive immune response, we used transgenic mice expressing the DTR under control of the CD11c promoter. When infected mice were subjected to diphtheria toxin (DT), the peptide-specific immune response in their spleen 12 days after infection was severely compromised, as measured using the ELISPOT assay (Fig. 2). These results strongly suggest that CD11c$^+$ cells are important for priming of peptide-specific cells following *T. cruzi* infection.

To further evaluate the subpopulation of CD11c$^+$ cells that could potentially be involved in the priming of the peptide-specific T cells, we stained LN and spleen cells with antibodies to CD11c and PDCA-1 and the activation markers CD40, CD40L, CD80, and CD86 at different times after challenge. As depicted in Fig. 3A, a clear upregulated pattern of expression of CD40, CD80 and CD86, but not CD40L, can be seen on the surface of CD11c$^+$PDCA-1$^+$ cells obtained from the LN. In contrast, we detect only the upregulation of CD40 on CD11c$^+$PDCA-1$^+$ splenocytes at day 10 after infection (Fig. 3B).

In addition, we also stained LN and spleen cells for CD11c expression in conjuction with CD8α in addition to the activation markers CD40, CD40L, and CD86 at different times after infection. A limited pattern of upregulation of expression of CD86 can be seen on the surface of CD11c$^+$CD8α$^+$ cells collected from the LN or spleen on days 3–7 following infection (Fig. 4A and B).

Similar analyses were also conducted for CD11C$^+$CD8a$^-$ cells collected from the spleen and LN, but we did not detect an upregulation of expression of the activation markers CD40, CD40L, CD80, or CD86 at any time point from 3 to 30 days in the spleen or LN (data not shown).

To determine whether indeed CD11c$^+$PDCA-1$^+$ cells could present antigen for specific CD8 lymphocytes, we purified CD11c$^+$PDCA-1$^+$. After sorting the cells from naïve or 5-day infected LN cells, we obtained cells that were 95.3 and 83% pure as determined by the PDCA-1 marker (Fig. 5A and B, respectively). For some unknown reason, during the purification process, some cells become negative for the marker for CD11c marker but still retained the PDCA-1 marker. The PDCA-1$^+$ cells obtained from mice that were infected expressed significantly higher amounts of MHC-II-IA^b and CD80 (Fig. 5C and D, respectively). PDCA-1$^+$ cells were used to stimulate purified CD8$^+$ splenic cells obtained from *T. cruzi* infected mice. As shown in Fig. 5E, IFN-γ producing cells were detected only when CD8$^+$ were incubated with PDCA-1$^+$ cells obtained from infected mice.

The fact that CD11c$^+$ cells from the spleen exhibit a limited activation phenotype suggested that perhaps most of the specific T cells found in the spleen might not be primed there. If this assumption is correct, the re-circulation of T cells could account for the CD8$^+$ T-cell mediated functions detected in this organ. To test whether lymphocyte re-circulation was responsible for the immune response observed in the spleen, we treated infected mice with FTY720. This immunosupressive drug inhibits S1P1 signalling, thus efficiently blocking re-circulation of naïve and activated T cells from the LNs into peripheral tissues, thereby preventing development peripheral T-cell responses [27–29].

Mice were infected with *T. cruzi* parasites and FTY720 or diluent were administered on the same day of challenge and every 2 days thereafter as described in Section 2. Two weeks later, the frequency of IFN-γ-producing cells were estimated *ex vivo* by using ELISPOT assay in the presence of peptides VNHRFTLV or TsKb-20. Alternatively, splenocytes were cultured in the presence or absence of peptides VNHRFTLV or TsKb-20 and the expression of surface CD107a, IFN-γ and TNF-α by ICS.

In infected mice, administration of FTY720 resulted in 2.52- or 3.05-fold increases in the frequency of IFN-γ-secreting cells from the LNs specific for VNHRFTLV or TsKb-20, respectively, as detected using the ELISPOT assay (Fig. 6). In contrast, this increase in the frequency IFN-γ-secreting peptide-specific cells in the LN was accompanied by a significant decrease of immune responses of splenic lymphocytes. Immune responses were initially determined by the frequency of IFN-γ-producing cells as measured by the ELISPOT assay (Fig. 7A). The frequency of IFN-γ-producing cells found in the spleen after FTY720 administration was reduced by 74.55% or 100% upon stimulation with peptides VNHRFTLV or TsKb-20, respectively (Fig. 7A).

