LFA-1 Mediates Cytotoxicity and Tissue Migration of Specific CD8+ T Cells after Heterologous Prime-Boost Vaccination against Trypanosoma cruzi Infection

Camila Pontes Ferreira1,2†, Leonardo Moro Cariste1,3†, Fernando Dos Santos Virgílio1,2, Barbara Ferri Moraschi1,2, Caroline Brandão Monteiro3, Alexandre M. Vieira Machado4, Ricardo Tostes Gazzinelli4,5, Oscar Bruna-Romero6, Pedro Luiz Menin Ruiz3, Daniel Araki Ribeiro3, Joseli Lannes-Vieira7, Marcela de Freitas Lopes8, Mauricio Martins Rodrigues1,2,3 and José Ronnie Carvalho de Vasconcelos1,2,3*

1Molecular Immunology Laboratory, Center of Molecular and Cellular Therapy, São Paulo, Brazil, 2Department of Microbiology, Immunology and Parasitology, Federal University of São Paulo (UNIFESP), São Paulo, Brazil, 3Department of Biosciences, Federal University of São Paulo, São Paulo, Brazil, 4René Rachou Research Center, FioCruz, Minas Gerais, Brazil, 5Division of Infectious Disease and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, MA, United States, 6Department of Microbiology, Immunology and Parasitology, Federal University of Santa Catarina, Florianópolis, Brazil, 7Biologie Interactions Laboratory, Oswaldo Cruz Institute, FioCruz, Rio de Janeiro, Brazil, 8Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

Integrins mediate the lymphocyte migration into an infected tissue, and these cells are essential for controlling the multiplication of many intracellular parasites such as Trypanosoma cruzi, the causative agent of Chagas disease. Here, we explore LFA-1 and VLA-4 roles in the migration of specific CD8+ T cells generated by heterologous prime-boost immunization during experimental infection with T. cruzi. To this end, vaccinated mice were treated with monoclonal anti-LFA-1 and/or anti-VLA-4 to block these molecules. After anti-LFA-1, but not anti-VLA-4 treatment, all vaccinated mice displayed increased blood and tissue parasitemia, and quickly succumbed to infection. In addition, there was an accumulation of specific CD8+ T cells in the spleen and lymph nodes and a decrease in the number of those cells, especially in the heart, suggesting that LFA-1 is important for the output of specific CD8+ T cells from secondary lymphoid organs into infected organs such as the heart. The treatment did not alter CD8+ T cell effector functions such as the production of pro-inflammatory cytokines and granzyme B, and maintained the proliferative capacity after treatment. However, the specific CD8+ T cell direct cytotoxicity was impaired after LFA-1 blockade. Also, these cells expressed higher levels of Fas/CD95 on the surface, suggesting that they are susceptible to programmed cell death by the extrinsic pathway. We conclude that LFA-1 plays an important role in the migration of specific CD8+ T cells and in the direct cytotoxicity of these cells.

Keywords: vaccination, Trypanosoma cruzi, migration, integrins, specific CD8+ T cells
INTRODUCTION

Chagas disease, caused by the intracellular parasite Trypanosoma cruzi, is a major public health problem, with about seven million people infected worldwide (1). CD8+ T cells are crucial for controlling the multiplication of intracellular pathogens such as T. cruzi. These cells control the infection by secreting cytokines such as IFN-γ and TNF-α, or by direct cytotoxicity against infected target cells (2). The heterologous prime-boost vaccination strategy has shown significant results in the induction of specific CD8+ T cells and the generation of an optimal protective immune response. Among several possible combinations of vectors for this type of immunization, we used a plasmid vector for priming and an adenovirus-Ad5 vector (replication-defective human Ad type 5) for boosting, both containing an insertion of the ASP-2 gene (T. cruzi's amastigote surface protein 2 gene). This type of immunization was capable of protecting A/Sn mice that are highly susceptible to experimental infection with T. cruzi (3, 4).

The results obtained in preclinical experimental models with heterologous prime-boost immunization have boosted recent clinical trials (5–10). In 2013, the first results of a Phase II clinical trial were published. In that study, a number of volunteers, who were vaccinated with plasmid DNA followed by immunization with Ad5, both encoding the genes of the apical membrane antigen 1 and the immunodominant surface protein of the Plasmodium falciparum circumsporozoite protein, developed immunity to malaria (11).

To the CD8+ T cells exert their effector function, these cells must migrate to non-lymphoid peripheral tissues where the infection occurs. Our group recently demonstrated that the protection generated by heterologous prime-boost immunization regimen depends on the recirculation of specific CD8+ T cells, since immunized and protected A/Sn mice became susceptible to the experimental challenge with T. cruzi after FTY720 drug treatment (12). This immunosuppressive drug reduces lymphocyte recirculation by altering T cell signaling via sphingosine-1-phosphate receptor-1 (S1P1r). This leads in sustained inhibition of S1P1r signaling, trapping T cells within the secondary lymphoid with no impairment of T cell activation (12, 13). Based on this knowledge, we hypothesized that other molecules, such as integrins, could be involved in the CD8+ T cell migration. The integrins are heterodimers that composed of an alpha and beta chain; LFA-1 is composed of αLβ2 (CD11a/CD18) chains, and VLA-4, of α4β1 (CD49d/CD29) chains. These molecules play an important role in the formation of immunological synapses and signal transduction, which result, for example, in cell migration, activation, and/or proliferation (14, 15). During transendothelial migration, chemokine-activated CD8+ T cells migrate on endothelial cells and, thus, migrate into the tissues (16). In β2 integrin-deficient mice, LFA-1 shows a significant reduction in the in vitro lymphocyte migration, strengthening the role of this molecule in leucocyte migration (17). The LFA-1 role in lymphocyte migration has also been demonstrated in the experimental autoimmune encephalomyelitis, in which regulatory CD4+ T cells can migrate to the CNS via LFA-1 (18). Its role has also been demonstrated in allografts, and the antagonism of this molecule is a very effective inhibitor of acute rejection, thus prolonging allograft survival in rodents (19). VLA-4 has also been studied in liver allograft rejections, where it seems responsible for the migration of effector CD8+ T cells and transplant rejection along with LFA-1 (20, 21). During infection by intracellular parasites such as T. cruzi, specifically by the Colombian strain, there is a predominance of effector CD8+ T lymphocytes (CTLs) with high expression of LFA-1 and VLA-4 in the myocardium of infected mice (22). In addition, the high expression of LFA-1 on the surface of PfαCD8+ T cells during the acute and chronic phases has been demonstrated (23). However, the dominance of these cells in cardiac tissue favors the progression of the inflammatory reaction, culminating in Chronic Chagas heart disease (24).

