Fas Ligand-Dependent Inflammatory Regulation in Acute Myocarditis Induced by *Trypanosoma cruzi* Infection

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Fas/Fas ligand (Fas-L) engagement, a potent inducer of apoptosis, is also important for cellular activation, regulation of effector and chemotactic activity, and secretion of chemokines and cytokines. We evaluated the relevance of Fas/Fas-L in the regulation of myocarditis induced by *Trypanosoma cruzi* infection and observed that in Fas-L^{-/-} mice (*gld/gld*), cardiac infiltration was significantly reduced, accordingly showing less cardiomyocyte destruction. Fluorescence-activated cell sorting analysis of cardiac inflammatory cells showed higher numbers of CD8⁺ T cells in BALB/c compared with *gld/gld* mice but similar levels of lymphocyte function-associated antigen-1, intercellular adhesion molecule, CD2, and CD69 expression; MAC-1⁺ myeloid cells and mast cells were increased in BALB/c mice, whereas *gld/gld* mice exhibited an enrichment of CD4⁺/low T cells. Intracellular labeling of cytokines revealed no clear cardiac skewing of Th1 or Th2 responses, but we found a higher number of interleukin-10⁺ cells in *gld/gld* mice and a deficient expression of vascular cell adhesion molecule-1 on cardiac endothelial cells in *gld/gld* mice. Finally, we found a population of CD3⁺ but CD4/CD8 double negative cardiac T cells in both groups of infected mice, but down-regulation of some adhesion molecules and surface receptors was only observed in *gld/gld* mice, indicating a targeted T-cell population mostly affected by the lack of Fas-L engagement. These results point to a role for myocarditis regulation by Fas/Fas-L beyond its possible direct relevance in cellular death. (Am J Pathol 2007, 171:79–86; DOI: 10.2353/ajpath.2007.060643)

Lymphocyte death triggered by antibody-induced crosslinked surface Fas molecules was described almost 20 years ago.¹ Since then, many groups have demonstrated the enormous importance of this molecular interaction, as well as downstream adaptor molecules, in embryonic tissue remodeling,² tumor surveillance,³ autoimmunity, and control of acquired immune responses.^{4,5} All these findings strengthened the notion that Fas/Fas-L (Fas ligand) engagement is necessary for regulated and physiological apoptosis in a number of systems. Although Fas signals have become inextricably associated to cell death, it is now clear that Fas-triggering induces cellular and immunological responses far beyond its relevance in apoptosis.⁶ Fas can induce cell activation, proliferation, differentiation, secretion of cytokines and chemokines, recruitment of inflammatory cells, cell survival, and more.⁷

Fas is a member of the tumor necrosis factor receptor superfamily of cytokine receptors that triggers intracellular signals through the death domain formed after engagement of two adjacent trimeric Fas-L.⁸ Intracellular effects of Fas engagement are mediated by a cascade of adaptor and effector molecules.⁷ FADD (Fas-associated death domain) couples Fas to oligomers of caspase 8, as well as caspase 10, for autocatalytic activation and cell death. However, FADD is associated to not only apoptosis. Activation-induced T-cell proliferation, thymic maturation in an age-dependent manner, and B-cell development are defective in the absence of FADD.⁹ Apparently, this balance of *proliferation/activation* × *apoptosis* is based in a single serine amino acid in the position 191, because this punctual mutation abrogates its role in proliferation without impairing its apoptotic function.¹⁰ Other intermediate molecules in the Fas-based pathways have also been depicted as nonexclusive apoptotic players,⁶ such as Daxx and RIP. These molecules trigger signal

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transduction pathways based in nuclear factor- κ B, extracellular signal-regulated kinase, or mitogen-activated protein kinase kinase for proliferation, transcription induction or repression, differentiation, and survival.⁷

In the case of inflammatory regulation, recent data indicate that acute inflammatory response is either prevented or attenuated after blockade of Fas/Fas-L. Although there are some controversial data regarding the primary importance of soluble Fas-L (sFas-L) versus membrane Fas-L (mFas-L),^{11,12} it is clear that Fas-based intracellular signaling is important for secretion of interleukin (IL)-1 β , IL-6, IL-17, IL-18, macrophage inflammatory protein MIP-2, MIP-1 α , MIP-1 β , IL-8, and chemotaxis of neutrophils.¹³ For example, *in vitro* experiments showed that sFas-L required a CD8⁺ memory T cell [also expressing natural killer (NK) cell markers] to recruit inflammatory cells. These cells were not conventional NK/T cells but were necessary for CC and CXC chemokine secretion and Fas-L-dependent neutrophil recruitment.¹¹ On the other hand, *in vivo* approaches have indicated that mFas-L and not sFas-L is responsible for its inflammatory activity.¹²

