Cytotoxic CD4⁺ T cells driven by T-cell intrinsic IL-18R/MyD88 signaling predominantly infiltrate *Trypanosoma cruzi*-infected hearts

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Abstract:

Increasing attention has been directed to cytotoxic CD4⁺ T cells (CD4CTLs) in different pathologies, both in humans and mice. The impact of CD4CTLs in immunity and the mechanisms controlling their generation, however, remain poorly understood. Here, for the first time, we showed that CD4CTLs abundantly differentiate during mouse infection with an intracellular parasite. CD4CTLs appear in the spleen in parallel to Th1 cells, display pathogen-derived peptide-specific cytotoxicity against antigen-presenting cells and express immunoregulatory and/or exhaustion markers. We demonstrated that CD4CTL absolute numbers and activity are severely reduced in both *Myd88*^{-/-} and *Il18ra*^{-/-} mice. Of note, the infection of mixed-bone marrow chimeras revealed that WT, but not *Myd88*^{-/-}, cells transcribe the CD4CTL gene signature and that *Il18ra*^{-/-}CD4⁺ phenocopy *Myd88*^{-/-}CD4⁺ T cells. Moreover, the adoptive transfer of WT CD4⁺GzB⁺ T cells to susceptible *Il18ra*^{-/-} mice increased their survival. Importantly, cells expressing the CD4CTL phenotype predominate among CD4⁺ T cells infiltrating the infected cardiac tissue, are increased in the circulation of Chagas patients and their frequency correlates with severe cardiomyopathy. Our findings describe CD4CTLs as a major player in immune response to a relevant human pathogen and disclose T-cell intrinsic IL-18R/MyD88 signaling as a key pathway controlling the magnitude of the CD4CTL response.

Introduction:

CD4CTLs are present in viral infections, cancer and autoimmunity (Cheroutre & Husain, 2013; Juno et al., 2017; Takeuchi & Saito, 2017). Although more frequently found in chronic conditions, CD4CTLs have also been described during acute viral infection (Brien et al., 2008; Brown et al., 2012; Fang et al., 2012; Jellison, et al., 2005; Wilkinson et al., 2012). At present, however, there are few studies on the CD4CTL response to infection with non-viral intracellular pathogens (Burel et al., 2016; Canaday et al., 2001; Kotov et al., 2018; Krueger et al., 2021). The role of CD4CTLs in immunity against intracellular parasites and the mechanisms operating in the generation and maintenance of this cell subset are poorly understood.

Following the recognition of the cognate antigen-MHC complex, and under the influence of cytokines secreted by antigen presenting cells (APCs), naïve CD4⁺ T cells proliferate and differentiate into diverse subsets, such as Tfh, Th1, Th2 or Th17, with varied effector functions. Different pathogen-derived molecular patterns (PAMPs) elicit distinct innate immune pathways, resulting in tailored Th responses against the infectious agent (Kara et al., 2014). Interestingly, increasing heterogeneity in the transcriptional profile of effector CD4⁺ T cells, dictated in a microbe-specific manner, has been recently found in the gut (Kiner et al., 2021). Additionally, tissue-derived factors can also be involved in defining CD4⁺ T cell phenotypes (Poholek, 2021). In this context, the mechanism regulating the differentiation of CD4CTLs remains an open and relevant question. Although some authors consider CD4CTLs functional variants of Th1 cells, there is also evidence that CD4CTLs might constitute a Th1-independent subset, directly differentiating from naïve CD4⁺ T cell precursors, or even a heterogeneous population congregating cytolytic cells that originate from different T-

cell subsets, such as Treg and Th2 cells [reviewed in (Cheroutre & Husain, 2013) and (Takeuchi & Saito, 2017)].

Receptors members of the TLR and IL-1R families are upstream of the MyD88 adaptor molecule and are expressed both in myeloid and lymphoid cell lineages. MyD88-dependent signaling in innate cells can influence Th1 cell differentiation, by inducing the release of IL-12 or IFN- γ (Joffre et al., 2009; Takeda et al., 1998). The T-cell intrinsic MyD88 adaptor function was also shown to be essential for the Th1 response in different contexts. The identity of the receptor acting upstream MyD88 on T cells, however, was not always determined (Frazer et al., 2013; LaRosa et al., 2008) and remains a matter of debate. Early *in vitro* studies have shown that IL-18 synergizes with IL-12 in the induction of IFN- γ production and T-bet expression by CD4⁺ T cells and, therefore, impacts on Th1 development (Robinson et al., 1997). Intriguingly, however, the IL-18 contribution for a robust Th1 response *in vivo* has not always been confirmed (Haring & Harty, 2009; Monteforte et al., 2000). ST2 (IL-33 receptor) and IL-1R have also been implicated in the establishment of Th1 responses to viral infection (Baumann et al., 2015) and immunization (Ben-Sasson et al., 2009; Schenten et al., 2014), respectively. Recently, our group has disclosed the crucial role of T cell-intrinsic IL-18R/MyD88 signaling for the reinforcement and maintenance of the Th1 program *in vivo*, in response to mouse infection with *Trypanosoma cruzi*, where Th1 cells have a key protective function (Oliveira et al., 2017; Rodrigues et al., 2012).

T. cruzi is an intracellular protozoan parasite and the etiologic agent of Chagas disease (*American trypanosomiasis*), one of the most important parasitic diseases in the Western Hemisphere. Chagas disease is an important cause of heart failure, stroke, arrhythmia, and sudden death, being the leading cause of cardiomyopathy among younger individuals in Latin America (Bern, 2015; Nunes et al., 2018). Here, we

showed that CD4⁺ T lymphocytes with CD4CTL phenotype are abundantly present in the spleen of mice infected with *T. cruzi*, displaying *in vitro* and *in vivo* Ag-specific cytotoxicity against APCs. Of note, we found that cells with CD4CTL phenotype predominate among CD4⁺ T lymphocytes infiltrating the infected cardiac tissue. Using mixed bone marrow (mix-BM) chimeras, we showed that T cell-intrinsic IL-18R/MyD88 signaling plays a crucial role on the expansion/survival of CD4⁺ T cells expressing the CD4CTL gene signature *in vivo*. In accordance with results obtained in mix-BM chimeras, we found that absolute numbers of CD4⁺ T cells displaying cytotoxic markers and activity were severely reduced in *Myd88*^{-/-} and *Il18ra*^{-/-} mouse lineages. Moreover, the adoptive transfer of WT CD4⁺GzB⁺ T cells to *Il18ra*^{-/-} mice increased the survival of this susceptible mouse strain to infection. Interestingly, the majority of CD4⁺GzB⁺ T cells in WT infected mice express high levels of immunoregulatory molecules such as Tim-3, Lag-3 and CD39. Finally, and importantly, we detected the presence of CD4⁺ T cells expressing perforin in the circulation of patients with the severe cardiac form of Chagas disease and correlation analyses point to a possible pathogenic role of these cells in the chronic phase of Chagas myocarditis. To our knowledge, the present study is the first to extensively characterize CD4CTLs generated in response to a protozoan pathogen and to elucidate a central mechanism that accounts for their expansion/survival. It is expected that pharmacological targeting of the IL-18R/MyD88 signaling pathway might contribute to the strategic development of new therapeutic and vaccine approaches aiming the control of the CD4CTL response.

Results:

CD4⁺ T cells with cytolytic phenotype are abundantly present in the spleen of mice infected with *T. cruzi*

CD4CTLs express granzyme B (GzB) and perforin (PRF), which are major constituents of CD8CTL and NK cell-granules that promote target cell death in a number of ways (Afonina et al., 2010). Here, we revealed the presence of a TCR $\alpha\beta^+$ CD8 α^- NK1.1 $^-$ CD4 $^+$ GzB $^+$ T cell population, which represents around 30% of CD4 $^+$ T cells in the spleen of C57BL/6 (B6) mice, in the acute phase of infection with *T. cruzi* (**Figures 1A-1B**). Equivalent percentages of CD4 $^+$ PRF $^+$ T cells were found (**Figure 1C**) and the majority of CD4 $^+$ GzB $^+$ T cells express PRF and the cytolytic marker 2B4 (CD244) (**Figures 1D-1E** and Figure 1-figure supplement 1A). Of note, most CD4 $^+$ GzB $^+$ T cells (> 80%) do not express IFN- γ at day 14 post-infection (pi), the peak time-point of the T cell response, and although CD4 $^+$ GzB $^+$ and CD4 $^+$ IFN- γ^+ cells develop similar kinetics in the spleen, the absolute number of CD4 $^+$ GzB $^+$ T cells was superior to that of CD4 $^+$ IFN- γ^+ cells, at every time point of infection (**Figures 1F-1G**). It is known that intracellular parasitism by *T. cruzi* Y strain in the spleen is at its highest point between day 12 and 15 pi, concomitant with the histologically observed massive destruction of parasitized splenic macrophages (Cordeiro et al., 1997). Therefore, there is a coincidence between parasite titers in the spleen and the frequency and absolute numbers of CD4 $^+$ GzB $^+$ T cells. We also observed an increase in the percentage of degranulating CD4 $^+$ T cells at day 14 pi, as inferred by staining with anti-CD107a (LAMP-1) mAb, comparable to the observed on CD8 $^+$ T cells (**Figure 1H**), although CD8CTLs are present at higher percentage (Figure 1-figure supplements 1B-E and 1G) and absolute number (Figure 1-figure supplements 1F and 1H). In summary, these data show the remarkable induction of CD4 $^+$ T cells with cytolytic potential during infection with *T. cruzi*, an effector subpopulation whose appearance in the spleen parallels and largely outnumbers that of classic GzB-negative IFN- γ^+ Th1 cells.