Subsequently, we estimated the immune response by the detection of peptide-specific CD8$^+$ cells that mobilized CD107a to their surface and expressed IFN-γ and TNF-α upon exposure to the

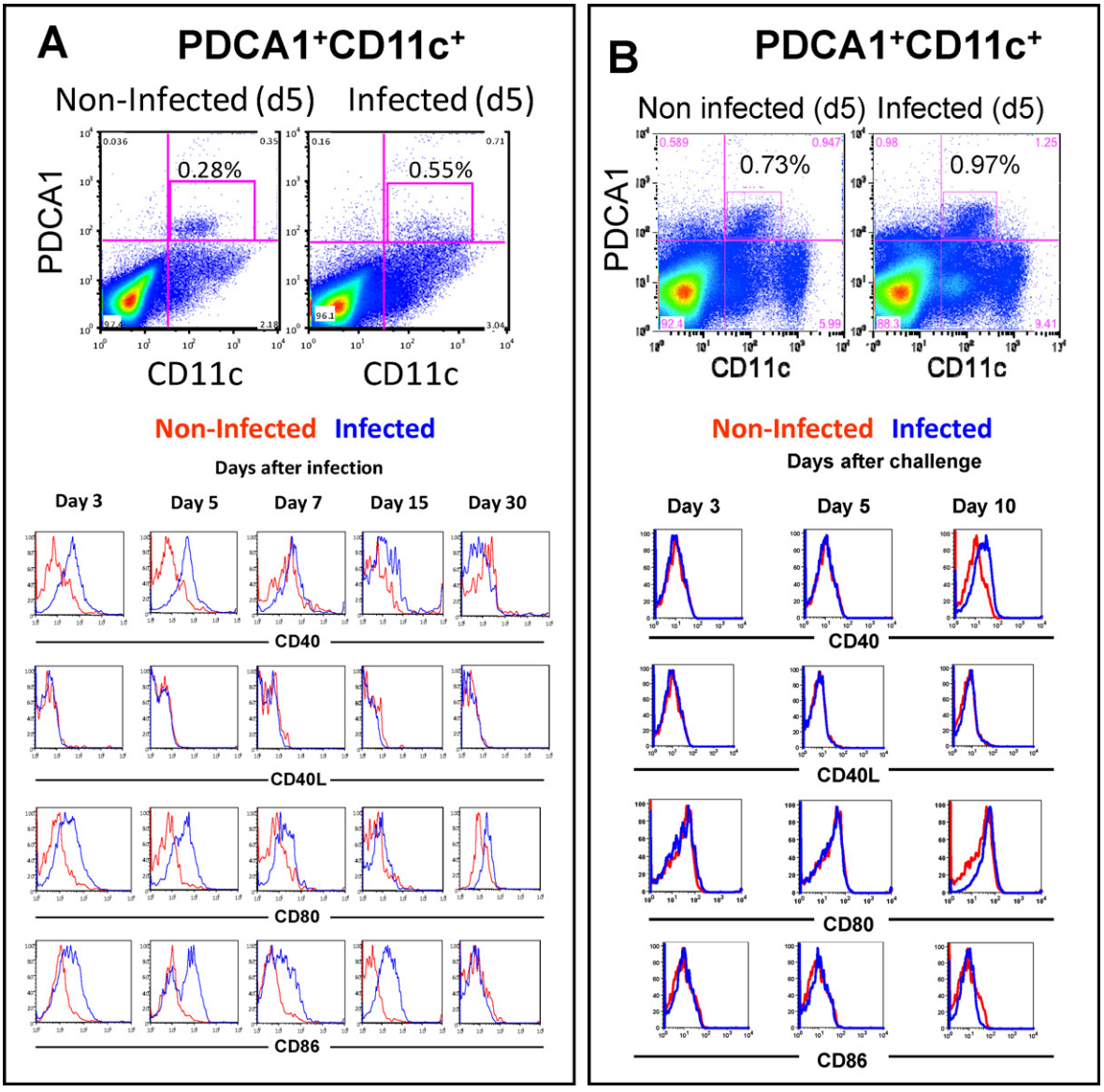

Fig. 3. Expression of activation markers on CD11c$^+$ and PDCA-1$^+$ DCs from the LNs or spleens from infected mice
WT C57BL/6 mice were infected s.c. with 10^4 *T. cruzi* blood parasites. At the indicated days after infection, LN (panel A) or splenic (Panel B) cells were collected and co-stained for the expression of CD11c, PDCA-1, and the indicated marker.
The expression of the different activation markers were compared between cells collected from infected (blue lines) and naïve (red lines) mice.

peptides *in vitro*. The frequency of CD8$^+$ cells that were CD107a$^+$, IFN-γ$^+$ or TNF-α$^+$ was reduced by 74.61% or 84.15% after stimulation with VNHRFTLV or TsKb-20, respectively (Fig. 7B). The reduction substantially affected all the different subpopulations of CD8$^+$ cells (Fig. 7C). The proportions of each population did not change significantly in the cells collected from infected mice that were administered or not with FTY720 (Fig. 7D).