Mice bearing a disrupted gene for Fas (*lpr*) or Fas-L (*gld*) have attenuated neutrophilic infiltration and fibrosis,¹⁴ with lessened aggressive idiopathic pulmonary fibrosis, silicosis, and acute lung injury.¹⁵ Besides this, local administration of adenovirus carrying the Fas-L gene into an inflamed joint protects or reduces the severity of systemic collagen-induced arthritis, where autoreactive T cells are recruited by Fas-L^{high} cells and destroyed.¹⁶ In the case of severe myocarditis induced by coxsackievirus B3, C3H/HE mice treated with anti-Fas-L, C3H/HE-*lpr/lpr*, or C3H/HE-*gld/gld* mice showed reduced inflammatory infiltration.^{17–19}

Based on these results, we evaluated the importance of Fas/Fas-L engagement in the regulation of myocarditis induced by *Trypanosoma cruzi* infection. The disease is characterized by an initial acute phase with trypomastigote forms in the blood and acute myocarditis,^{20,21} followed by a silent, indeterminate period with subpatent levels of parasitemia. Approximately 30% of infected human subjects progress to a severe, diffuse, and fatal chronic cardiomyopathy.²² It is mostly accepted that mononuclear cells, mainly T lymphocytes, are responsible for cardiomyocyte destruction and heart failure in an antigen-dependent manner. However, autoimmunity toward cardiac components²³ and microvascular lesions have also been purposed to be involved in the pathophysiology of chagasic heart disease.²⁴

In the last few years, many attempts have been made to characterize the phenotype of cardiac inflammatory cells and molecular mechanisms involved in cellular recruitment and cytotoxic events in *T. cruzi* infection. The predominance of CD8⁺ T cells in cardiac inflammatory foci has been reported by different groups,²⁵ especially in the chronic phase, but only recently has the phenotype of these cells been assessed by flow cytometry. Cardiac CD8⁺ T cells have been discerned as L-selectin^{low} (CD62-L^{low}), leukocyte function-associated antigen 1^{high} (LFA-1^{high}), and very late antigen 4^{high} (VLA-4^{high}).²⁶ These cells do express activated/memory cell surface

markers (CD44⁺/CD11a⁺) in the chronic phase but have sharply attenuated effector functions, as ascertained by cytotoxic assays and production of interferon- γ (IFN- γ).²⁷ This may be, at least in part, one of the mechanisms by which *T. cruzi* persists in the heart tissue, causing a long-lasting myocarditis.

A predominant Th1 response is of central importance to controlling parasitism and mounting a protective immune response in *T. cruzi* infection.²⁸ In the early acute phase, activated macrophages secrete inflammatory cytokines, especially tumor necrosis factor- α and IL-12, which in turn stimulate the secretion of IFN- γ by NK cells,²⁹ although CD4⁺ and CD8⁺ $\alpha\beta$ T cells are also important sources of IFN- γ .³⁰ Control of acute inflammation in nonlymphoid tissues and avoidance of secondary damage are believed to be based on a subsequent wave of anti-inflammatory cytokines, such as transforming growth factor- β and IL-10, but also IL-4.³¹

The particular aspect relating to cytotoxic cells and molecular pathways directed against cardiomyocytes is one of the most obscure points of the infection. Perforin-deficient mice infected with *T. cruzi* exhibited cardiac cellular inflammatory infiltration and cardiomyocyte destruction much more intensely than infected counterparts.³² These data indicate that perforin has an important role in the regulation of inflammatory response but not in the direct destruction of cardiac cells.