CD4⁺ T cells from infected spleen exert Ag-specific cytotoxicity

To ascertain the presence of CD4⁺ T lymphocytes with cytolytic function, we performed a redirected cytotoxicity assay, where anti-CD3 mAb-coated B cell blasts were used as target cells and co-incubated with either highly purified effector (CD44^{hi}) CD4⁺ T cells sorted from the spleen of infected mice, or naive (CD44^{lo}) CD4⁺ T cells from non-infected controls. The CTL activity was observed only when effector CD4⁺ T cells were employed (**Figure 2A**). Antigen-specific cytotoxicity using highly purified CD4⁺ T cells was also assayed *in vitro* against an I-A^{b+} macrophage cell line (IC-21), loaded or not with *T. cruzi* amastigote total protein extract. Results shown in **Figure 2B** demonstrate that CD4CTLs with cognate cytotoxic activity are present in the spleens of infected mice. The FasL molecule is expressed on CD8⁺ and CD4⁺ T cells at the immunological synapse following Ag-triggered degranulation and participates in CD4CTL-mediated lysis of target cells (Bossi & Griffiths, 1999; Brown et al., 2009; Kotov et al., 2018). As shown in **Figure 2B**, addition of anti-FasL mAb to the assay significantly inhibited the specific lysis of target cells, evidentiating the involvement of the FasL/Fas pathway in the killing process.

Then, we tested the *in vivo* killing of target splenocytes loaded with class II-restricted peptides (SA85-1.1c and d), derived from a protein of the *T. cruzi* transialidase superfamily (Millar & Kahn, 2000) (**Figure 2C**). The detailed Ag repertoire of CD4CTLs could not be further assessed because of the scarce definition of parasite-derived CD4 epitopes in the literature. To circumvent this, we infected mice with ovalbumin (OVA)-expressing Y strain trypomastigotes (Y-OVA) (Gomes-Neto et al., 2018) and compared the *in vivo* cytotoxic response against known OVA-derived CD4 and CD8 epitopes: OVA₃₂₃₋₃₃₉ and OVA₂₅₇₋₂₆₄, respectively. As shown in **Figure 2D**, the same level of specific killing was attained against targets loaded with class I-

or class II-restricted OVA peptides. Together, these experiments confirmed the presence of a CD4⁺ T cell subpopulation with Ag-specific cytolytic function against MHC class II-restricted epitopes in the spleen of mice infected with *T. cruzi*.

CD4⁺GzB⁺ T cells express transcription factors typical of CTLs

We then investigated the expression of transcription factors (TFs) typical of cytotoxic cell subsets, such as CD8⁺ T cells and Tregs. T-bet and Eomesodermin (Eomes) bind to the promoters of the *Gzmb* and *Prfl* genes (Glimcher et al., 2004; Intlekofer et al., 2005; Pearce et al., 2003) and are expressed in CD4CTLs (Curran et al., 2013; Eshima et al., 2012; Qui et al., 2011). **Figures 2E and 2F** show that the vast majority of CD4⁺GzB⁺ T cells express T-bet, but do not express FoxP3. In accordance with the cytolytic potential of CD4⁺GzB⁺ T cells in our model, 60% of the CD4⁺GzB⁺ T cells express Eomes, while only a small percentage of GzB-negative CD4⁺ T cells are Eomes⁺ (**Figure 2G**). Of note, at day 14 pi, the vast majority of both GzB⁺ and GzB-negative CD4⁺ T lymphocytes are effector/memory cells CD44^{hi}CD62L^{lo} (Figure 2-figure supplement 2).

CD4CTLs migrating to the gut gradually stop expressing ThPOK, while acquiring the expression of RUNX3 (Mucida et al., 2013; Reis et al., 2013), which is known to drive the CTL gene program in CD8⁺ CTLs (Cruz-Guilloty et al., 2009; Egawa et al., 2007). The long-form transcript encoding RUNX3 (*Runx3d*) is also expressed in Th1 cells, but not in Th2 or naïve cells (Djuretic et al., 2007). As shown in **Figure 2H**, besides *Runx3d* mRNA, we found that CD4⁺GzB⁺ T cells, sorted from the spleen of tamoxifen-treated and infected *Gzmb*CreER^{T2}/ROSA26EYFP reporter mice (Bannard et al., 2009) (Figure 2-figure supplement 3A), express ThPOK (*Zbtb7b* gene)-coding RNA comparable to naïve CD4⁺ T cells, at day 14 pi, when their number and frequency are

at the highest levels in this organ (**Figure 1G**). The absence of CD8 α -coding transcripts confirms the purity of sorted CD4⁺GzB⁺ T cells (Figure 2-figure supplement 3B). These data show that CD4⁺GzB⁺ T cells generated in response to infection with *T. cruzi*, besides expressing, cytolytic markers, also express T-bet, Eomes and RUNX3, typical TFs found in cytotoxic T cells.

CD4⁺GzB⁺ T cells express cytotoxic markers and immunoregulatory molecules

Tr1 cells are a subset of IL-10-producing CD4⁺FoxP3⁻ T cells that express GzB and PRF and exert a potent suppressive function both in humans and mice (Roncarolo et al., 2018). Since a high percentage of CD4⁺GzB⁺ T cells in our system express Eomes, which drives the development of Tr1 cells (Zhang et al., 2017), we investigated whether *T. cruzi*-induced CD4⁺GzB⁺ T cells would share other phenotypic characteristics with Tr1 cells. We detected a very low frequency of IL-10-producing TCR $\alpha\beta$ ⁺CD4⁺ T cells in the spleens of mice infected with *T. cruzi*, in agreement with previous data (Jankovic et al., 2007), although IL-10⁺ cells were found to be more frequent among CD4⁺GzB⁺ T cells (**Figures 3A** and Figure 3-figure supplement 1A-B). The co-expression of Lag-3 and CD49b was described to define both human and mouse Tr1 cells (Gagliani et al., 2013). Although most splenic CD4⁺GzB⁺ T cells express Lag3, only a minor percentage of these cells co-express CD49b (**Figures 3B-3C** and Figure 3-figure supplement 1C). On the other hand, a substantial fraction of splenic CD4⁺GzB⁺ T cells express the Tim-3 immunoregulatory molecule and downregulate CD27 (**Figures 3D-3E**), indicating that these might be terminally differentiated T cells, as suggested in other models (Anderson et al., 2016; Takeuchi & Saito, 2017).

Natural killer group 2 (NKG2) members A, C, D and E are C-type lectin-like receptors and cytotoxic markers expressed by NK, NKT, $\gamma\delta$ and activated CD8⁺ T

cells (Gunturi et al., 2004; Stojanovic et al., 2018). Recently, NKG2C/E and NKG2D molecules were shown to identify a CD4CTL population, present in mouse lungs during influenza A virus (IAV) infection (Marshall et al., 2017). As shown in **Figures 3F-3G**, the fraction of cells expressing NKG2A/C/E and NKG2D was increased among splenic CD4⁺GzB⁺ T cells, when compared either to GzB-negative CD4⁺ T cells of infected mice, or with naive CD4⁺ T cells of non-infected controls (Figure 3-figure supplement 2A-B). The class I-restricted T cell associated molecule (CRTAM) was described as being critical to instruct the differentiation of CD4CTLs (Takeuchi et al., 2016; Takeuchi & Saito, 2017). **Figure 3H** shows that the frequency of CRTAM-expressing cells was also increased among splenic CD4⁺GzB⁺ T cells of *T. cruzi*-infected mice, when compared with GzB-negative CD4⁺ T cells. The frequencies of CRTAM⁺, NKG2A/C/E⁺ and NKG2D⁺ CD8⁺GzB⁺ T cells in infected mice are shown in Figure 3-figure supplement 3. Since at day 14 pi, both GzB⁻ and GzB⁺ CD4⁺ T cells are in their majority effector/effector memory cells (Figure 2-figure supplement 2), equivalent results were obtained gating on CD44^{hi} cells or not, as shown in Figure 3-figure supplement 4. Together, these results show that a significant fraction of the CD4⁺GzB⁺ T cell subpopulation found in this unicellular parasite infection model complies with the general phenotype of CD4CTLs and CD4CTL-precursors reported in response to viral infection.