Herein, we tested whether LFA-1 and VLA-4 integrins were key mediators for T cell-mediated protective immunity against T. cruzi infection. For that purpose, mice were vaccinated with heterologous prime-boost vaccine (recombinant plasmid DNA/AdHu5), challenged and treated with blocking antibodies to LFA-1 and/or VLA-4. Our results demonstrate that LFA-1, but not VLA-4, is essential for protective immune response of highly susceptible mice against T. cruzi infection. Also, the study demonstrated that LFA-1 mediates CD8+ T cells migration into infected tissues, such as the heart, and plays an important role in CD8+ T cells cytotoxicity for parasite clearance.

MATERIALS AND METHODS

Ethics Statement
This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory mice of the Brazilian National Council of Animal Experimentation (http://www.mctic.gov.br/mctic/opencms/textogeral/conceas.html). The protocol was approved by the Ethical Committee for Animal Experimentation at the Federal University of Sao Paulo (Id # CEP 7559051115).

Mice and Parasites
Female 5- to 8-week-old A/Sn or C57BL/6 mice were purchased from the Federal University of São Paulo. ICAM-1-deficient mice were kindly supplied by Dr. João Santana, Ribeirão Preto School of Medicine-FMSP. Parasites of the Y strain of T. cruzi were used in this study (2, 3). Blood trypomastigotes of the Y strain of T. cruzi were maintained by weekly passages in A/Sn mice at the Xenodiagnosis Laboratory of Dante Pazzanese Cardiology Institute. Bloodstream trypomastigotes were obtained from mice infected 7–28 days earlier with parasites of the Y strain. For in vivo experiments, each mouse was inoculated with 150 trypomastigotes (A/Sn) or 105 trypomastigotes (C57BL/6) diluted in 0.2 mL phosphate-buffered saline (PBS) and administrated subcutaneously (s.c.) in the base of the tail. Parasitemia was determined by collecting 5 μL of blood, and parasites were counted on the light microscope (25).
Immunization Protocol
In this study, we used the heterologous prime-boost immunization protocol with plasmid pgSPC1.9 and the human replication-defective adenovirus type 5 containing the ASP-2 gene, as described previously (3, 26). Briefly, this immunization consists of a dose of plasmid DNA as a prime (pcDNA3 control or pgSPC1.9). The mice were intramuscularly inoculated (i.m.) with 50 µg of plasmid DNA into each tibialis anterior muscle. Three weeks after the first immunization, mice were boosted with 2 × 10⁶ plaque-forming units of the adenoviral vectors Adβ-gal or AdASP-2. Both injections were performed via intramuscular route (tibialis anterior muscle).

Peptide
TEWETGQI peptide was synthesized by GenScript and obtained at purity higher than 95%. The TEWETGQI epitope expressed on ASP-2 surface is target of CD8+ T cells and was identified previously (27). It was used for specific CD8+ T cell stimulation in vitro and ex vivo. The H2Kβ-TEWETGQI multimer, labeled with fluorophore APC, was purchased from Immudex (Copenhagen, Denmark) and used for specific CD8+ T cell detection in tissues.

Treatment with Monoclonal Antibodies
Anti-LFA-1 (anti-CD11a, clone M17-4) and anti-VLA-4 (anti-CD49d, clone PS/2) monoclonal antibodies were purchased from BioXcell; in addition, we used Rat IgG2a (clone 2A3) isotype control. The in vivo treatment was performed with 10 i.p. injections of 250 µg of mAb/mouse (every 48 h after infection, until day 20 after infection). The concentration of LFA-1 used for in vivo treatment was the same used by Reisman et al. (28). To evaluate the efficiency of LFA-1 integrin blockade, C57BL/6 mice were infected with 10⁴ trypomastigote forms of Y strain, and 12 days post infection, the splenocytes were harvested and incubated in vitro for 24 h at 30°C with monoclonal 250 µg/mL of 2A3 isotype control or anti-LFA-1 in complete medium [1% NEAA, 1% l-glutamine, 1% vitamins and 1% pyruvate, 0.1% 2-ME, 10% fetal bovine serum (FBS) (HyClone)]. After incubation, splenocytes were washed and labeled with anti-CD8 PerCP (clone 53-6-7, BD) and anti-CD11a FITC (clone 2D7, BD), fixed with 1% paraformaldehyde and analyzed by flow cytometry. Concomitantly, we also evaluated the blockade of the LFA-1 molecule stimulating splenocytes in vitro with 1 µg/mL anti-CD3 (clone 145-2C11, eBioscience) in complete medium for 72 h at 37°C and 5% CO₂. On the second day of incubation, 250 µg of 2A3 isotype control or anti-LFA-1 monoclonal antibodies were added to the culture. On the third day of culture, cells were harvested and labeled with anti-CD8 PerCP and anti-CD11a FITC for flow cytometric analysis. LFA-1 expression was performed on gated CD8+ T cells, according to Figures S1A,B in Supplementary Material, treatment with monoclonal anti-LFA-1 blocked most LFA-1 molecule expressed on activated CD8+ T cells and after anti-LFA-1 FITC staining there was a lower CD11a MFI on the surface of these cells, indicating that there is competition between anti-LFA-1 monoclonal antibodies used for in vivo blocking (clone M17-4) and anti-CD11a FITC (clone 2D7, BD) used for flow cytometry labeling.