Our present results using Fas-L-deficient mice indicate for the first time that this molecule, just as perforin, plays a role in the regulation of cardiac inflammatory response induced by *T. cruzi* infection. We observed reduced myocarditis in *gld/gld* mice, as well as an enrichment of cardiac IL-10-producing cells and weak expression of vascular cell adhesion molecule (VCAM)-1 on endothelial cells. We observed fewer cardiac CD8⁺ T cells, MAC-1⁺ myeloid cells, and mast cells in *gld/gld* mice and a targeted T-cell population CD3⁺/CD4⁺/CD8⁺ that was phenotypically down-regulated by the absence of Fas-L. The refined balance between pro- and anti-inflammatory mediators, now including Fas/Fas-L, is possibly the key to the control of the infection and maintenance of asymptomatic status.

Materials and Methods

Mice

Male, specific pathogen-free Fas-L^{-/-} (*gld/gld*) mice (6 to 7 weeks old) and their counterparts isogenic BALB/c, perforin^{-/-} (C57Bl/6 background), and C57Bl/6 mice were purchased from the Breeding Laboratory Animal Center at Fundação Oswaldo Cruz. Mice were housed for 7 to 10 days in the Laboratory of Cellular Biology, Division of Animal Experimentation, under environmental factors and sanitation conforming to the guide for the Care and Use of Laboratory Animals. This project was approved by Fiocruz Committee of Ethics in Research (0099-01), according to resolution 196/96 of the National Health Council of Brazilian Ministry of Health.

Parasites and Infection

T. cruzi Y strain was maintained *in vivo* by passage in nonsyngeneic Swiss Webster mice. Infected blood was collected, and trypomastigote forms were isolated as previously described.³³ Thereafter, parasites were diluted in saline and counted in a hemacytometer to adjust the inocula to 1×10^3 parasites per 200 μ l for intraperitoneal injection in both groups of mice. Noninfected mice received 200 μ l of saline.

Parasitemia, Mortality, and Blood Samples

Individual parasitemia was counted in 5 μ l of blood collected from tail snips on indicated days postinfection (dpi), and mortality was scored daily. For plasma analysis, 100 μ l of blood samples were also collected from tail snips in heparinized capillaries and centrifuged. For creatine kinase-MB activity (CK-MB, the cardiac isoform of CK), plasma from noninfected and infected BALB/c and *gld/gld* mice was collected on 0, 8, and 15 dpi and analyzed using commercially available kits (Merck KGaA, Darmstadt, Germany) as described elsewhere.³⁴ This quantitative assay is used as a marker of cardiomyocytes damage, and it is expressed as a rate of NADPH increase ($\Delta E/min$) in seven sequential readings in a spectrophotometer (Molecular Devices, Sunnyvale, CA) at 340 nm.

Histopathological Analysis

Mice were euthanized with CO₂ on 0, 8, and 15 dpi, tissue fragments were fixed in Millonig-Rosman solution (10% formaldehyde in phosphate-buffered saline), and paraffin-embedded samples were further processed and stained with hematoxylin and eosin in 3- μ m-thick slices. The number of inflammatory foci (composed by ≥ 10 inflammatory cells), inflammatory cells per foci, *T. cruzi* parasite nests, and number of parasites per nest were determined by scanning the whole-tissue slice in a total of around 300 individual microscopic fields for each parameter and time point (one field = 0.196 mm²). For VCAM-1- or MAC-1-based immunohistochemistry, we used primary monoclonal antibodies (mAbs) (Southern Laboratories, Birmingham, AL) and secondary goat anti-rat conjugated to horseradish peroxidase (Sigma) in 1-hour incubation and revealed with 3-amino-9-ethylcarbazole (AEC; Sigma) as described elsewhere.²⁶ Direct quantification of mast cells was performed by toluidine blue staining in cardiac slices 16- μ m thick to confirm fluorescence-activated cell sorting analysis (see below).

Inflammatory Cell Harvesting

Hearts from four to five infected BALB/c and *gld/gld* mice were collected on 15 dpi and cut into fragments of 1 to 2 mm in ice-cold phosphate-buffered saline (Sigma, St. Louis, MO). Fragments were transferred to a solution of collagenase type IV 0.2% (lot 51K8610, 2.6 units/mg solid; Sigma) and submitted to four to five cycles of enzymatic digestion of 20 minutes at 37°C. Isolated cells

were centrifuged and immediately transferred to ice-cold Dulbecco's modified Eagle's medium (Sigma) plus 10% of fetal calf serum (Sigma) and maintained on ice. Cellular debris were removed by fetal calf serum centrifugation at $25 \times g$ for 5 minutes, and before phenotypic labeling, the cells were incubated for 20 minutes at 4°C with 10% solution of inactivated normal sheep serum to block Fc receptors.