CD4CTLs are present in lower numbers in Myd88^{-/-} infected mice

To extend our understanding on the requirements for the differentiation of CD4CTLs and their relationship to Th1 cells, we next investigated the role of IL-12, IFN- γ and MyD88 in their generation. IL-12 and IFN- γ are the major cytokines driving Th1 differentiation and T-bet expression, but their requirement for CD4CTL

generation is controversial (Brown et al., 2009; Cooper et al., 2004; Curran et al., 2013; Hua et al., 2013; Kotov et al., 2018; Krueger et al., 2021; Tagawa et al., 2016; Xie et al., 2010). Here, we observed that *Il12p40*^{-/-} mice infected with *T. cruzi* displayed lower percentages of T-bet⁺CD4⁺ and IFN-γ⁺CD4⁺ T cells and exhibited very low levels of CD4⁺GzB⁺ T cells (**Figure 4A**). Interestingly, in infected *Ifng*^{-/-} mice, despite the lower frequency of T-bet⁺CD4⁺ T cells, CD4⁺GzB⁺ T cells attained the same frequencies as observed in the spleens of WT animals (**Figure 4B**). However, absolute numbers of both total CD4⁺ and CD4⁺GzB⁺ T cells were lower in *Ifng*^{-/-} mice when compared to WT controls. Thus, both IL-12p40 and IFN-γ play important but distinct roles in the differentiation and expansion/survival of CD4⁺GzB⁺ T cells.

The above results obtained in *Il12p40*^{-/-} and *Ifng*^{-/-} mice, together with the fact that *Myd88*^{-/-} mice have lower levels of Th1 cells and are highly susceptible to infection with *T. cruzi* (Campos et al., 2004; Oliveira et al., 2010), prompted us to address the role of MyD88-dependent pathways in the generation of CD4CTLs. Although infected *Myd88*^{-/-} mice displayed equivalent absolute numbers and percentages of total splenic CD4⁺ T cells when compared to WT mice (Figure 4-figure supplement 1A), the frequencies and absolute numbers of CD4⁺GzB⁺ T cells were significantly lower in this mouse strain (**Figures 4C-4E**). Furthermore, absolute numbers of CRTAM⁺, NKG2D⁺ and NKG2A/C/E⁺ CD4⁺GzB⁺ T cells were severely diminished in *Myd88*^{-/-} mice (**Figures 4C-4E**). This picture does not result from lower numbers of total activated CD4⁺ T cells in infected *Myd88*^{-/-} mice, as shown by the percentages and absolute numbers of CD44^{hi} cells, which are equivalent to WT animals (Figure 4-figure supplement 2). On the other hand, absolute numbers of CRTAM⁺, NKG2D⁺ and NKG2A/C/E⁺ CD8⁺GzB⁺ T cells were not affected in *Myd88*^{-/-} mice (Figure 4-figure supplement 1C-D). Concerning the expression of perforin, both the percentages and absolute numbers of PRF⁺CD4⁺ T

cells were also severely decreased in *Myd88*^{-/-} compared to WT mice (**Figure 4F**). The same was observed when analyzing the frequency and absolute numbers of PRF⁺CD4⁺ T cells among GzB-expressing cells (**Figure 4G**). In order to establish to what extent the absence of MyD88 expression would impact on cytotoxicity mediated by CD4⁺ T splenocytes, we sorted activated CD4⁺CD44^{hi} T cells from both WT and *Myd88*^{-/-} infected mice to a high purity level and performed redirected cytotoxicity assay. Lower levels of cytotoxicity were observed when sorted *Myd88*^{-/-} CD4⁺CD44^{hi} T cells were employed, as compared to assays using equal numbers of sorted WT CD4⁺CD44^{hi} T cells (**Figure 4H**). Together, these results demonstrate the important role of MyD88 signaling for the establishment of a robust cytotoxic response mediated by CD4CTLs.

T cell-intrinsic IL-18R/MyD88 signaling plays a crucial role in the establishment of a CD4⁺ T cell subpopulation bearing the CD4CTL gene signature

MyD88 deficiency results in lower IL-12 production by APCs (Campos et al., 2004) and this could explain the diminished numbers of CD4CTLs found in infected *Myd88*^{-/-} mice. However, we have recently shown that IL-18R/MyD88 signaling intrinsic to CD4⁺ T cells is critical for Th1 cell proliferation and resistance to apoptosis in response to infection with *T. cruzi* (Oliveira et al., 2017). If CD4CTLs herein described share a common early differentiation process with (or derive from) Th1 cells, it is reasonable to expect that besides IL-12 and IFN- γ , T-cell-intrinsic MyD88-dependent signaling would also be important for the generation of a robust CD4CTL response. In order to test this hypothesis, we constructed mix-BM chimeras, in which WT (B6 x B6.SJL) F1 mice were irradiated and reconstituted with a 1:1 mix of WT and *Myd88*^{-/-} BM cells. After reconstitution, mix-BM chimeras were infected with *T. cruzi* and, at day 14 pi, we sorted WT (CD45.1) and *Myd88*^{-/-} (CD45.2) CD44⁺CD4⁺ T cells to a high purity level

for transcriptome analysis. We have then investigated whether the transcriptional signature of CD4CTLs (Donnarumma et al., 2016) was present in sorted CD4⁺ T splenocytes and to what extent the absence of T-cell intrinsic MyD88-dependent signaling would impact on the acquirement of this transcriptional program. This signature, previously described in the experimental model of Friend virus infection, distinguishes CD4CTLs from other CD4⁺ T cell subsets, including Th1 and Tfh cells (Donnarumma et al., 2016). Importantly, gene set enrichment analysis (GSEA), comparing the transcriptome of sorted WT and *Myd88*^{-/-} CD4⁺ T cells, revealed that 51 genes of the CD4CTL signature were upregulated in WT CD4⁺ T cells, while 78 were downregulated in *Myd88*^{-/-} CD4⁺ T cells (**Figures 5A-5C**). We confirmed, by flow cytometry, the upregulation of Blimp-1 (*Prdm1* gene) TF and of the ecto-nucleotidase CD39 (*Entpd1*) in WT CD4⁺GzB⁺ T cells (**Figures 5D-5E**). Of note, Blimp-1 is required for the cytotoxic function of CD4CTLs (Hua et al., 2013) and CD39 expression level was found to be higher in CD4⁺GzB⁺ T cells than in Tregs (**Figure 5E**).

In order to identify the receptor signaling upstream MyD88 responsible for the establishment of a robust CD4CTL response, we further constructed three groups of mix-BM chimeras, by mixing 1:1 WT and (i) *Il1r1*^{-/-}, (ii) *Il18ra*^{-/-} or (iii) *Myd88*^{-/-} BM cells. A fourth group of mix-BM chimeras, 1:1 WT(B6):WT (B6.SJL) control was also generated. After infection with *T. cruzi*, the percentages and absolute numbers of GzB⁺ cells among CD4⁺ T cells were analyzed in each chimeric group (gate strategy in Figure 5-figure supplement 1). **Figure 5F** shows that the lack of IL-18R signaling in T cells, but not of IL-1R, phenocopied the absence of T cell-intrinsic MyD88 signaling, revealing that IL-18R is a critical pathway upstream MyD88 necessary for the establishment of the CD4⁺GzB⁺ T cell population.