To evaluate the influence of restricting T-cell re-circulation on the outcome of infection, we also monitored the parasitemia levels and survival of mice that were and were not subjected to FTY720 over the course of infection. We found that drug exposure resulted in increased parasitemia and accelerated mortality of infected mice (Fig. 8A and B, respectively). Therefore, we concluded that lymphocyte re-circulation is indeed important for the acquired protective immune response in this mouse model of acute infection.

We then sought to test the same hypothesis by applying a distinctly different approach. In this case, we used highly susceptible A/Sn mice that were genetically vaccinated by priming with plasmid pIgSPCl.9 followed by a booster immunization with AdASP-2. We previously showed that this heterologous prime-boost regimen reproducibly conferred protective immunity against a lethal challenge with *T. cruzi* [25]. Immunity was mediated by CD8$^+$ T cells as depletion of these T cells renders these mice completely susceptible to infection. These CD8$^+$ T cells are specific for the ASP-2 H-K^k restricted epitopes TEWETGQI, PETLGHEI or YEIVAGYI [31]. Prior to challenge, these mice exhibit a strong immune response to all three epitopes [31].

Following infection (s.c.), some of these vaccinated mice were subjected to FTY720. We then monitored the parasitemia levels and survival. We observed that drug exposure caused an increase in the parasitemia levels and accelerated the mortality of vaccinated mice (Fig. 9A and B, respectively). This observation indicates that vaccinated mice still require lymphocyte re-circulation to mount an effective immune response on subsequent challenge. This finding further corroborated our initial conclusions regarding the importance of re-circulation activity, even for the vaccine-supported