Flow Cytometry

For phenotypic analysis by flow cytometry, we performed four-color labeling of the samples for 30 minutes at 4°C with anti-CD3 (Southern Laboratories), anti-CD8, and anti-CD4 (Southern) monoclonal antibodies and then with anti-adhesion molecules intercellular adhesion molecule (ICAM)-1 or LFA-1 (Southern Laboratories), co-receptor CD2, or early activation T-cell marker CD69. For analysis in all T-cell subpopulations (CD3⁺/CD4⁺; CD3⁺/CD8⁺ and CD3⁺/CD4⁻/CD8⁻), we first gated CD3⁺ cells, opened the CD4 $\times$ CD8 dot plot, and after gating all subpopulations, we performed the phenotypic determination. We also used anti-CD45R (B220) anti- $\gamma\delta$ T cells, and anti-MAC-1. All monoclonal antibodies were purchased from BD Pharmingen (San Jose, CA), except when indicated. SSC^{interm} $\times$ MAC-1⁺ were considered MAC-1⁺-myeloid cells (macrophages, neutrophils, and dendritic cells), and mast cells were SSC^{high} $\times$ MAC-1⁺. For cytokine detection, we labeled the cells with anti-CD3, fixed the samples with formaldehyde 1% (Sigma), and permeabilized plasma membranes with saponin 0.2% (Sigma). We then used anti-IL-2, -IL-4, -IL-10, or -IFN- γ , washed, and acquired in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Data analysis was performed using CellQuest software version 3.2 (Becton Dickinson).

Statistical Analysis

The Mann-Whitney nonparametric test was used to compare two sets of data. *P* values were as indicated in figure legends. Mortality rate was evaluated according to the log-rank test with *P* = 0.0002.

Results

Parasitemia was consistently similar in both BALB/c and *gld/gld* mice in the early acute phase after infection with Y strain of *T. cruzi* (Figure 1A). This is in accordance with our previous data where we also observed equivalent levels of parasitemia in the first 2 weeks of infection.²⁸ We observed 20% mortality at 19 dpi in BALB/c mice and 80% at 20 dpi (Figure 1B). *gld/gld* mice, in turn, showed 75% mortality at 15 dpi and 100% at 18 dpi (Figure 1B). Although *gld/gld* mice died before 20 dpi, approximately 20% of BALB/c mice reached the chronic phase (data not shown). We then thought that possibly, contrary to other inflammatory models that are ameliorated in the absence of Fas engagement, the cardiac inflammatory infiltration induced by *T. cruzi* infection was more intense in *gld/gld*

Figure 1. Parasitemia and cumulative mortality. Parasitemia was counted in 5 μ l of blood collected from tail snips and scored at indicated time points (A). Cumulative mortality was followed daily (B), and results are representative of 50 to 80 mice per group, $P = 0.0002$.

mice. Histopathological analysis at 0 and 8 dpi showed no morphological alterations of cardiac fibers in either lineage (Figure 2, A–D) and, most importantly, no cellular infiltration (Figures 2, A–D, and 3, A and B). However, at 15 dpi, BALB/c mice presented significantly higher numbers of cellular inflammatory infiltration as well as inflammatory cells per focus when compared with *gld/gld* mice (Figures 2, E and F, and 3, A and B). The quantification of parasites in the cardiac tissue in both groups of mice revealed similar numbers of parasite nests (Figure 3C) and parasites per nest (Figure 3D). Besides this, parasite nests in *gld/gld* mice were not usually associated or had few adjacent inflammatory cells (Figure 2F). Therefore, we decided to evaluate cardiomyocyte death by dosing enzyme CK-MB activity as a marker of myocardium damage³⁴ (Figure 3, E and F). At 8 dpi, BALB/c mice had higher levels of CK-MB activity when compared with *gld/gld* mice, and although at 15 dpi both lineages had increased the tracer activity, BALB/c mice again showed higher levels when compared with *gld/gld* mice (Figure 3, E and F), in accordance with the magnitude of cellular infiltration. Taken together, these data indicate that in *T. cruzi* infection, the absence of Fas/Fas-L decreases myocarditis. In addition, the data indicate that cardiac cellular inflammatory infiltration and cardiomyocytes destruction were probably not the primary cause of the mortality observed in *gld/gld* mice.