In order to further consolidate the role of IL-18R signaling in the generation of a strong CD4CTL response, we analyzed the expression of PRF as well as of CRTAM, NKG2D and NKG2A/C/E cytotoxic markers on CD4⁺GzB⁺ T cells from the spleens of *Il18ra*^{-/-} mice. **Figure 6A** shows that both the frequency and absolute number of PRF⁺CD4⁺ T cells were significantly diminished in the spleens of infected *Il18ra*^{-/-} compared to WT mice. The same was true for PRF-expressing cells among GzB⁺CD4⁺ T lymphocytes (**Figure 6B**). As observed in infected *Myd88*^{-/-} mice, the frequency of GzB⁺CD4⁺ T cells and their absolute number were also decreased in infected *Il18ra*^{-/-} compared to WT mice (**Figures 6C-6E**). Absolute numbers of CRTAM⁺, NKG2D⁺ and NKG2A/C/E⁺ GzB⁺CD4⁺ T cells were significantly diminished in *Il18ra*^{-/-} mice, as well, again phenocopying results obtained in *Myd88*^{-/-} mice (**Figures 6C-6E**). The cytotoxic potential of CD4⁺ T splenocytes from *Il18ra*^{-/-} infected animals was also evaluated by performing redirected cytotoxicity assays with equal numbers of CD4⁺CD44^{hi} T cells purified from infected *Il18ra*^{-/-} or WT mice. Again, as shown for *Myd88*^{-/-}CD4⁺ T cells, lower levels of target killing were observed when employing purified *Il18ra*^{-/-}CD4⁺CD44^{hi} T cells as effectors (**Figure 6F**). Collectively, the present data demonstrate that T-cell intrinsic IL-18R/MyD88 signaling support the generation and/or survival of CD4⁺ T cells with cytotoxic potential in response to parasite infection, revealing a parallel between the requirements for the development of robust Th1 and CD4CTL responses.

The adoptive transfer of CD4⁺GzB⁺ T cells to *Il18ra*^{-/-} mice increased survival to infection

In vivo studies addressing the function of CD4⁺GzB⁺ T cells have been hampered by the absence of a stable and abundantly expressed surface-marker of cytotoxicity,

which would allow purifying these cells alive. To circumvent this, we employed infected and tamoxifen-treated $GzmbCreER^{T2}/ROSA26EYFP$ reporter mice, from whose spleens $CD4^+EYFP^+$ (GzB^+) cells were highly purified by flow cytometry and adoptively transferred to infected $Il18ra^{-/-}$ mice, following the scheme shown in Figure 6-figure supplement 1. The $Il18ra^{-/-}$ mouse lineage was previously shown to be very susceptible to infection with *T. cruzi* (Oliveira et al., 2017), and display lower numbers of $CD4^+GzB^+$ and $CD4^+PRF^+$ T cells with cytotoxic potential, as demonstrated above. The transfer of highly purified $CD4^+GzB^+(EYFP^+)$ T cells to $Il18ra^{-/-}$ mice significantly extended their survival rate without, however, decreasing the parasite load in the hearts (**Figure 6G**). In a previous work, we have demonstrated that the transfer of total WT $CD4^+$ T cells protects infected $Il18ra^{-/-}$ mice, and this property was attributed to Th1 cells, which are also diminished in this mouse lineage (Oliveira et al., 2017). Since infected $Ifng^{-/-}$ mice have the same frequency of $CD4^+GzB^+$ T cells as WT mice in their spleens (**Figure 4B**), we tested whether the adoptive transfer of $CD4^+$ T cells sorted from $Ifng^{-/-}$ mice would also be able to provide some degree of protection to $Il18ra^{-/-}$ mice. As shown in Figure 6-figure supplement 2, indeed, this was the case. Although IFN- γ expression by $CD4^+$ T cells is necessary for decreasing parasitemia (Oliveira et al., 2017), this result indicated that the secretion of IFN- γ by adoptively transferred $CD4^+$ T cells is not absolutely required for increasing the survival time of infected $Il18ra^{-/-}$ recipients. Together, these data demonstrate the protective function of the $CD4^+GzB^+$ T cell subset against mortality caused by an intracellular parasite, which is independent of decreasing heart parasitism.

The great majority of $CD4^+$ T cells in the infected myocardium express the $CD4CTL$

phenotype and the frequency of CD4⁺PRF⁺ T cells correlates with cardiomyopathy in Chagas patients

Previous studies in viral infection models suggested that CD4CTLs are present in tissues where pathogen replication occurs (Marshall et al., 2017; Takeuchi et al., 2016). Since *T. cruzi* heavily infects the heart, we performed intravascular staining with anti-CD4 mAb and demonstrated that tissue-infiltrating CD4⁺ T cells are abundant in the myocardium of infected mice (Figure 7-figure supplement 1A-B). As shown in **Figures 7A-7B**, CD4⁺GzB⁺ T cells infiltrating the cardiac tissue constitute the vast majority of CD4⁺ T cells. At the peak time-point of parasite load in the heart, CD4⁺GzB⁺ T cells in the myocardium express the LAMP-1 degranulation marker at levels comparable to those of CD8⁺ T cells (**Figures 7C-D** and Figure 7-figure supplement 1C). Importantly, the great majority of heart-infiltrating CD4⁺GzB⁺ T cells expresses PRF, as well as the CRTAM and NKG2D cytotoxic markers (**Figure 7E-7F**). On the other hand, only few CD4⁺GzB⁺ T cells express NKG2A/C/E in the myocardium (Figure 7-figure supplement 1D, left), although high percentages of NKG2A/C/E⁺CD8⁺GzB⁺ T cells were found both in the heart (Figure 7-figure supplement 1D, right) and in the spleen (Figure 3-figure supplement 3A) of infected mice.

Finally, we investigated the presence of PRF⁺CD4⁺ T cells in the circulation of patients infected with *T. cruzi* and suffering from chronic Chagas cardiomyopathy (CC). In order to ascertain the specificity of CD4⁺ T cells against *T. cruzi*, PBMC from CC patients or from healthy donors (HD) were incubated (or not) with trypomastigote-derived antigens and analyzed for the expression of perforin by flow cytometry (Figure 7-figure supplement 2). As shown in **Figures 7G and 7H**, after treatment, both the expression level of PRF (MFI) and the percentage of PRF⁺CD4⁺ T cells increased

among CD4⁺ T cells from CC patients, but not from HD controls. Of note, CD4⁺ T cells expressing PRF were found at higher frequency in CC patients and their level of PRF expression was also higher than the observed in CD4⁺ T cells of HD (**Figure 7I**). We next performed correlative analyses between the frequency of PRF-expressing CD4⁺ T cells in PBMC from CC patients and two echocardiographic parameters, usually employed to evaluate cardiac function in CC patients: left ventricular ejection fraction (LVEF) and left atrium diameter. As shown in **Figure 7J**, a clear inverse correlation between the frequency of CD4⁺PRF⁺ T cells and LVEF, as well as a direct correlation between the frequency of these cells and the left atrium diameter was found. These correlations suggest that CD4CTLs may play a pathogenic role in the chronic phase of Chagas disease. Together, data obtained here in CC patients and in the mouse experimental model point to the heart as an important target-organ for CD4⁺ T cells with cytolytic potential generated in response to infection with *T. cruzi*.

Discussion:

Here we show, for the first time, that CD4CTLs are abundantly present in the spleen of mice infected with *T. cruzi* and that cells with CD4CTL phenotype predominate among CD4⁺ T cells infiltrating the infected cardiac tissue. The expression of LAMP-1 degranulation marker by CD4⁺GzB⁺ T cells in the heart and in the spleen indicates that these cells may play important effector functions in both organs. These data suggest that CD4⁺GzB⁺ T cells with cytotoxic potential are attracted from the spleen to the cardiac tissue and preferentially accumulate there. CD4⁺GzB⁺ T cells present in the *T. cruzi*-infected hearts express PRF, CRTAM and NKG2D cytotoxicity markers, but differently from CD4CTLs found in the lungs of mice infected with IAV (Marshall et al., 2017), do not express NKG2A/C/E molecules. This interesting

dissimilarity requires further investigation and could be due to differential regulation exerted by tissue-specific factors or, alternatively, by different innate responses influencing CD4CTL differentiation, which would be distinctly triggered by viral and parasite pathogens.

CD4⁺ T cells purified from the spleen of infected mice killed APCs presenting parasite-derived antigens *in vitro*. *In vivo* Ag-specific cytotoxicity against I-A^b-restricted epitopes was also observed. Killing infected APCs in the spleen would lead to a decrease in the activation of effector T cells, dampening the exuberant immune response against the parasite. Therefore, the action of CD4CTLs may contribute to immunoregulation, avoiding the development of exacerbated immunopathology, which can cause mortality in the acute phase of the infection with *T. cruzi*. Favoring this hypothesis, we showed that the adoptive transfer of CD4⁺GzB⁺ T cells to susceptible *Il18ra*^{-/-} mice, which have lower levels of these cells, significantly increased their survival to infection, without affecting parasite loads. Together with our previous work (Oliveira et al., 2017), the current results point to possible complementary roles of CD4CTL and Th1 cells. Both cell types might be required for survival against infection with *T. cruzi*, working together to establish a balance between the control of parasite load on one side, and dampening of the immunopathology resulting from this process, on the other. This equilibrium is associated with the establishment of the asymptomatic form of chronic infection (Nunes et al., 2018), in which increased levels of CD4⁺GzB⁺ T cells have been found in humans (Keesen et al., 2012). In accordance with a putative regulatory role played by CD4⁺GzB⁺ T cells, we showed here that these cells express surface co-inhibitory receptors at particularly high levels, such as Lag-3 and Tim-3, as well as the ecto-ATPase CD39. These immunoregulatory molecules, typically

expressed by Treg and Tr1 cells, have been implicated in suppressive function (Anderson et al., 2016; Mascanfroni et al., 2015; Park et al., 2019).