Real-time PCR
Hearts, livers, and spleens from the T. cruzi-infected, immunized, and/or treated mice with anti-LFA-1 A/Sn were used for extracting DNA. The extraction protocol, the specific primers for a satellite DNA region of the parasite, and the RT-PCR reaction using the TaqMan Universal Master Mix II with UNG were adapted from Piron and colleagues (29). For the race plates, we used StepOnePlus (Applied Biosystems®), and distilled water for negative control reaction.

Enzyme-Linked Immunospot (ELISPOT) Assay
Sterile PBS containing 10 µg/mL of anti-mouse IFN-γ monoclonal antibody (clone R4-6A2, Pharmingen) was added onto nitrocellulose 96-well flat-bottom plates; after 24 h, the plates were washed with RPMI and blocked with RPMI containing 10% FBS for 2 h. Following, 1 × 10⁶ responder cells from spleen, liver, or lymph node were incubated with 3 × 10⁵ antigen-presenting cells in complete medium [1% NEAA, 1% l-glutamine, 1% vitamins and 1% pyruvate, 0.1% 2-ME, 10% FBS (HyClone), and 20 U/mL mouse recombinant IL-2 (SIGMA)]. The plate was incubated in the presence or absence of 10 µM of peptide TEWETGQI. After 24 h, the plates were washed three times with PBS, and five times with PBS–TWEEN 20 (0.05% Tween). Each well received biotinylated anti-mouse monoclonal antibody (clone XMG1.2, Pharmingen) diluted in PBS-0.05% TWEEN 20 at a final concentration of 2 µg/mL. The plates were incubated with streptavidin-peroxidase (BD) and developed by adding peroxidase substrate (50 mM Tris–HCl, pH 7.5, containing 1 mg/mL DAB and 1 µL/mL 30% hydrogen peroxide, both from SIGMA). The number of IFN-γ-producing cells was determined using a stereoscope.

Intracellular Cytokine Staining
Two million cells from the spleen, lymph node, or liver were treated with ACK buffer (NH₄Cl, 0.15 M; KHCO₃, 10 mM; Na₂-EDTA 0.1 mM; pH = 7.4). ICS was performed in vitro culture of splenocytes in presence or absence of 10 µM of peptide TEWETGQI as described previously (25). Cells were washed three times in plain RPMI and resuspended in cell culture medium consisting of RPMI 1640 medium supplemented with 10 mM HEPES, 0.2% sodium bicarbonate, 59 mg/L of penicillin, 133 mg/L of streptomycin, 10% HyClone FBS, 1 mM sodium pyruvate, 55 µM 2-mercaptoethanol. The viability of the cells was evaluated using 0.2% trypan blue exclusion dye to discriminate between live and dead cells. Cell concentration was adjusted to 2 × 10⁶ cells/mL in cell culture medium containing CD107α FITC antibody (clone 1D4B, BD), anti-CD28 (clone 37.51, BD), BD Golgi-Plug (1 µL/mL), and monensin (5 µg/mL) and incubated no longer than 12 h in V-bottom 96-well plates (Corning) in a final volume of 200 µL in duplicate, at 37°C in a humid environment containing 5% CO₂. After 12 h incubation, cells were stained for surface markers with anti-CD8 PERCP antibody (clone 53-6-7, BD) on ice for 30 min. To detect IFN-γ, TNF or granzyme B by intracellular staining, cells were then washed twice in buffer containing PBS, 0.5% bovine serum albumin, and re-stained with anti-IFN-γ Alexa Fluor 700 (clone XMG1.2, BD), anti-TNF FITC (clone TPB1, BD), or anti-granzyme B PE (clone 43H, BD). The number of IFN-γ, TNF or granzyme B-producing cells was determined using a stereoscope.
albunin (BSA), and 2 mM EDTA, fixed and permeabilized with BD perm/wash buffer. After being washed twice with BD perm/wash buffer, cells were stained for intracellular markers using APC-labeled anti-IFN-γ (clone XMG1.2, BD), PE-labeled anti-TNF-α (clone MP6-XT22, BD), and anti-granzyme B PE (clone GB11, INVITROGEN) for 20 min on ice. Finally, cells were washed twice with BD perm/wash buffer and fixed in 1% PBS−paraformaldehyde. At least 700,000 cells were acquired on a BD FACS Canto II flow cytometer and analyzed with FlowJo.

**Purification of Liver and Heart Lymphocytes**

The perfused liver was lysed with collagenase buffer composed of 0.2 mg/mL collagenase IV (SIGMA), 0.02 mg/mL DNase (SIGMA), and 5% FBS. The leukocytes were separated on a 35% Percoll gradient (GE Healthcare), followed by centrifugation at 600 × g for 20 min and at 4°C. The pellet was suspended in RPMI 1640 (SIGMA) with 10% FBS (30). For the purification of the lymphocytes of the heart, we followed the protocol of Gutierrez et al. (31). Briefly, hearts collected from five mice at day 20 d.p.i. were minced, pooled, and incubated for 1 h at 37°C with RPMI 1640, supplemented with NaHCO₃, penicillin−streptomycin gentamicin, and 0.05 g/mL of liberase blendzyme CI (Roche, Basel, Switzerland). The organs were processed in a Medimachine (BD Biosciences) in PBS containing 0.01% BSA. After tissue digestion and washes, cell viability was assessed by trypan blue exclusion, counted in a hemocytometer.