We then evaluated the phenotype of major cardiac inflammatory populations and endothelial cells, in regards the expression of some adhesion molecules and/or secretion of cytokines that could provide some clues

Figure 2. Histopathological analysis. BALB/c (left) and *gld/gld* mice (right) were euthanized at 0 (A and B), 8 (C and D), and 15 (E and F) dpi with *T. cruzi*. Hearts were collected for further paraffin-embedded processing and staining with H&E. **Arrows** indicate cellular inflammatory foci, and **arrowheads**, parasite nests; magnifications, $\times 400$. Results are representative of three independent experiments with five to eight *T. cruzi*-infected mice per time point/experiment.

Figure 3. Cardiac inflammatory response and cardiomyocytes damage. H&E-stained cardiac slices were analyzed, and the number of inflammatory foci (A), inflammatory cells per foci (B), parasite nests (C), and parasites per nest (D) were counted in at least 300 microscopic fields for each parameter and time point. The quantification was done by scanning the whole tissue in BALB/c (black bars) and *gld/gld* (open bars) mice at indicated time points after infection with *T. cruzi*. ^a $P < 0.05$. $\Delta E/min$ expresses cardiac tissue damage evaluated by creatine kinase cardiac isotype MB (CK-MB) activity in plasma samples from BALB/c (E) and *gld/gld* (F) mice. Results are representative of three independent experiments with five to eight *T. cruzi*-infected mice per time point/experiment. ^{*} $P < 0.01$ when comparing BALB/c with *gld/gld* at 8 or 15 dpi, and ^{*} $P < 0.01$ when comparing the same mouse lineage at 8 and 15 dpi.

Figure 4. Major cardiac inflammatory cells harvested from infected *T. cruzi* mice. Perforin^{+/+} (A and B, hachured curves), perforin^{-/-} (A and B, black line), BALB/c (C–H, hachured curves), and *gld/gld* (C–H, black lines) mice were euthanized at 15 dpi of *T. cruzi* infection and cardiac tissue dissociated by enzymatic solution. Cells were labeled with indicated mAbs, and numbers indicate marker-delimited positive cells. Results are representative of three independent experiments with five *T. cruzi*-infected mice pooled/experiment.

about the inferior gravity of cardiac inflammatory reaction in *gld/gld* mice. We first isolated cardiac inflammatory cells from *T. cruzi*-infected perforin^{-/-} and perforin^{+/+} mice and observed an enrichment of CD8⁺ (Figure 4A) over CD4⁺ T cells in perforin^{-/-} (Figure 4B), confirming our previous results based on immunohistochemistry.³² When we isolated inflammatory cells from BALB/c and *gld/gld* mice at 15 dpi, we observed a different pattern, with predominance of CD8⁺ T cells in BALB/c mice

(Figure 4C) but a predominance or equivalent levels of CD4^{+/low} over CD8⁺ T cells in *gld/gld* mice (Figure 4D). We found very few B220⁺ cells and virtually no γδ T cells in either group of mice (Figure 4, E and F) but higher numbers of MAC-1⁺ myeloid cells (Figure 4G) and mast cells (Figure 4H) in BALB/c mice. Toluidine blue staining of cardiac mast cells confirmed fluorescence-activated cell sorting analysis, where we found 0.612 cells/mm² in BALB/c mice and only 0.281 cells/mm² in *gld/gld* mice at 15 dpi (data not shown). Regarding the profile of cytokines produced by cardiac lymphocytes, BALB/c and *gld/gld* mice showed no clear skewed Th1 or Th2 immune responses; both showed IFN-γ, IL-2, and IL-4, but IL-10, a potent anti-inflammatory cytokine, was only detected in *gld/gld* mice (Figure 5A). These results corroborate previous data obtained with splenocytes from *T. cruzi*-infected *gld/gld* mice, where we also observed higher levels of IL-10 by ELISA.²⁸ VCAM-1-based immunohistochemistry showed visible labeling of activated endothelial cells in cardiac slices from infected BALB/c mice (Figure 5B) but a weak labeling in *gld/gld* mice (Figure 5C), confirmed by negative controls (Figure 5, D and E).