Chronic Chagas cardiomyopathy usually develops in around 30% of infected individuals after decades, probably reflecting an imbalance between parasite and host immune response (Nunes et al., 2018). Although the exact mechanism remains to be elucidated, the association of NK, CD8CTL and GzA-expressing CD4⁺ T cells with the development of chronic carditis has been indicated (Ferreira et al., 2017; Menezes et al., 2012; Nunes et al., 2018). Since CD4CTLs recognize the cognate antigen in the context of MHC class II molecules, the lysis of infected MHC II⁺ endothelial cells might trigger immunopathology (Reis et al., 1993). In the present study we found that the frequency of parasite-specific CD4⁺PRF⁺ T cells is increased in the circulation of chronic cardiac patients with Chagas disease (CC) and that PRF expression in CD4⁺ T cells is higher in the CC group when compared to healthy donors. A correlation between the frequency of PRF⁺CD4⁺ T cells and two echocardiographic parameters employed for evaluating myocarditis in CC patients was also encountered. It is tempting to speculate that the loss of regulatory function exerted by different CD4⁺ T cell subsets, including CD4CTLs, might lead to the chronic pathology. Therefore, further studies are necessary for fully defining the role of tissue infiltrating CD4CTLs in Chagas disease. Interestingly, NKG2D, expressed by most CD4CTLs in the heart, can mediate both activating and inhibitory signals (Wensveen et al., 2018). Importantly, a polymorphism in one of the NKG2D ligands, the major histocompatibility complex class I-related chain A gene (MICA) molecule, was associated with left ventricular systolic dysfunction in patients with chronic Chagas myocarditis (Ayo et al., 2015).

We have shown here that CD4⁺GzB⁺ T cells originating in response to *T. cruzi* infection largely outnumber IFN- γ -producing CD4⁺ T cells and that their appearance

in the spleen follows the same kinetics of classic Th1 cells, indicating thereby that differentiation of CD4CTLs is not a late event during infection. Of note, more than 60% of CD4⁺GzB⁺ T cells have downregulated CD27 expression in the spleen at day 14 pi, a characteristic of terminally differentiated T cells (Takeuchi & Saito, 2017). We have also shown here that the majority of CD4⁺GzB⁺ T cells express T-bet, Eomes and Blimp-1. Although Eomes seems not to be essential for CD4CTLs in certain studies (Donnarumma et al., 2016; Kotov et al., 2018), it was proposed to be the master regulator for CD4CTLs and Tr1 cells by others (Curran et al., 2013; Eshima et al., 2012; Qui et al., 2011; Zhang et al., 2017). The CD4⁺GzB⁺ T cell population described herein presents some characteristics of Tr1 cells. However, we only detected a very low frequency of CD4⁺GzB⁺ T cells co-expressing Lag-3 and CD49b (Gagliani et al., 2013) or IL-10. In this regard, data from *T. cruzi* infection differs from those observed during infection with other intracellular parasites such as *T. gondii* and certain *L. major* strains, in which high frequencies of CD4⁺ T cells producing both IL-10 and IFN- γ were described (Anderson et al., 2007; Jankovic et al., 2007). In contrast with ThPOK-negative CD4CTLs that accumulate in the gut (Cheroutre & Husain, 2013; Mucida et al., 2013; Reis et al., 2013), CD4⁺GzB⁺ T cells in the *T. cruzi*-infected spleen express ThPOK at the peak of infection. Interestingly, the absence of ThPOK downregulation was also observed in mouse (Mittal et al., 2018) and human CD4CTLs (Serroukh et al., 2018). Thus, different subsets of CD4CTL possibly exist, in which the interplay between ThPOK and RUNX3 expression might be differentially regulated.

Although many authors consider CD4CTLs merely functional variants of Th1 cells, in some models CD4CTLs fail to fit Th1-defining criteria, such as the dependence on IL-12, STAT4 or T-bet for differentiation (Brown et al., 2009; Curran et al., 2013; Hua et al., 2013; Kotov et al., 2018). Nonetheless, in agreement with other studies (Cooper

et al., 2004; Krueger et al., 2021; Tagawa et al., 2016; Xie et al., 2010), we showed here that CD4⁺GzB⁺ T cells are scarcely generated in the absence of IL-12p40. Importantly, we demonstrated that the expansion/survival of CD4CTLs also depends on T-cell intrinsic IL-18R/MyD88 signaling, which we have previously shown to induce cell cycling, anti-apoptotic and Th1-signature transcripts (Oliveira et al., 2017). Notably, several genes of the CD4CTL signature, which distinguishes CD4CTLs from Th1 and Tfh cells (Donnarumma et al., 2016), were expressed by WT, but not by *Myd88*^{-/-} CD4⁺ T cells from infected mix-BM chimeras. Therefore, analogous to its action on Th1 cells, we showed here that T-cell intrinsic IL-18R/MyD88 signaling contributes for the reinforcement and/or stabilization of CD4⁺ T cell commitment into the CD4CTL phenotype. IFN-γ is another key cytokine involved in Th1 differentiation and *Ifng*^{-/-} mice are extremely susceptible to infection with *T. cruzi* (Campos et al., 2004). Interestingly, we found no difference in the frequency of CD4⁺GzB⁺ T cells between WT and *Ifng*^{-/-} infected mice, in accordance with a study showing that IFN-γ had no impact on the ability of CD4 effectors to acquire cytolytic activity (Brown et al., 2012). However, both GzB⁺ and total CD4⁺ T-cell numbers were significantly lower in the infected *Ifng*^{-/-} animals, indicating that IFN-γ might also play a role in the expansion/survival of both Th1 and CD4CTLs in our system. It is known that IFN-γ and IL-18R participate in a reciprocal positive feedback loop in Th1 differentiation process: while IFN-γ is required for IL-12-driven upregulation of IL-18R expression (Nakahira et al., 2001; Smeltz et al., 2001), IL-18 is a major IFN-γ-inducing factor. Therefore, the absence of IL-18R upregulation in CD4⁺ T cells of *Ifng*^{-/-} mice would prevent the expansion of both CD4⁺ T cell subpopulations. Thus, in *T. cruzi* infection model, IL-12, IFN-γ and IL-18R/MyD88 signaling act as key pathways at early check-points in the

generation of CD4⁺GzB⁺ T cells with cytotoxic potential, suggesting that these cells might share common early differentiating steps with the Th1 subset. It is not excluded, however, that different precursors of CD4CTL and Th1 cells might exist (Takeuchi & Saito, 2017). A cartoon summarizing our results and cited works published by others is shown in Figure 7-figure supplement 3. Of note, in humans, the higher production of IL-18 due to gene polymorphisms has been correlated to protection against chronic Chagas cardiomyopathy (Leon Rodriguez et al., 2016; Nogueira et al., 2015).

It is possible that differentiation into CD4CTLs could also be influenced by pathogen-derived factors. Very recently, Krueger *et al.* have shown that a subset of IL-12-dependent Th1-derived cytotoxic cells was present in the spleen of *Il18*^{-/-} mice at later stages of infection with *Salmonella enterica* (Krueger et al., 2021). Whether that result was due to compensatory mechanisms, such as the known exacerbated IL-12 production by infected *Il18*^{-/-} mice (Monteforte et al., 2000), or to distinct innate responses, differentially induced by phagosomal bacteria and cytoplasmatic parasites, remains to be determined. Of note, other discrepancies between results obtained with *Il18*^{-/-} versus *Il18ra*^{-/-} mouse lineages have been described in different models in the past (Gutcher et al., 2006; Lewis & Dinarello, 2006). It is known that different classes of pathogens can trigger diverse APC responses and, therefore, elicit pathogen-tailored Th programs (Kara et al., 2014; Kiner et al., 2021). Previous works have reported the immunoregulatory action of *T. cruzi*-derived PAMPs on CD4⁺ T cell activation, either by directly acting as co-stimulatory signals for T cells, or by modulating APC functions (Bellio et al., 1999; DosReis et al., 2002; Medeiros et al., 2007). It would be interesting to test *T. cruzi*-derived molecules on their capacity to favor the generation of CD4CTLs.