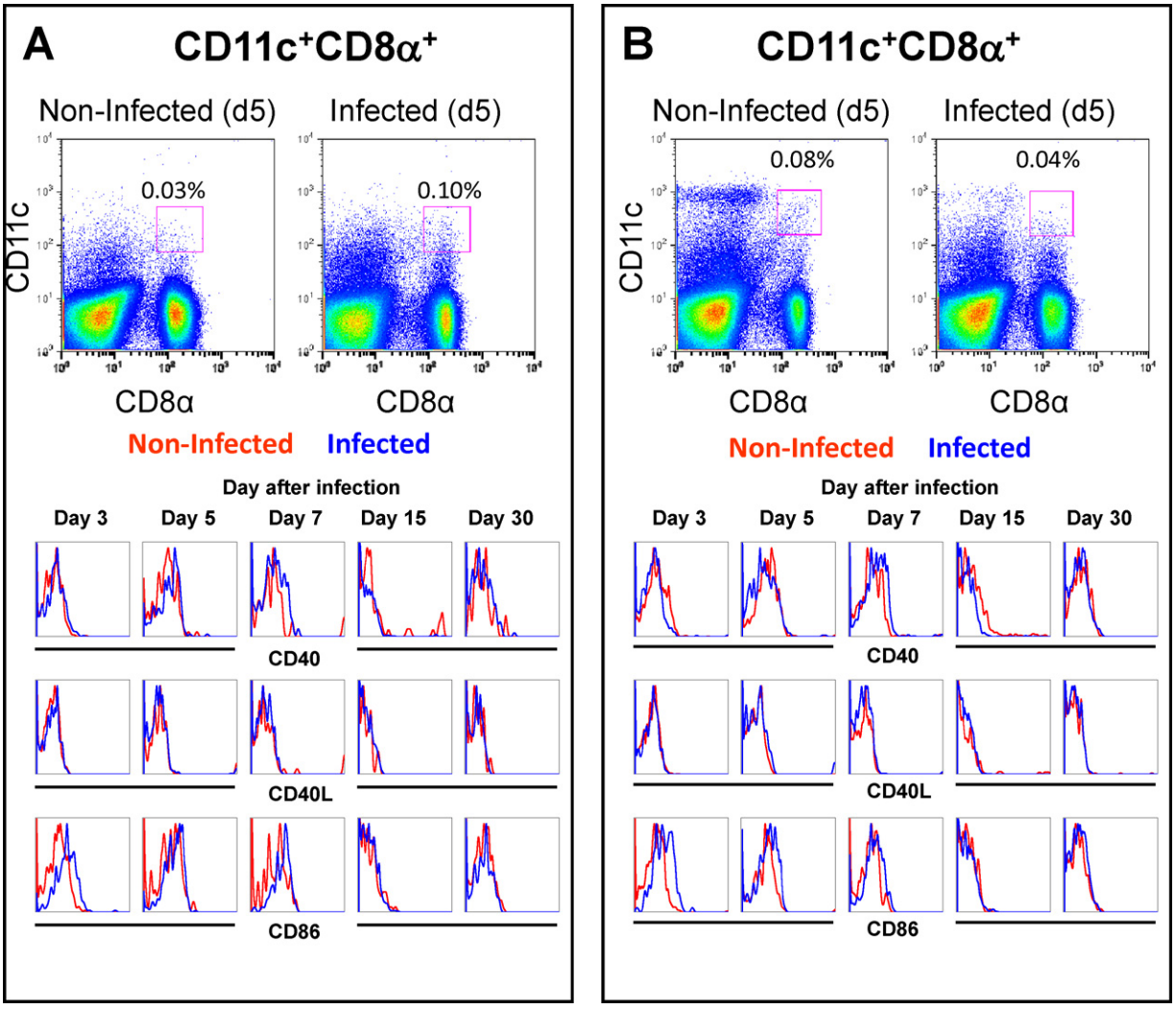

Fig. 4. Expression of activation markers on CD11c$^+$ and CD8-α$^+$ DCs from the LNs or spleens of infected mice
WT C57BL/6 mice were infected s.c. with 10^4 *T. cruzi* blood parasites. At the indicated days after infection, LN (panel A) or splenic (Panel B) cells were collected and co-stained for the expression of CD11c, CD8-α, and the indicated marker.
The expression of the different activation markers were compared on cells collected from infected (blue lines) and naïve (red lines) mice.

protective immune response, as seen in this second mouse model of acute infection.

4. Discussion

The CD8$^+$ T-cell immune response elicited by *T. cruzi* infection in most inbred mouse strains can control multiplication of this intracellular pathogen and preclude acute-phase pathologies such as death [1,10–17]. The time at which acquired immunity develops is highly dependent on the parasite load [12,32]. In our model, with the Y strain of *T. cruzi*, we observed that the CD8$^+$ T-cell immune response is only triggered at the time of the peak parasitemia [10,12]. Because the number of circulating parasites at this time is high, antigen presentation could occur in the draining LN or the spleen. However, the results of our experiments that involved the use of the immunosupressive drug FTY720, in combination with the identification of activated CD11c$^+$ cells, found mostly in the LN, clearly demonstrated that the LNs draining the parasite entrance are where the specific CD8$^+$ T cells are primed. Then, they exit the LN and reach the spleen. Our results are similar to those of experimental vaccination studies with radiation-attenuated malaria parasites [33]. In this case, the CD8$^+$ T-cell response originates in the LN draining site at the site of parasite entrance in the skin, and then these cells migrate to other peripheral organs. Similar to our results, exposure to FTY720 led to accumulation of specific T cells in the draining LN and a ~85% reduction of the specific CD8$^+$ T cells in the spleen [33]. Together, these results provide compelling evidence that the priming of CD8$^+$ T cells can take place in the local lymphoid tissue during protozoan infection/vaccination and that a rapid re-circulation to the spleen is likely to occur. As in our case, the authors conclude that this rapid re-circulation during infection was critical for protective immunity mediated by malaria-specific CD8$^+$ T cells [33].