By fluorescence-activated cell sorting, infected BALB/c mice were enriched with CD3⁺/CD8⁺ T cells when compared with *gld/gld* mice, with no major alterations in either group of mice in regards to the expression of not only adhesion molecules ICAM and LFA-1 but also CD2 and CD69 (Figure 6), as observed in CD3⁺/CD4⁺ cells as well (data not shown). For example, we observed approximately 24.4% of CD8⁺ cells in BALB/c mice and only 7.6% in *gld/gld* mice. Despite the reduced number of this subpopulation, almost all CD8⁺ T cells were ICAM-1⁺ in infected BALB/c or *gld/gld* mice (Figure 6, top panels), and this distribution was observed in all labeling. After four-color labeling, we detected a particular population of T cells CD3⁺/CD4⁻/CD8⁻ (Figure 7) that seemed to be a targeted T-cell population in the heart,

Figure 5. Cytokines produced by cardiac lymphocytes and VCAM expression on endothelial cells. Cardiac cells were collected from BALB/c (solid bars) or *gld/gld* (open bars) mice infected with *T. cruzi* at 15 dpi and labeled with mAb anti-cytokines as indicated (A). Analysis was performed in the morphological gate enriched in lymphocytes and in CD3⁺ cells, and results are representative of two independent experiments with five *T. cruzi*-infected mice pooled/experiment. VCAM-1 labeling was analyzed also at 15 dpi in BALB/c (B) or *gld/gld* (C) cardiac slices and compared with negative controls (D) and (E); magnifications, ×400.

Figure 6. Partial phenotypic characterization of $CD3^+/CD8^+$ cardiac cells. BALB/c (left) and *gld/gld* (right) mice were euthanized at 15 dpi with *T. cruzi* and labeled with indicated mAbs. Cells were gated in the morphological region enriched in lymphocytes (FSC×SSC) and restricted to $CD3^+$ T cells. Results are representative of three independent experiments with five *T. cruzi*-infected mice pooled/experiment.

mostly affected by the lack of Fas/Fas-L engagement. These $CD3^+/CD4^-/CD8^-$ cells from infected *gld/gld* mice showed down-regulation in the expression of all those molecules when compared with BALB/c mice (Figure 7). At 15 dpi we observed 30 to 40% of $CD3^+/CD4^-/CD8^-$ T cells in the cardiac tissue by fluorescence-activated cell sorting analysis, but at 17 dpi, they were less than 15% (data not shown). We are now investigating by adoptive transfer the possible role played by this population in the regulation of early acute phase-induced by *T. cruzi* infection.

Discussion

A number of inflammatory diseases such as idiopathic pulmonary fibrosis,¹⁹ silicosis,³⁵ acute lung injury,³⁶ coxsackievirus infection,¹⁸ control of *Leishmania major* replication,³⁷ and other infections indicate the importance of Fas/Fas-L in the regulation of inflammatory responses.

Figure 7. Partial phenotypic characterization of $CD3^+/CD4^-/CD8^-$ cardiac cells. Cardiac cells were collected from *T. cruzi*-infected BALB/c (right) and *gld/gld* (left) mice at 15 dpi. Analysis was performed after four-color labeling with mAbs anti-CD3, CD4, CD8, and x-axis-indicated molecules. Histogrammed $CD3^+$ cells are shown regarding the expression of CD4 and CD8 molecule (A and B), and analysis of C–J is restricted to region R1 indicated ($CD3^+/CD4^-/CD8^-$ cells). Results are representative of three independent experiments with five *T. cruzi*-infected mice pooled/experiment.

This role is mediated by Fas-triggered intracellular signal transduction pathways, which are not necessarily based on apoptotic events but mainly on cytokine and chemokine secretion.

In accordance with previous data on other infectious diseases, our results showed that Fas/Fas-L interaction is important for controlling cardiac inflammatory response in *T. cruzi* infection, because there was a very modest myocarditis induced by the infection in *gld/gld* mice. Regardless of this, Fas-L-deficient mice were susceptible to the infection, and our recent results suggest renal collapse as a primary cause of infected *gld/gld* death (G.M. Oliveira, O.M. Masako, N.N. Rocha, W.S. Batista, R.L. Diniz, R.S. Santana, M.M. Batista, T.C. Araújo-Jorge, A. Henriques-Pons, manuscript in preparation).