In summary, the present work is the first to described a CD4CTL subset abundantly present in the *T. cruzi*-infected cardiac tissue, bringing novel pieces to the scenario of

the immune response against a major human intracellular parasite. Importantly, our data disclose a mechanistic framework involved in the generation of a robust CD4CTL response, whose control and optimization may provide new therapeutic and vaccination strategies to combat infection with intracellular pathogens and chronic diseases resulting thereof.

Materials and Methods

Mice. C57BL/6, B6.SJL and F1 (B6 x B6.SJL) mice were from the Universidade Federal Fluminense. *Myd88*^{-/-} mice were generated by Dr. S. Akira (Osaka University, Japan); *Il12p40*^{-/-} and *Ifng*^{-/-} mice were purchased from USP; *Il18ra*^{-/-} and *Il1r1*^{-/-} mice from JAX Mice, USA. GzB reporter mice, *GzmbCreER*^{T2}/ROSA26EYFP, previously described (Bannard et al., 2009) were obtained from Dr. Ricardo Gazinelli (UFMG, BH, Brazil). Mice were on the C57BL/6 background, except *GzmbCreER*^{T2}/ROSA26EYFP, which were on mixed Sv129/B6 background and backcrossed to B6 for 5 generations. In each experiment, only male mice of the same age (6- to 8-week-old) were used. Mice were housed at a maximum of 5 animals per cage, in forced-air-ventilated micro-isolation rack system, in a room at 21–23°C under a 12/12 h light/dark cycle and provided sterilized water and chow ad libitum. All mouse experiments were conducted in accordance with guidelines of the Animal Care and Use Committee of the Federal University of Rio de Janeiro (Comitê de Ética do Centro de Ciências da Saúde CEUA - CCS/UFRJ, license: IMPPG022).

Experimental infection. Bloodstream trypomastigotes of the Y strain of *T. cruzi* (2 x 10³ cells/0.2 mL) were inoculated intraperitoneally (i.p.). Y-OVA strain trypomastigotes (Gomes-Neto et al., 2018) were obtained from LLC-MK2 infected cultures and 2 x 10⁶ trypomastigotes (in 0.2 mL PBS) were inoculated i.p. Rare outliers (mice in which

the infection was not properly established) were identified by the lower size of the spleen (total cell number) and lower percentage of GzB⁺CD8⁺ T cells (inferior of 50%) in the spleen. These outlier mice were excluded from all analyses.

Generation of BM chimeras. Acidic drinking water (pH 2.5-3.0) was given for 8 days to recipient mice prior to irradiation with a lethal single dose of 800 rad (TH780C - Theratronics, Canada). The day after irradiation, BM cells were transferred and mice were treated with neomycin sulfate (2 mg/mL) in the drinking water for 15 days. Marrow was harvested from the femur and tibia of B6.SJL (CD45.1⁺) and B6, *Myd88*^{-/-}, *Il1r1*^{-/-} or *Il18ra*^{-/-} (CD45.2⁺) mice. For mixed BM chimeras, CD45.1⁺ and CD45.2⁺ BM cells were transferred in a 1:1 ratio (2 x 10⁶ cells each) to irradiated mice. In all experiments, mice were infected 6 to 8 weeks after cell transfer, when reconstitution was achieved.

Human population. A total of thirteen volunteers were enrolled in this study. Nine volunteer patients had positive specific serology for *T. cruzi* and displayed the chronic Chagas cardiomyopathy (CC) with right and/or left ventricular dilation, global left ventricular dysfunction, alterations in the cardiac electric impulse generation and conduction upon electrocardiogram, chest x-rays and echocardiography; four individuals displayed negative specific serological tests for Chagas disease and were included as control group (healthy donors, HD). Patients were from Chagas disease endemic regions within Minas Gerais, Brazil, and were evaluated at the outpatient clinic of the Universidade Federal de Minas Gerais. Healthy donors were from Belo Horizonte, which is a non-endemic region for Chagas disease. We excluded from our study individuals with any other chronic inflammatory diseases, diabetes, heart/circulatory illnesses or bacterial infections. All individuals included in this work were volunteers and treatment and clinical care were offered to all patients, as

needed, despite their enrollment in this research project. All participants provided written informed consent for participation in the study. This cross-sectional study is part of an extended project evaluating biomarkers of cardiomyopathy development in Chagas disease, which has the approval of the National Committee of Ethics in Research (CONEP#195/2007) and are in accordance with the Declaration for Helsinki.

PBMC flow cytometry. Peripheral blood samples were collected in heparin tubes. PBMCs were obtained by separating whole blood over Ficoll (Sigma-Aldrich, St. Louis, MO), as previously described (Menezes et al., 2012). Cells were washed and resuspended in RPMI 1640 medium (Gibco, Rockville, MD) supplemented with 5% heat-inactivated AB human serum, antibiotics (penicillin, 200 U/ml; and streptomycin, 0.1 mg/mL) and L-glutamine (1 mM) (Sigma-Aldrich) at a concentration of 1×10^7 cells/mL. Cells from each volunteer were cultured on 96-well plates (Costar, Corning Incorporated, Corning, NY) in 200 μ L of medium alone (ctr) or containing 20 μ g/mL of trypomastigote antigen (trypo) for 18h. Trypo was obtained from trypomastigotes purified from VERO cell cultures: parasites were washed, freeze-thaw for 3 cycles, followed by 5 cycles of 5 minutes each of sonication using 30 seconds between them. Samples were ultracentrifugated at 5,000g for 60 min, at 4°C. Supernatants were collected, dialyzed for 24h and protein quantification performed by Bradford method. Cells (2×10^5) were incubated with PerCP-anti-CD4 (clone A161A1) antibody (Biolegend) for 15 min at 4°C, washed in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), and fixed by a 20 min incubation with a 2% formaldehyde. After washing with PBS, cells were permeabilized with 0.5% saponin for 15 min and intracellularly stained with PE-anti-perforin (clone B-D48) antibody (Biolegend) for 20 minutes, washed twice with 0.5% saponin solution, resuspended in

PBS and analyzed. A minimum of 100,000 gated events from each sample were collected and analyzed using FlowJo software.

Flow cytometry of mouse cells. Reagents and antibodies: PB-anti-CD45.1, APC-Cy7-anti-CD45.2, FITC- PE- or BV-605-anti-CD4, PE-anti-CD8, PE-Cy7-anti-NK1.1, BV421-anti-TCR β , PE-anti-2B4, FITC-anti-CD107a, PE-Cy7-anti-CD39, PE-anti-FasL, PE-Cy7-anti-CRTAM, PE-anti-NKG2D, PE-anti-NKG2A/C/E, FITC- or AlexaFluor647-anti-GzB, PE-anti-Eomes, PE- or A647-anti-T-bet, PE-anti-FoxP3, AlexaFluor647-anti-Blimp1 and PE-anti-PRF were used. All mAbs were purchased from Biolegend, BD Biosciences or eBioscience/Thermo Fisher Scientific. Spleen cells were stained after red blood cell lysis with ACK buffer. Hearts were perfused with 20 mL of cold PBS, minced into small fragments and treated with collagenase D (1.0 mg/mL) and DNase I (0.1 mg/mL), for 1 hour at 37°C. Collagenase D, DNase I, monensin and saponin were purchased from Sigma-Aldrich. Single cell suspensions were incubated with anti-CD16/CD32 (FcR block) for 5 min and then surface stained for 30 min on ice. For IL-10 and IFN- γ intracellular staining, 2×10^6 spleen cells were cultured in the presence of PMA (16 nM), ionomycin (0.5 μ M), monensin (5 μ M) and brefeldin A (3.0 μ g/mL) for 4 hours. After staining for surface markers, cells were fixed with paraformaldehyde 1% for 1 h, permeabilized with saponin 0.2% for 20 min and stained with PE-Cy7-anti-IFN- γ mAb and/or PE-anti-IL-10 mAbs (Biolegend). For TF and GzB/PRF staining, the FoxP3 staining buffer kit (eBioscience, ThermoFisher Scientific) was employed. At least 20,000 events gated on CD4⁺ T cells were acquired. Analytical flow cytometry was conducted with MoFlo (Beckman Coulter/Dako-Cytomation), FACS Canto, FACS Calibur or BD LSRFortessa (BD Bioscience) and data analyzed with Summit V4.3 software (Dako Colorado, Inc).