**Flow Cytometry Analysis**

Splenocytes were treated with ACK buffer for red cell lysis and washed with RPMI with 10% FBS. The spleen, heart, lymph node, and liver cells were stained with H2K<sup>+</sup>−TEWETGQI multimer for 10 min at RT. The cell surface was stained for 30 min at 4°C. The following antibodies were used for surface staining: anti-CD3 APCc7 (clone 145-2C11, BD), anti-CD8 PERCP or anti-CD8 PACIFIC BLUE (clone 53-6.7, BD), anti-CD11a FITC (clone 2D7, BD), anti-CD11c APCc7 (clone HL3, BD), anti-CD44 FITC (clone IM7, BD), anti-CD62L PE (clone MEL-14, BD), anti-CXCR3 PERCP/Cy5.5 (clone 173, BioLegend), anti-CD27 FITC (clone LG3A10, BD), anti-CD4 PEcy7 (clone RM4-5, BD), anti-CD122 FITC (clone 2F1, eBioscience), anti-CD49d PEcy7 (clone R1-2, BD), anti-CD69 PERCP (clone H1.F23, BD), anti-CD34 PEcy7 (clone H71, BioLegend), anti-CD95 PEcy7 (clone J02, BD), anti-CD25 FITC (clone LG3A10, BD), anti-CD127 PE (clone SB/199, BD), anti-CD122 FITC (clone TM-β1, BD), anti-CD38 PE (clone 90, BD), anti-β2 PERCP (clone FIB27, BioLegend), anti-CD31 FITC (clone MEC 13.3, BD), anti-CD272 PE (clone 8F4, eBioscience), anti-CD272 FITC (clone J43, eBioscience), anti-CTLA-4 PE (clone UC10-4B9, eBioscience), and anti-CCR7 PE (clone 4B12, BD). At least 500,000 cells were acquired on a BD FACs Canto II flow cytometer and analyzed with FlowJo 8.7.

**In Vivo Proliferation Assay**

A/Sn were immunized with ASP-2 using the heterologous “prime-boost” vaccination regimen and infected with 150 trypomastigotes forms of *T. cruzi*. At the moment of infection, mice were treated with monoclonal antibodies (LFA-1 or 2A3 isotype control) and 2 mg of BrdU (5-bromo-2′-deoxyuridine, SIGMA) by route i.p., at every 48 h, until the 20th day after challenge. Then, 2 × 10<sup>6</sup> splenocytes were treated with ACK buffer for red cell lysis, washed with RPMI plus 10% FBS, and stained with H2K<sup>+</sup>−TEWETGQI multimer and anti-CD8 antibody. The specific CD8<sup>+</sup> T cells were stained according BrdU-FITC Kit protocol (BD Pharmingen) for analysis of BrdU incorporation. A minimum of 700,000 cells were acquired on a BD FACs Canto II flow cytometer and analyzed with FlowJo 8.7.

**Histology and Immunohistochemistry**

The mice’s heart, spleen, and liver were fixed in 10% formalin, and then dehydrated, embedded in paraffin blocks, and sectioned on a microtome. Staining was obtained with hematoxylin and eosin, and the number of amastigotes nests was quantified using a light microscope with 40x objective lens. Overall, 50 fields/group were counted. For immunohistochemistry the hearts of the animals were removed and frozen in Tissue-Tek O.C.T. (Sakura Finetek), and the 7 µm thickness cuts were made in the cryostat (Leica) and then fixed in ice-cold acetone for 15 min. The samples were stained with 20 µg of the biotinylated anti-CD8 antibody (clone 53-6.7, RD systems) for 12 h in the wet chamber, and after incubation was labeled with streptavidin Alexa Fluor<sup>®</sup> 488 (Thermo Fischer) at the concentration of 0.5 mg/mL, diluted 1:100 for 1 h and room temperature. The DAPI (4’,6-diamidino-2-phenylindole, SIGMA) dye was used for labeling the 5 mg/mL cell nucleus, diluted 1:1,000 for 15 min at room temperature. The images were acquired in the Confocal Leica TCS SP8 CAR5 microscope of the Institute of Pharmacology and Molecular Biology (INFAR).
of the Paulista School of Medicine of the Federal University of São Paulo. The images were obtained using the 63x objective and processed by the ImageJ program.

Statistical Analysis
The number of parasites/mL corresponding to the peak of parasitemia, the number of IFN-γ-producing cells (ELISPOT), and the absolute number of CD8+ T cells were compared by analysis of unidirectional variance (ANOVA); subsequently, the Tukey's HSD test was used. To compare the survival of mice after challenge with *T. cruzi*, we used the Log-rank test. The receptor expression was compared using MFI (mean fluorescence intensity), and the naïve group MFI was taken as the baseline. MFI was determined by the FlowJo software. Differences were considered significant when P value was <0.05.

RESULTS

LFA-1 Is Essential for Survival of A/Sn Mice during the Experimental Challenge with *T. cruzi*

Previously, we demonstrated that treatment with FTY720, which retains CD8+ T cells in the lymph nodes via blockade of receptor S1Pr1, culminates in death of immunized mice. As LFA-1 and VLA-4 integrins were expressed on those specific of receptor S1Pr1, culminates in death of immunized mice. As LFA-1 and VLA-4 integrins were expressed on those specific CD8+ T cells were compared by analysis of unidirectional variance (ANOVA); subsequently, the Tukey's HSD test was used. To compare the survival of mice after challenge with *T. cruzi*, we used the Log-rank test. The receptor expression was compared using MFI (mean fluorescence intensity), and the naïve group MFI was taken as the baseline. MFI was determined by the FlowJo software. Differences were considered significant when P value was <0.05.