Both studies used parasites that infect mice (*T. cruzi* or *Plasmodium yoelii*). Nevertheless, it is important to highlight that only *T. cruzi* infects humans. Also, the studies of malaria used radiation-attenuated parasites as vaccine because they do not cause infection. Therefore, it is unknown whether the same occurs during acquired immunity to experimental infection as in our case. These observations with *T. cruzi* and malaria parasites stand in contrast to other pathogens. Several studies describe re-circulation mediated by S1P1 is not critical for the protective immune responses to certain viral, bacterial and protozoan

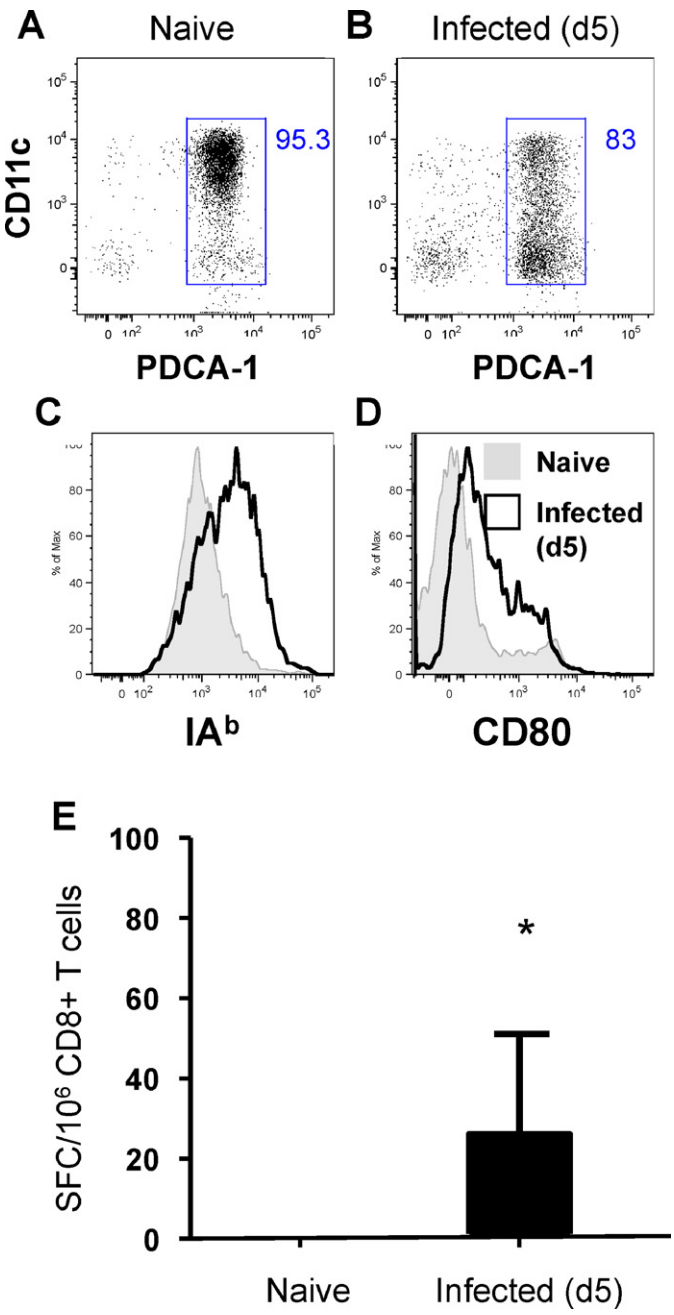

Fig. 5. PDCA-1$^+$ cells purified from infected mice present antigen to specific CD8$^+$ cells.
PDCA-1$^+$ cells were sorted from LN cells from mice infected 5 days earlier s.c. with *T. cruzi* parasites as described in Section 2. As controls, we used PDCA-1$^+$ cells isolated from LN of naïve mice.
(A and B) Sorted cells from naïve or infected mice were stained with antibodies to CD11c and PDCA-1. Numbers represent the frequency of cells in each gate.
(C and D) PDCA-1$^+$ cells were stained with antibodies to IA^b or CD80.
(E) Purified PDCA-1$^+$ cells from naïve or infected mice were used in quadruplicate cultures to stimulate specific CD8$^+$ cells. Asterisk denotes the that number of SFCs of CD8$^+$ cells stimulated with PDCA-1$^+$ cells purified from infected mice were significantly higher the one stimulated with PDCA-1$^+$ cells from naïve animals ($P < 0.01$).