***In vitro* and *in vivo* cytotoxicity assays.** For redirected cytotoxic assay, sorted B cells (B220⁺), previously activated by LPS (InvivoGen) for 48 h and then incubated for 1 h with anti-CD3ε antibody (10 µg/mL), were employed as target cells. FACS-sorted naïve (CD44^{low}CD4⁺) and effector/memory (CD44^{hi}CD4⁺) T cells were purified from the spleen of non-infected and infected mice, respectively. Sorted T cells were coincubated at different E:T ratios, with 10⁴ target B cells in round-bottom microplates (Costar-Corning) for 14 h, at 37°C in a 5% CO₂ incubator and in triplicates. After incubation, living target cells were quantified by flow cytometry, after staining with propidium iodide at 0.5 µg/ml (Sigma-Aldrich). For *T. cruzi*-specific cytotoxicity assays, CD4⁺ T cells were obtained from infected B6 mice, at day 14 pi, and purified by FACS sorting (MoFlo cell sorter, Beckman Coulter/Dako-Cytomation). IC-21 macrophage cell lineage (ATCC) were used as targets *in vitro*. For this, IC-21 cells were counted and split into two tubes: half of the target cells were incubated for 45 min at 37° C in 5% CO₂, in RPMI containing *T. cruzi* amastigote protein extract (30 µg/mL) obtained as previously described (Oliveira et al., 2010). Ag-loaded cells were then stained with 2.5 µM CFSE (CFSE^{Hi}) (Invitrogen, Life Technologies), and control cells with 0.5 µM CFSE (CFSE^{Low}) in 5 mL of warm PBS for 10 min at 37° C. After two washes in RPMI 1640 (10% FCS), cells were resuspended at a concentration of 2 x 10⁵ cells/mL each and mixed at 1:1 ratio (CFSE^{Hi}:CFSE^{Low}). Purified CD4⁺ T cells were added at different E:T ratios. Cells were seeded in duplicate to round-bottom 96-well plate in a final volume of 200 µL. After ON incubation, cells were harvested and incubated with 0.5 µg/mL of propidium iodide and PI⁺ cells were excluded from FACS analysis. The percentage of specific lysis was calculated using the formula: % of specific lysis = 100x [(CFSE^{Low} / CFSE^{Hi}) ctr – (CFSE^{Low} / CFSE^{Hi}) effector] x (CFSE^{Low} / CFSE^{Hi}) ctr⁻¹. For *in vivo* cytotoxicity assays, splenocytes from non-infected B6 mice were

employed as target cells after being divided in 3 subpopulations and stained with 10.0 (High), 2.5 (Int) and 0.625 (Low) μ M of CFSE or of CTV (Invitrogen, Life Technologies). CFSE^{Int} and CFSE^{Low} cells were loaded with one of the following peptides: SA85c (SHNFTLVASVIIIEEA), SA85d (LVASVIIIEEAPSGNT), OVA₃₂₃₋₃₃₉, OVA₂₅₇₋₂₆₃ or PA8 (VNHRFTLV) at 2 μ M, all from GenScript, EUA. CFSE^{Hi} cells were not loaded with any peptide and used as internal control. For calculating the percentage of specific lysis, CFSE^{Hi} and CFSE^{Int} (or CFSE^{Low}) were gated together in order to constitute 100% of cells. CFSE^{Hi} and CFSE^{Int} (or CFSE^{Low}) populations were then individually gated and specific lysis was calculated using the following formula: % of specific lysis = 100x [(CFSE^{Low or Int} / CFSE^{Hi}) ctr – (CFSE^{Low or Int} / CFSE^{Hi}) effector] x (CFSE^{Low or Int} / CFSE^{Hi}) ctr⁻¹.

Real time RT-PCR. RNA was extracted using the RNeasykit (QIAGEN). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Invitrogen, Life Technologies), following the manufacturer's instructions. Gene expression was analyzed using primers in combination with SYBR Green® on a StepOneReal Time PCR System (Applied Biosystems, Life Technologies). The expression level of the genes of interest was determined relative to the expression of 18S rRNA. Primers: *Runx3* Fwd.: 5'- ACA GCA TCT TTG ACT CCT TCC -3'; *Runx3* Rev.: 5'- GTT GGT GAA CAC GGT GAT TG- 3'; *Thpok* Fwd: 5'- ATG GGA TTC CAA TCA GGT CA- 3'; *Thpok* Rev.5'- TTCTTC CTA CAC CCT GTG CC -3'; *CD8a* Fwd: 5' - ACT GCA AGG AAG CAA GTG GT – 3'; *CD8a* Rev: 5'-CAC CGC TAA AGG CAG TTC TC- 3'; 18S-Fwd.: 5'- GGG CGT GGG GCG GAG ATA TGC - 3' and 18S-Rev.: 5'- CG CGG ACA CGA AGG CCC CAA A -3'. For parasite load quantification, hearts of infected mice were perfused, minced and immediately homogenized in 1.0 ml of 4.0 M Guanidine thiocyanate (SIGMA-Aldrich) containing 8.0 μ L/mL of β -mercaptoethanol

and processed for DNA extraction. Generation of PCR standards and detection of parasite tissue load by real-time PCR was carried out as described (Oliveira et al., 2017); briefly, primers amplify a repeated satellite sequence of *T. cruzi* DNA of 195 base-pairs: TCZ-Fwd.: (5'-GCTCTTGCCCACAAGGGTGC-3') and TCZ-Rev.: (5'-CCAAGCAGCGGATAGTTCAGG-3'). Reactions with TNF- α -Fwd: (5'-CCTGGAGGAGAAGAGGAAAGAGA-3') and TNF- α -Rev.: (5'-TTGAGGACCTCTGTGTATTTGTCAA-3') primers for *Mus musculus* TNF- α gene were used as loading controls.

Gene-expression analysis. Gene expression data were extracted with GeneSpring software GX 11.0 software using default parameters. Data were then normalized by subtracting the average from all genes from each gene intensity and dividing the result by the standard deviation of all gene intensities (i.e. z-score normalization). Z ratios were calculated as previously described (Cheadle et al., 2003) and used to compare the difference in expression between WT samples and *Myd88*^{-/-} samples. Gene Set Enrichment Analysis (GSEA) (<http://www.broadinstitute.org/gsea/index.jsp>) was performed on the genes pre-ranked by Z ratios (1,000 permutations and Weighted Enrichment Statistic) using as data set 82 differentially expressed genes of sorted WT and *Myd88*^{-/-}CD4⁺ T cells (GEO accession no. GSE57738) (Oliveira et al., 2017); and using as gene set the CD4⁺Gzmb⁺ (CD4CTL) transcriptional signature (159 upregulated genes) previously described (<https://www.ebi.ac.uk/ena/data/view/PRJEB14043>).

Adoptive CD4⁺ T cell transfer assay. Eight-week-old male GzmbCreER^{T2}/ROSA26EYFP mice were infected with 2 x 10³ trypomastigotes and treated with 4 mg/animal/day of tamoxifen citrate (Sandoz-Novartis) from day 7 to 11 pi by gavage. Spleen cells were harvested and treated with ACK for red blood cells lysis at day 13 pi. Alternatively, CD4⁺ T cells were purified from the spleen of non-treated

and infected *Ifng*^{-/-} mice. In both cases, spleen cells were labeled with anti-B220, anti-CD11b, anti-NK1.1, anti- $\gamma\delta$ TCR, anti-CD8 and biotinylated PE-conjugated anti-CD4 antibodies for 30 minutes on ice, followed by staining with PE-Cy7-conjugated Streptavidin for 20 min. Residual PE-Cy7⁺ cells were excluded and EYFP⁺PE⁺ (CD4⁺GzB⁺ T cells) or PE⁺CD4⁺ (*Ifng*^{-/-}CD4⁺ T cells) were sorted using MoFlo cell sorter (Beckman Coulter/Dako-Cytomation). CD4⁺GzB⁺ T cells (2.8 x 10⁵ cells/animal) or *Ifng*^{-/-} CD4⁺ T cells (1.3 x10⁶ cells/animal), both presenting purity higher than 98%, were adoptively transferred by *iv* injection into *Il18ra*^{-/-} recipient mice, previously infected with 1 x 10³ trypanomastigotes (day 7 pi). Control groups of *Il18ra*^{-/-} and *Myd88*^{-/-} infected mice received PBS. Survival was accompanied daily.

Statistics. Statistical analyses were performed using GraphPad Prism version 6 for Mac OS (GraphPad Software, San Diego California USA, www.graphpad.com). Sample sizes were chosen to allow the detection of statistical differences with $p < 0.05$ using Student's *t* test, or the Log-rank (Mantel-Cox) test for mouse survival curves. Correlation analysis employed Pearson correlation test. When comparing two groups, the minimum number was n=3 per group. In some experiments, the number was increased to n= 9 to permit $p < 0.05$ even considering the possibility of a single outlier in a population. Data are expressed as mean $\pm$ SEM and considered statistically significant if *p* values were < 0.05 .