LFA-1 Blockade Increases Tissue Parasite of Immunized and Infected A/Sn Mice

Since anti-VLA-4 treatment did not interfere in the mice parasitemia and/or survival, all following experiments were performed by blocking LFA-1 integrin only. As the LFA-1 blockade leads to increased blood parasitemia and rapid death of the mice, we investigated whether the parasitic increase also occurs in the tissues of infected, immunized, and/or anti-LFA-1-treated A/Sn mice. The heart, liver, and spleen of these mice were extracted after the 20th day of infection for quantification of the parasite's DNA by real-time PCR; in addition, the number of amastigote nests in the heart was quantified using hematoxylin–eosin staining. There was a statistical increase in the number of amastigote nests in the hearts of the LFA-1-treated mice compared with the immunized and infected group, and the largest amount of nests was found in the hearts of mice solely infected (Figures 2A,B). In addition, LFA-1 blockade resulted in the increase of parasites in the tissues analyzed compared with immunized, infected mice. The spleen showed higher parasite increase, followed by hearts and livers respectively (Figure 2C). These results demonstrate that treatment with anti-LFA-1 increases blood parasitemia, which will reflect on increased tissue parasite burden.

LFA-1 Blockade Increases the Expression Level of the Fas/CD95 Molecule on the Surface of Effector CD8+ T Cells

We analyzed whether LFA-1 blockade affects effector phenotype and activation of specific CD8+ T cells. To this end, splenocytes were stained with anti-CD8 and H2K^b TEWETQGI-multimer, and surface markers. In previous results obtained by our group, we demonstrated that immunization followed by infection induces specific CD8+ T cells with the phenotype of effector cells (TE), which is characterized by the expression of CD11a^{high}, CD44^{high}, CD62L^{low}, and CD127^{low} (25, 26). Anti-LFA-1 treatment increased
Fig. 1 | LFA-1 blockade increases blood parasitemia and culminates in the death of A/Sn and C57BL/6 mice. A/Sn-, C57BL/6-, and intercellular adhesion molecule (ICAM)-deficient mice were immunized with plasmids pcDNA3 and/or plgSPclone9 and adenoviral vectors Adβgal and AdASP-2. Fifteen days after the last immunization dose, mice were infected with 150 (A/Sn) and/or 10^4 (C57BL/6 and ICAM mice) blood trypomastigotes of the Y strain of Trypanosoma cruzi.

Anti-VLA-4 and/or LFA-1 treatment was performed every 48 h after infection until the 20th days after infection. (A) Blood parasitemia was assessed, and values were log-transformed and parasitemia data for each mice group are represented as mean ± SD for each group (n = 4). Values from peak parasitemia (day 12) were compared by one-way ANOVA and Tukey’s HSD tests, and the results were as follows: Significant differences were found between the red in green groups (P < 0.05). There were no significant differences between the blue group and the green group (P > 0.05).

(B) Kaplan–Meier curves for survival of the different groups were compared using the log-rank test. The results of the comparisons were as follows: The mice in red group survived longer than the mice in green group. The survival rate of mice in the blue group was similar to the mice in the green group. (C) Log parasitemia data for each mice group (C57BL/6 mice infected and/or treated with anti-LFA-1) are represented as mean ± SD for each group (n = 4). Values from peak parasitemia (day 9) were significantly different across the groups (P > 0.05). On day 14, significant difference was found between the infected and treated with anti-LFA-1 mice and infected and treated with the anti-2A3 isotype control mice (P < 0.05). (D) Kaplan–Meier curves for survival of the different groups were compared using the log-rank test. The results of the comparisons were as follows: the mice in yellow group survived longer than the mice in blue group. (E) Log parasitemia data for each mice group (ICAM-deficient and C57BL/6 mice) are represented as mean ± SD for each group (n = 4). Peak parasitemia values (day 9) were significantly different across C57BL/6 groups and in ICAM-deficient mice groups (P > 0.05).

The frequency and the absolute number of specific CD8+ T cells in the spleen (Figures 3A, B). In addition, as there is competition between the antibodies used for in vivo blockade and flow cytometric labeling, we observed a decrease in CD11a MFI in the anti-LFA-1-treated groups (Figure 3C). In general, the markers whose expression levels increased in the anti-LFA-1-treated group (Gr.3) in comparison with the infected group (Gr.1) or the immunized and infected group (Gr.2) were as follows: CD27, CD43, CD69, and CD95 (Figure 3C). Our group has also demonstrated that the cells generated by immunization followed by infection expressed lower levels of CD95 on the surface compared with infected only group, and these cells were also resistant to death induced by anti-CD95 (12). Moreover, markers CD183, CD38, and PD-1 also displayed increased expression levels on the
Figure 2 | Treatment with anti-LFA-1 increases tissue parasitemia. A/Sn mice were immunized with ASP-2 using the heterologous “prime-boost” vaccination regimen, infected with 150 trypomastigotes forms of Trypanosoma cruzi and treated with anti-LFA-1 or isotype control until the 20th after infection. In this day, spleens, hearts, and livers of this mice were taken for quantification of the parasite DNA and count of amastigote nests in the heart. (A) Histological section of the heart; the arrows indicate the amastigote nests in each group. (B) Quantification of heart amastigote nests performed from 50 fields/group. (C) Quantification of the parasite DNA by real-time PCR in the different groups (Log) obtained from 50 ng of tissue DNA. Results represent mean ± SD for each group (n = 4) and to a single experiment. Asterisks represent statistical differences among the groups. Statistical analysis was performed using one-way ANOVA (*P < 0.001, **P = 0.000331, ***P = 0.006602, and ****P < 0.01).

surface of specific CD8+ T cells after treatment with anti-LFA-1 (Gr.3) compared with Gr.2 (Figure 3C). KLRG1, however, had similar MFI among the three groups, and these groups showed low expression of markers CD122, BTLA, CTLA-4, and CD25. These results suggest that anti-LFA-1 treatment does not impair specific CD8+ T cells in the spleen. Instead, there is a greater frequency and absolute number of these cells. In addition, the treatment did not alter the phenotype of effector CD8+ T cells (TE); however, we found in the spleen of treated mice that those CD8+ T cells expressed high levels of CD95.