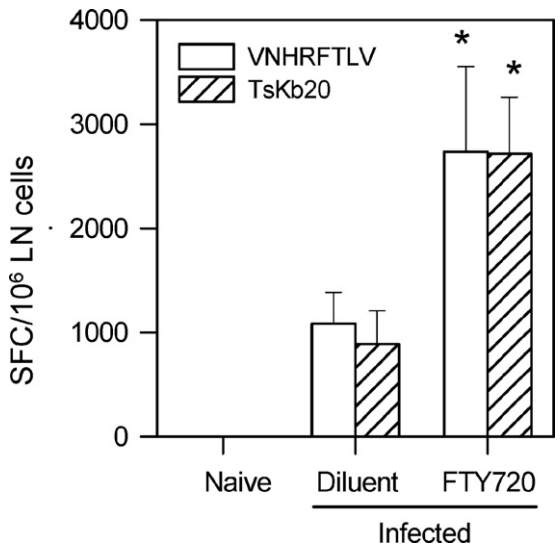

Fig. 6. Administration of FTY720 increases the number of peptide-specific cells in the LN of mice infected with *T. cruzi*.
C57BL/6 mice were infected s.c. with 10^5 *T. cruzi* blood-borne parasites. Mice were administered on the same day of challenge and every 2 days thereafter 20 μg of FTY720 or diluent i.p. Two weeks later, the frequency of IFN-γ-producing cells were estimated *ex vivo* by using ELISPOT assay in the presence of peptides VNHRFTLV or TsKb-20. The results are presented as means ± SD of 4 mice per group and are representative of experiments performed at least twice with similar results. Asterisks denote the number of SFCs from infected mice given FTY720 were significantly higher than infected mice treated with the diluent only ($P < 0.01$).

infections [34–36]. The precise reasons for these divergent responses are not clear but probably reflect differences in the priming sites as well as, the immunopathologies caused by the different infectious agents.

In addition to the role of S1P1-dependent circulation during protective immunity acquired during *T. cruzi* infection, we also observed that previously vaccinated mice became more susceptible to infection when subjected to FTY720 exposure. For vaccination, we used a heterologous prime-boost regimen consisting of an initial immunization with plasmid DNA and a booster immunization with a replication-defective recombinant human adenovirus type 5 (HuAd5), both encoding the *asp-2* gene. Immunity elicited by this vaccination protocol is long lived and mediated by Th1 CD4$^+$ as well as CD8$^+$ Tc1 cells [25,31,37].

The heterologous prime-boosting regimen of vaccination using plasmid DNA and replication-defective recombinant HuAd5 provides protective immunity in some other important pre-clinical experimental models such as SIV, malaria, Ebola, and Marburg viruses [38–45]. Based on these pre-clinical experimental models, human trials have been initiated [46–49]. Our observation that S1P1 is important for protective activity of T cells in previously vaccinated animals is completely new and should be studied further in these experimental models.

Although we measured only CD8 T-cell mediated immune responses only, it is highly possible that the same pattern would happen to specific CD4$^+$ T cells. This T-cell sub-population is very important for protective immunity during to *T. cruzi* infection [25]. The absence of re-circulation of both types of lymphocytes probably account for the sub-optimal protective immunity observed after administration of FTY720. Possibly, both cells promote the processes required for parasite elimination on the tissue.

The fact that FTY720 interfere with S1P1 activation makes it theoretically capable of act on other cells types that express this receptor. However, the effect on other cell types is poorly known at present. It has been previously described that FTY720 administration may increase or reduce the activity of regulatory T cells [50,51]. A recent study indicated that this drug act on astrocytes S1P1 to reduce experimental allergic encephalomyelitis clinical scores [52]. Whether these or other cell types play a role in our system is currently unknown.