The reduced cardiac cellular inflammatory reaction observed in *gld/gld* may be based in at least three major mechanisms: 1) reduced endothelial cell activation, 2) production of IL-10 by the few cardiac inflammatory T cells, and 3) reduction of MAC-1⁺ cells in the tissue. Mast cells and other MAC-1⁺ myeloid cells, which could be either macrophages, neutrophils, or dendritic cells, are inflammatory cells of central importance in secretion of cytokines, chemokines, and inflammatory mediators that are necessary for endothelial cell activation, recruitment/

activation of inflammatory cells, and destruction of intracellular parasites. We do not know why these cells are reduced in the cardiac tissue of infected *gld/gld* mice. It could be possible that Fas/Fas-L interaction is necessary for sustained expression of MAC-1 or even mast cell and other myeloid cell survival during infection. The increase in IL-10 observed in inflamed hearts, and also in the spleen,²⁸ of *gld/gld* mice may be one of the major causes of reduced cellular cardiac inflammation, down-regulating proinflammatory cells, as well as inhibiting endothelial cell activation and recruitment of inflammatory blood cells in *gld/gld*.

We observed in both BALB/c and *gld/gld* mice a CD3⁺/CD4⁻/CD8⁻ double negative (DN) T-cell population representing 30 to 40% of the CD3⁺ T cells harvested from cardiac tissue. Apparently, this population is present in the heart only in the early acute phase, because at 17 dpi they were barely detected. This could explain why they had not yet been observed, because other groups that isolate cardiac T cells from *T. cruzi*-infected mice do it at later time points. We are now investigating the immunological relevance of these cells, in regards to secretion of cytokines and adoptive transfer. We believe that Fas/Fas-L interaction is important for a given effector and/or regulatory function, because DN cells from infected *gld/gld* down-regulate some important co-receptors and adhesion molecules for regular T cells.

It has long been known that *gld/gld* mice, and *lpr/lpr* mice, have B220⁺/CD3⁺ CD4 and CD8 DN cells.³⁸ We observed this population in spleens of *gld/gld* but not in the heart, because less than 10% were B220⁺ cardiac cells. It may be possible that these cardiac DN cells were previously the B220⁺/CD3⁺ population that down-regulated the B220⁺ marker. However, not only infected BALB/c mice but also Swiss Webster, C3H, and C57Bl/6 have the DN population at 15 dpi (R.L.D. and A.H.-P., manuscript in preparation). These data suggest that DN cells compose a particular population of T cells, not expressing NK cell markers, possibly with no previously described functions in myocarditis induced by *T. cruzi* infection.

CD3⁺/CD8⁺ and CD3⁺/CD4⁺ cardiac T cells collected from BALB/c and *gld/gld* mice showed no relevant alterations in the expression of ICAM-1, LFA-1, CD2, and CD69, although with clearly less CD8⁺ T cells in *gld/gld* mice. It seems that, in both groups of mice, these cells are compatible with the phenotype of effector/activated T cells. In addition, the enrichment of CD4⁺/IL-4^{low} T cells in *gld/gld* mice observed in most experiments could be caused by the lack of normally observed activation-induced cell death of CD4⁺ cells by Fas-L, as observed in spleens.²⁸ This activation-induced cell death deficiency, altering that subpopulation of cardiac T cells, could be central to controlling pathogenic cells, as observed in coxsackievirus B3 infection. In this model of myocarditis, *gld/gld* mice also have modest cardiac inflammatory responses based on selective destruction of T cells. Wild-type mice have a predominant Th1-driven immune response with high levels of IFN- γ , whereas Fas-L- or Fas-deficient mice have a dominant CD4⁺/IL-4⁺ (Th2) T-cell population. Adoptive transfer of $\gamma\delta$ T cells bearing

Fas-L to *gld/gld* mice and cytotoxic assays have indicated that cardiac subpopulation V γ 4⁺ cells selectively kill virus-specific Th2 CD4⁺ T cells in a Fas-based pathway, enriching the inflamed heart in pathogenic Th1 cells.¹⁷

The delicate balance between a protective inflammatory response, controlling parasite burden and infection, and a harmful infiltration causing secondary damage to the tissue is certainly based on a number of cellular populations, soluble inflammatory mediators, and membrane receptors. Taken together, our results point to a relevant role of Fas/Fas-L interaction in the control of myocarditis induced by *T. cruzi* infection and add more evidence of molecular triggering pathways that are important in the control of this important pathology.

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