Specific CD8+ T Cells Accumulate in Secondary Lymphoid Organs and Decrease Migration into the Heart after LFA-1 Blockade

After demonstrating that anti-LFA-1 treatment increases blood and tissue parasitemia and leads to mice death, our hypothesis was that specific CD8+ T cells cannot migrate into the infected tissues since LFA-1 is associated with leukocyte migration. To test our hypothesis, we measured in the spleen, lymph, blood, liver, and heart the frequency of specific CD8+ T cells. For that propose, the cells were labeled with anti-CD8 and H2Kb-TEWETGQI multimer. We found higher frequency and increment in the absolute numbers of specific CD8+ T cells in spleens and lymph nodes, but not in the blood and liver of the anti-LFA-1-treated group when compared with the infected and immunized group (Figures 4A–F). A dramatic influx reduction of specific CD8+ T cells after anti-LFA-1 treatment (Figure 4A) was observed in group 3 hearts. In addition, we found that specific CD8+ T cells in the spleen, blood, and heart of the infected group (Gr.1) and the immunized and infected group (Gr.2) expressed high levels of CD11a, whereas the cells in the immunized, infected, and anti-LFA-1-treated group (Gr.3) showed decreased CD11a MFI due to the competition described earlier (Figure 4G). We also evaluated the frequency of total CD8+ T cells in the spleen, blood, and heart and, as shown in Figure S2 in Supplementary Material, there was a reduction in the frequency of CD8+ T cells in the heart of the anti-LFA-1-treated group (Figures S2A–D in Supplementary Material). Furthermore, by immunohistochemistry, there is a decrease in the number of CD8+ T cells in the heart of animals treated with anti-LFA-1 (Figures S2E,F in Supplementary Material). The number of CD8+ T cells in the heart of the treated group was similar to the infected group, and in relation to the immunized group, treatment with anti-LFA-1 decreased the migration of CD8+ T cells to the heart, observed...
Figure 3 | Effector CD8+ T lymphocytes express high levels of CD95 on the surface after anti-LFA-1 treatment. A/Sn mice were immunized with ASP-2 using the heterologous “prime-boost” vaccination regimen, infected with 150 trypomastigotes forms of Trypanosoma cruzi and treated with anti-LFA-1 or isotype control until the 20th after infection. In this day, the splenic cells were labeled with H2K-KTEWETGQI multimer, anti-CD8 and surface markers.

(A) The frequency of specific CD8+ T cells in the spleen.

(B) Absolute number of specific CD8+ T cells in the spleen.

(C) Histograms with MFI of each marker analyzed in different groups. The red line represents the naive group, whereas the blue line represents groups 1, 2, and 3. Results in panels (A,B) are individual values with the mean ± SD of groups (n = 4), while in panel (C) representative analyses are shown for four mice per experiment. The experiment was performed two or more times with similar results. Statistical analysis was performed using the one-way ANOVA. Asterisks denote statistically significant differences between the groups 2 and 3 (P < 0.05).
Figure 4 Continued

A. Spleen

Gr.1 pcDNA3+Adβgal+T. cruzi
Gr.2 pigSPclone9+AdASP-2+T.cruzi+2A3 Isotype control
Gr.3 pigSPclone9+AdASP-2+T. cruzi+αLFA-1

B. Specific CD8+ T cells in the spleen x 10^6/mL

C. Specifically CD8+ T cells in the lymph node x 10^6/mL

D. Specifically CD8+ T cells in the blood x 10^6/mL

E. Specifically CD8+ T cells in the heart x 10^6/mL

F. H2Kk/TEMTETGCL

G. CD11a

naive
Gr.1
Gr.2
Gr.3
by the low number of these cells in the cardiac tissue. These results corroborate the decrease in specific CD8\(^+\) T cells in those organs. Therefore, during LFA-1 blockade, specific CD8\(^+\) T cells accumulate in the secondary lymphoid organs, such as spleen and lymph node, and cannot migrate into the heart, as observed by the lower frequency of these cells in that organ.

**Specific CD8\(^+\) T Cells Degranulate and Produce IFN-\(\gamma\) and TNF-\(\alpha\) after Anti-LFA-1 Treatment**

Having in mind the important role of IFN-\(\gamma\) during infection by *T. cruzi* (2), we analyzed whether the anti-LFA-1 treatment may alter the production of IFN-\(\gamma\) by specific CD8\(^+\) T cells. We also analyzed the effector function of specific CD8\(^+\) T cells in the spleen, liver, and lymph nodes regarding TNF-\(\alpha\) production and indirect cytotoxicity involving cell surface mobilization of CD107a. The gate strategy used to evaluate the production of cytokines and the polyfunctionality of specific CD8\(^+\) T cells is illustrated in Figures S3A,B in Supplementary Material. In the spleen of the anti-LFA-1-treated group, compared with the immunized and infected group, there was an increase in the percentage of polyfunctional specific CD8\(^+\) T cells, that is, cells that are capable of degranulating and, at the same time, producing IFN-\(\gamma\) and TNF-\(\alpha\). Such increase in polyfunctionality of specific CD8\(^+\) T cells after anti-LFA-1 treatment was also observed in lymph nodes and liver (Figures 5A,B). In addition, anti-LFA-1 treatment also culminated in an increase in the amplitude of the immune response, i.e., the percentage of specific CD8\(^+\) T cells producing IFN-\(\gamma\) or TNF-\(\alpha\) or degranulating, in the spleen, lymph node, and liver (Figure 5C). The number of specific CD8\(^+\) T cells producing IFN-\(\gamma\) was higher in the anti-LFA-1-treated group (Gr.3), when compared with Gr.2, and this increase occurred in the spleen, the lymph node, and liver (Figure 5D). Altogether, these results show that LFA-1 blockade does not affect the effector function of specific CD8\(^+\) T cells regarding IFN-\(\gamma\) and TNF-\(\alpha\) secretion and degranulation. The increase in the effector function is probably due to the accumulation of specific cells in spleen and lymph node after treatment with anti-LFA-1.