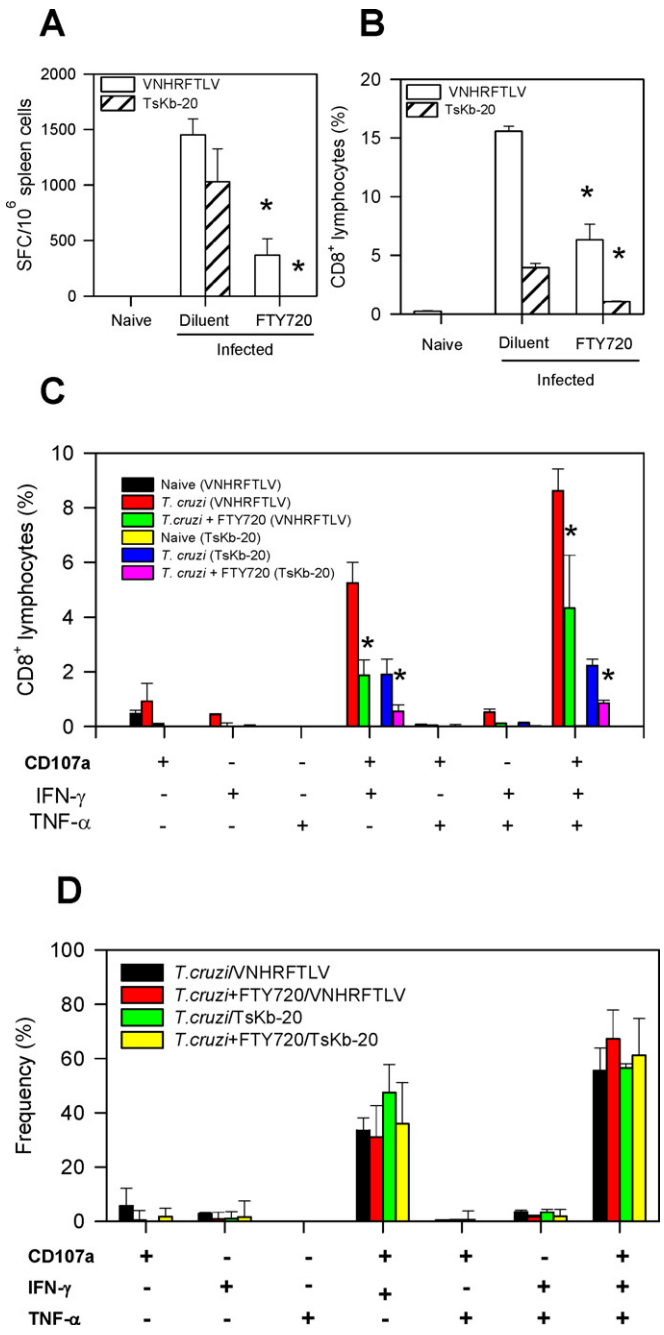

Fig. 7. Administration of FTY720 reduces the number of peptide-specific cells in the spleen of mice infected with *T. cruzi*
C57BL/6 mice were infected s.c. with 10^4 *T. cruzi* blood-borne parasites. Mice were administered on the same day of challenge and every 2 days thereafter 20 μg of FTY720 or diluent i.p. Two weeks later, the frequency of IFN-γ-producing cells were estimated *ex vivo* by using ELISPOT assay in the presence of peptides VNHRFTLV or TsKb-20 (Panels A and B).
Alternatively, splenocytes from these mice were cultured in the presence of anti-CD107a and anti-CD28, with or without the peptides VNHRFTLV or TsKb-20. After 12 h, the cells were stained to detect CD8, IFN-γ, and TNF-α (Panels C and D). The results are presented as means ± SD of 4 mice per group and are representative of experiments performed at least twice with similar results. Asterisks denote that the number of SFCs from infected mice receiving FTY720 were significantly lower than the infected mice treated with the diluent alone ($P < 0.01$).

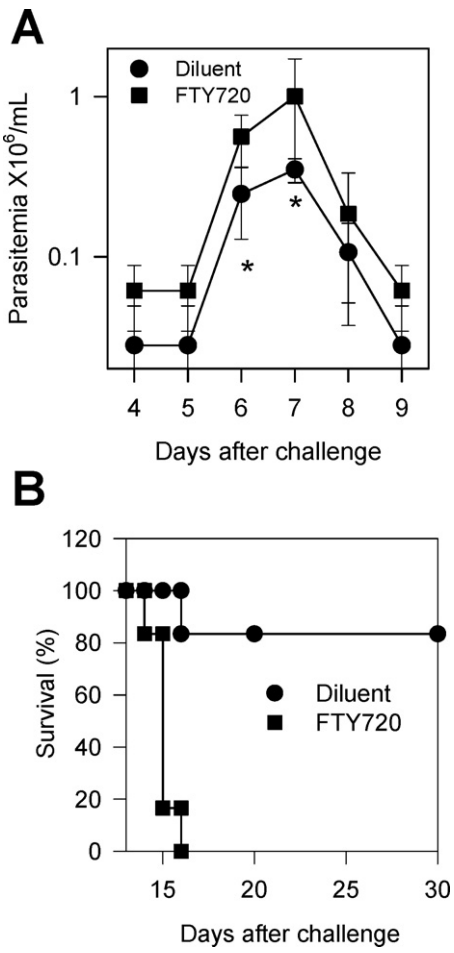

Fig. 8. Administration of FTY720 increases susceptibility to *T. cruzi* infection
C57BL/6 mice were infected s.c. with 10^5 *T. cruzi* blood parasites. Mice were treated on the same day of challenge and every 2 days thereafter with 20 μg of FTY720 i.p. or diluent only. (A) Parasitemia levels for each mouse group is presented as the mean ± SD ($n = 6$). Asterisks denote that mice from groups injected with diluent only exhibited significantly lower parasitemia ($P < 0.01$) than animals receiving FTY720. (B) Kaplan–Meier survival curves for the mouse groups treated as described above ($n = 6$). Mice from groups injected with diluent only survived longer than animals receiving FTY720 ($P < 0.01$, Logrank test). No animals died after the 30th day until the termination of the experiment. The results are representative of 2 independent experiments.