**Specific CD8\(^+\) T Cells Reduce Direct Cytotoxicity against Target Cells after Treatment with Anti-LFA-1**

First, we tested whether treatment had impaired the frequency and absolute numbers of specific CD8\(^+\) T cells. As we can see, there was an increase in the frequency and absolute numbers of specific CD8\(^+\) T cells in the anti-LFA-1-treated group (Figures 6A,B). We concluded that the treatment did not interfere with specific CD8\(^+\) T cell expansion. Specific CD8\(^+\) T cells are capable of secreting IFN-\(\gamma\) and TNF-\(\alpha\) after LFA-1 blockade in the spleen, we assessed whether the treatment had impaired specific CD8\(^+\) T cells proliferative capacity. The proliferation of specific CD8\(^+\) T cells in the spleen was analyzed in vivo by thymidine BrdU analog incorporation. We found that a similar proportion of the H2K\(^{K-}\)-TEWETGQI CD8\(^+\) T cells incorporated BrdU in vivo in non-treated or treated mice indicating that the proliferative capacity of these cells was not significantly different (Gr. 2 and Gr. 3, Figure 6C). However, the infected mice have a greater proliferation in comparison with groups 2 and 3 (Figures 6C,D).

Another effector function triggered by specific CD8\(^+\) T cells is the direct cytotoxicity against the target cells. Here, we analyzed whether this function had been affected by the treatment. For that purpose, we used in vivo cytotoxicity assay. Figure 7A shows representative histograms containing two populations P1 (CFSE\(^{low}\)) and P2 (CFSE\(^{high}\)) showing the specific lysis of H2K\(^{K-}\)-TEWETGQI peptide-labeled CFSE\(^{low}\) cells from Gr.1, Gr.2, and Gr.3 groups (Figure 7A). Surprisingly, we observed that the immunized mice treated with anti-LFA-1 had significantly decreased cytotoxicity when compared with the immunized group (Figure 7B). The specific CD8\(^+\) T cells from the immunized mice have 80% cytotoxicity whereas the anti-LFA-1-treated cells showed a 29% percentage drop. Figure 7C shows that the numbers of CFSE\(^{low}\) cells were similar across groups 1, 2, and 3, while the number of cells CFSE\(^{high}\) decreased in groups 2 (non-treated) and 3 (treated) compared with group 1 (Figure 7D).

The reduction of cytotoxicity did not affect the amount of granzyme B produced by specific CD8\(^+\) T cells in the anti-LFA-1-treated group. We observed that MFI and the percentage of specific CD8\(^+\) T cells that produce granzyme B were similar across the groups (Figures 7E,F). These results demonstrate that treatment with LFA-1 directly affects the cytotoxicity of specific CD8\(^+\) T cells and the impairment of this function may be one of the factors responsible for the reversal protection generated by immunization observed after LFA-1 blockade.

**DISCUSSION**

Our group previously demonstrated that the recirculation of specific CD8\(^+\) T cells generated by heterologous prime-boost immunization and *T. cruzi* infection is of paramount importance to the protection of A/Sn mice, which are highly susceptible to infection by *T. cruzi* (12, 13). Given that integrins play an important role...
FIGURE 5 | Continued
in cell–cell and cell–extracellular matrix interactions, and that these interactions are responsible for the intracellular signal transduction that culminates in cell migration (14) and formation of the immunological synapse (15), the aim of this study was to analyze the role of LFA-1 and VLA-4 integrins in specific CD8+ T cells migration. During activation of specific CD8+ T cells, there was an increase in the expression level of CD11a (LFA-1) and CD49d (VLA-4) chains (12). In addition, an increase in effector CD8+ T cells that express molecules LFA-1/ICAM-1 and VLA-4/VCAM-1 occurred in the hearts of mice infected with T. cruzi, suggesting the role of these molecules in the migration of specific CD8+ T cells into infected tissues (22, 23, 32). Here, we investigated the hypothesis that these molecules participate in the migration of specific CD8+ T cells generated by immunization and infection. LFA-1 blockade, but not VLA-4, makes vaccinated A/Sn and infected C57Bl/6 mice susceptible to infection with T. cruzi. The increased susceptibility of A/Sn mice was accompanied by increased parasitemia and tissue parasite burden, as well as rapid death of these mice. Even though VLA-4 does not play any role in our model, it participates in the migration of specific CTLs into the heart of C3H/He mice when infected with the Colombian strain of T. cruzi (22). Because we did not see any role of VLA-4 in the mice parasitemia and survival our study was conducted toward LFA-1 role.

To analyze the role of ICAM-1, a major ligand for LFA-1, we used ICAM-1 KO mice and challenged them with blood...
The lower expression of CD95 in the cells induced upon vaccination was the main difference to CD8+ T cells generated by the infection, which had higher levels of CD95 (25). There is an increase in the expression of Fas/CD95 on some specific CD8+ T cells after immunization and treatment with anti-LFA-1. This result suggests that specific CD8+ T cells in mice treated with anti-LFA-1 may be more susceptible to programmed cell death by the extrinsic pathway. Similar results were shown by Borisch and colleagues, who found that LFA-1 blockade reduces the survival of T lymphocytes, thus suggesting the important role of LFA-1 in survival signals during the process of T cells migration (39). Otherwise, reduction of migration to tissues upon LFA-1 blockade might increase effector CD8+ T-cells expressing Fas in the secondary lymphoid organs.