A current limitation of this experimental model for *T. cruzi* infection is the lack of information on where $CD8^+$ T cells encounter and eliminate parasite-infected cells; this is an aspect that may be critical to fully understand immune responses. Considering that *T. cruzi* can infect many cell types and cause systemic infection, it is plausible that many tissues may serve as sites of infection and for parasite/T-cell encounters. Supporting this hypothesis, we observed that vaccinated animals were resistant to *T. cruzi* challenge by different routes of infection (i.p. and s.c. [25,37]).

The finding that the administration of FTY720 significantly reduces protective immunity against *T. cruzi* infection and impairs the protective immunity afforded by vaccination may also have clinical implications for the use of this immunosuppressive drug. Certainly, its use in regions where Chagas disease is endemic should be done with caution considering the potential increase in susceptibility of treated individuals. Finally, treatment of organ-transplanted patients with FTY720 may interfere with immunity elicited by previous vaccination.

In conclusion, our study provides useful information on the importance of S1P1 for resistance against experimental infection with human protozoan parasites.

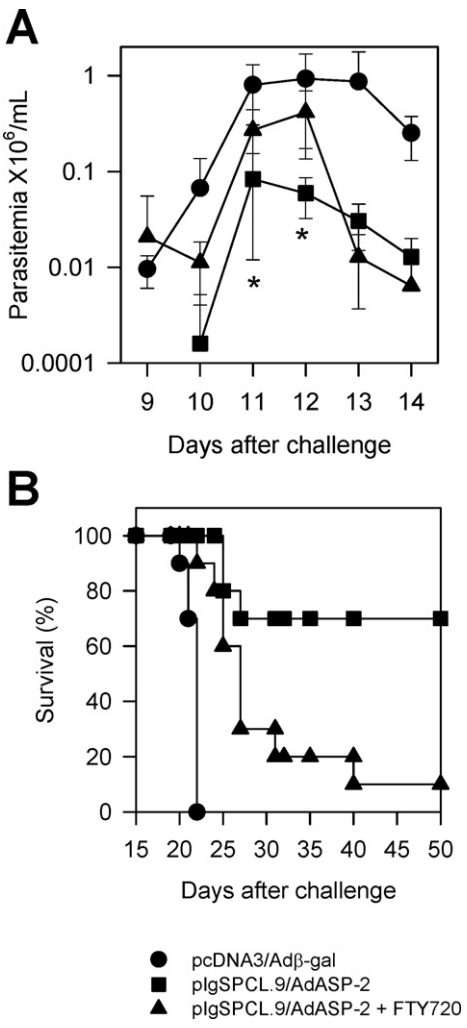

Fig. 9. Administration of FTY720 increases susceptibility to *T. cruzi* infection in previously vaccinated A/Sn mice

A/Sn mice were primed i.m. with 100 μg of plasmids, pcDNA3 or pIgSPCl.9. Three weeks later, these mice were boosted i.m. with 2×10^8 pfu Adβ-gal or AdASP-2. All mice were challenged s.c. with 150 bloodstream trypomastigotes. Half of the vaccinated mice received 20 μg of FTY720 every other day, and the other half received diluent only. (A) Parasitemia levels for each mouse group is presented as the mean ± SD ($n = 5$). Asterisks denote that mice from groups immunized with pIgSPCl.9/AdASP-2 and who received diluent only had significantly lower parasitemia than other mouse groups ($P < 0.01$). (B) Kaplan–Meier survival curves for the mouse groups immunized and challenged as described above ($n = 10$). Mice immunized with pIgSPCl.9/AdASP-2 and receiving diluent only survived significantly longer than those immunized with pIgSPCl.9/AdASP-2 and administered FTY720 ($P < 0.01$). The results shown are combined from 2 experiments. No animals died after the 50th day of challenge.

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