Indeed, there is an impaired migration of specific CD8+ T cells after LFA-1 blockade. The number of these cells was quantified in the spleen, heart, liver, lymph nodes, and blood after anti-LFA-1 treatment. The treatment led to an increase in the frequency and absolute number of specific CD8+ T cells in the spleen and lymph nodes, and a decreased frequency mainly in the heart. However, despite the apparent decrease in the overall number of CD8+ T cells in blood, this decrease was not statistically significant (Figure S2 in Supplementary Material). We have previously described that specific-peptide CD8+ activation occurs in the lymph nodes after subcutaneous infection by *T. cruzi* and also in vaccinated model (12, 13). We approached this subject by administering the immunosuppressive drug FTY720 (12, 13). In both models, this drug reduced lymphocyte recirculation by interfering with T cell signaling via S1Pr1. This

![FIGURE 7](image)
interference resulted in inhibition of S1Pr1 signaling, effectively trapping T cells within the lymph node without inhibiting T cell activation. FTY720 administration significantly impaired protective immunity supporting the hypothesis that T cell recirculation is critical for the protective immunity they mediate (12, 13). Here, we have not addressed how these cells accumulate more in the lymph after treatment with anti-LFA-1. However, our data confirm that recirculation of these cells is necessary to exert their effector function in the peripheral tissues. Thus, blocking the integrin LFA-1, we observed the same accumulation not only in the lymph node but also in the spleen. However, in our immunization, infection, and treatment model, there was no change in the number of specific CD8+ T cells in the peripheral blood (Figure S2 in Supplementary Material), probably because of the increased accumulation of CD8+ T cells in the spleen and lymph node. This phenomenon may be specific to our immunization and infection model but has not yet been explored in details or found in another model. One explanation might be that the blockage of LFA-1 integrin expressed by CD8+ T cells prevents the interactions with its ligand might be required to exit from lymph node. Another explanation might be due the decreased speed or movement of these cells (40). These issues need to be further addressed in our vaccination and infection model. These results confirm that LFA-1 is important to the migration of specific CD8+ T cells into infected tissues such as the heart, and the decline of these cells should be one of the causes for increased parasitemia in that organ. Recent studies have shown that LFA-1 blockade, and not VLA-4, reduces migration speed of T lymphocytes, leading to decreased antigen scanning by T cells (41) and, hence, lower immune response and higher parasitemia.

We also evaluated whether anti-LFA-1 treatment would affect the effector function of specific CD8+ T cells, since these cells accumulated in the spleen are incapable of controlling the number of parasites. We analyzed the production of pro-inflammatory cytokines, crucial for controlling T. cruzi multiplication, such as IFN-γ and TNF-α, and observed that there was accumulation of polyfunctional specific CD8+ T cells capable of degranulating and simultaneously producing TNF-α and IFN-γ in the spleen, lymph node and liver. As the LFA-1 blockade retained CD8+ T cells in the spleen and lymph node, we believe that this accumulation led to increased effector function of these cells. In the liver, in which there was no accumulation of such cells, the increase in intracellular cytokine production can be explained by the fact that LFA-1 blockade does not affect the production of those cytokines. In addition, it has been shown that high doses of anti-LFA-1 were required for impairing the production of those mediators (42). Finally, specific CD8+ T cells proliferated after LFA-1 blockade, and this result was consistent with the data obtained by Gérard et al. (38).

Another important function triggered by specific CD8+ T cells is the direct cytotoxicity against target cells. NK cytotoxic cells and CTLs of chagasic patients express perforin and granzyme B, suggesting the importance of these mediators to the host immune response (43). In addition, specific CD8+ T cells induced by immunization are cytotoxic and can produce perforin (2). Anti-LFA-1 treatment decreased the 80% cytotoxicity of specific CD8+ T cells in the immunized group to 60% after treatment. Similar results were obtained by Petit et al., whereas LFA-1 blockade was responsible for a 50% decrease of direct cytotoxicity triggered by CD8+ T cells (42). In addition, the decrease in cytotoxicity is independent of cytotoxic granule production and degranulation because there was no decrease in the amount of granzyme B and CD107a in specific CD8+ T cells treated with anti-LFA-1. We believe that cytotoxicity is impaired not because of the reduction of cytotoxic granules but because LFA-1 is important to maintain stability between target and cytotoxic cells. The role of LFA-1 in maintaining a stable contact between the cells was demonstrated by blocking β2 chain of LFA-1, since absence of such molecule impaired the formation time of cell protrusions, as well as the stability of the immunological synapse (44–47). Also, it has been shown that LFA-1 blockade impairs close contact between effector T cells and antigen-presenting cells (48).

The reduction of direct cytotoxicity may explain why specific CD8+ T cells cannot control the number of parasites in the spleen even if accumulation of these cells occurs during LFA-1 blockade. In addition, our results suggest that LFA-1 plays an important role in the migration of specific CD8+ T cells into the heart and the survival of these cells. Finally, we believe that impairment of direct cytotoxicity and lower migration of specific CD8+ T cells into the heart are the major causes of lack of protection to T. cruzi infection upon immunization and treatment with anti-LFA-1.

ETHICS STATEMENT

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory mice 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 7559051115).

AUTHOR CONTRIBUTIONS

CF, LC, and JV conceived and designed the experiments. CF, LC, FV, BM, CM, PR, and DA performed the experiments. CF and LC analyzed the data and prepared the figures. AM, RG, O-BR, and MR contributed with reagents and materials. CF and JV wrote the manuscript. ML, JL-V, CM, and BM performed the final review of the article. All the authors read and approved the final article.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01291/full#supplementary-material.
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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