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Figure Legends:

Figure 1: CD4⁺GzB⁺PRF⁺ T cells are present at high frequency in the spleen of mice infected with *T. cruzi*. (A) CD4⁺ T cell gating strategy (B and C) Representative flow-cytometry dot plots of GzB (B) or PRF (C) staining, gated on CD4⁺ T cells of non-infected and infected B6 mice (left) and mean frequency of CD4⁺GzB⁺ (or CD4⁺PRF⁺) T cells in the spleen (right). (D) Representative dot plots of PRF and GzB expression in CD4⁺ T cells gated as in (A) and mean frequencies of PFR⁺ among GzB⁺ CD4⁺ T cells (isotype controls on Figure 1-figure supplement 1A). (E) Representative histogram of 2B4 (CD244) expression on CD8⁺ (blue), CD4⁺GzB⁺ (red) or CD4⁺GzB⁻ T cells (pink), and correspondent mean MFI values; (F) Representative dot plots of GzB and IFN- γ staining in gated CD4⁺ T splenocytes (left), mean frequencies of GzB⁺IFN- γ ⁻ (red), GzB⁺IFN- γ ⁺ (purple) and GzB⁻IFN- γ ⁺ (blue) subsets among total CD4⁺ (center) or gated on CD4⁺GzB⁺ T cells (right). (G) Kinetics of the mean frequency of CD4⁺IFN- γ ⁺ (purple) and CD4⁺GzB⁺ (red) (left), and corresponding absolute numbers (right). (H) Frequency of LAMP-1⁺(CD107a⁺) cells among CD4⁺GzB⁺ (red) or CD8⁺GzB⁺ (blue) T cells in the infected spleen. Data obtained from individually analyzed mice (n=4), at indicated or 14 day pi. Error bars= SEM. ND= non-detected. *ns*= non-significant; **= $p \leq 0.01$ (Student t test). All data are representative of 3 or more independent experiments.

Figure 2: CD4 T cell-mediated Ag-specific cytotoxicity and TF expression by CD4⁺GzB⁺ T cells. (A) Mean frequencies of dead target cells (B blasts) in redirected cytotoxicity assay employing sorted naive (blue) or *in vivo* activated (red) CD4⁺ T cells as effectors, at different E:T ratios, in triplicates. (B) Specific lysis of IC-21 macrophages (T) loaded with amastigote antigens, coincubated with highly purified

CD4⁺ T cells (E) at the indicated E:T ratios, in the presence of anti-FasL mAb (red) or isotype control mAb (blue). Mean values of triplicates $\pm$ SEM are shown in (A) and (B); * $p \leq 0.05$ (two-tailed Student t-test). Data are representative of 3 independent experiments. Gating strategy on Figure 2-figure supplement 1A. (C and D) Percentages of Ag-specific cytotoxicity *in vivo*; bars represent mean values + SEM (error bars) of individually analyzed mice (n=3) infected with *T. cruzi* Y in (C) or Y-OVA strain in (D); class I-restricted (PA8 or OVA₂₅₇₋₂₆₄) and class II-restricted (SA851.1c+d or OVA₃₂₃₋₃₃₉) peptides were employed. Data are representative of 2 independent experiments. Cell survival was measured by flow cytometry (Figure 2-figure supplement 1B) and specific lysis calculated as described in Methods. (E and F) Representative dot plots of CD4⁺ T cells (gated as in Figure 1A) and corresponding mean frequencies of gated subpopulations: (E) GzB and FoxP3 staining and (F) GzB and T-bet staining. (G) Representative histogram of Eomes expression in CD4⁺ T splenocytes of non-infected mice (ctr, gray) and in GzB⁻ (black) and GzB⁺ (red) CD4⁺ T cells from infected mice, gated as in Figs. 1A and 1B (left) and mean frequencies of Eomes⁺ cells among these different subpopulations (right). All experiments were done at day 14 pi. Bars represent mean values of non-infected ctr group n=3 or infected group, n=3 to 5, individually analyzed mice. Error bars= SEM. (H) Runx3d and ThPOK (*Zbtb7b*) mRNA expression estimated by qRT-PCR in CD4⁺EYFP⁺ T cells sorted from infected and tamoxifen-treated GzmbCreER^{T2}/ROSA26EYFP mice, as described in Methods and shown in Figure 2-figure supplement 3A; ND = not detected. Data are representative of 3 independent experiments.

Figure 3: Expression of IL-10, immunoregulatory molecules and cytotoxic markers by CD4⁺GzB⁺ T cells. (A) Representative dot plots of IL-10 staining and

mean frequencies of IL-10⁺ cells among non-infected (ctr, gray) or GzB⁻ (black) and GzB⁺ (red) CD4⁺ T cells from infected mice. **(B)** Representative histogram of Lag-3 staining and mean frequency of Lag-3⁺ cells. **(C)** Representative dot plots of Lag-3 and CD49b staining and mean frequency of different cells subsets. **(D)** Representative histogram of Tim-3 staining and mean frequency of Tim-3⁺ cells among different cells subsets. **(E)** Representative histogram of CD27 staining and mean frequency of CD27⁺ cells. **(A-E)** CD4⁺GzB⁺ T cells gated as in Figures 1A-1B; staining controls in Figure 3-figure supplement 1. **(F-H)** Representative dot plots and mean frequencies of **(F)** NKG2A/C/E, **(G)** NKG2D and **(H)** CRTAM expression among gated CD4⁺ T splenocytes. All experiments were done at day 14 pi. Non-infected ctr group, n=3; infected group, n=3 to 5 mice, individually analyzed. Error bars= SEM; *p≤ 0.05; ***p≤ 0.001; ****p≤ 0.0001 (two-tailed Student t-test). Data are representative of 3 independent experiments. Staining controls are shown on Figure 3-figure supplement 2.

Figure 4: CD4CTLs are severely reduced in *Myd88*^{-/-} mice. **(A-B)** Representative dot plots of GzB and T-bet staining on gated CD4⁺ T splenocytes (left panels) and mean frequencies of T-bet⁺, IFN-γ⁺ and GzB⁺ CD4⁺ T cells and absolute numbers of GzB⁺CD4⁺ T cells (on the right) from **(A)** WT (B6) and *Il12p40*^{-/-} mice on day 14 pi and **(B)** WT and *Ifng*^{-/-} mice on day 11 pi. **(C)** Representative dot plots and gating strategy, **(D)** mean frequencies and **(E)** absolute numbers of GzB⁺ or NKG2A/C/E⁺, NKG2D⁺ and CRTAM⁺ cells among GzB⁺CD4⁺ T cells in the spleen of WT (blue) and *Myd88*^{-/-} (red) mice at day 13 pi. Bars represent mean values in each group; n= 5 individually analyzed mice. Error bars= SEM; ns= non-significant; *p≤ 0.05; **p≤ 0.01; ****p≤ 0.0001 (Student t-test). Representative dot plots of GzB, NKG2A/C/E, NKG2D and CRTAM staining on CD4⁺ T cells from non-infected animals are shown

in Figure 4-figure supplement 1B. **(F-G)** Frequencies (left) and absolute numbers (right) of PRF⁺ cells among CD4⁺ T cells **(F)** and of PRF⁺ cells among GzB⁺CD4⁺ T cells **(G)** in the spleen of WT (blue bars) and *Myd88*^{-/-} (red bars) mice. GzB⁺CD4⁺ T cells were gated as shown in Figs. 1A, 1B and 1G. Data in **(F)** and **(G)** are compiled from four independent experiments with n= 3 animals in each group, each symbol represents an individual analyzed mice. **(H)** Frequency of dead target cells (B cell blasts) in cytotoxic assay, after 14 h of co-culture with CD4⁺CD44^{hi} T cells sorted from WT (blue line) or *Myd88*^{-/-} (red line) infected mice at day 13 pi. B cells were coated with anti-CD3 (left) or not (right). Mean of triplicates +/- SEM for each E:T ratio point are shown. Data are representative of 3 independent experiments.

Figure 5: The CD4CTL gene program is absent in MyD88-deficient CD4⁺ T cells.

(A) GSEA performed using pre-ranked list of genes expressed in WT or *Myd88*^{-/-} CD4⁺ T cells sorted from mix-BM chimeras using the CD4CTL gene signature previously described as gene set (66). **(B)** Venn diagram of CD4CTL genes upregulated in WT and downregulated in *Myd88*^{-/-} CD4⁺ T cells sorted from chimeric mice. **(C)** Heat map of normalized CD4CTL-signature gene expression in WT or *Myd88*^{-/-} CD4⁺ T cells sorted from chimeric mice: values indicate mean Z-score, upregulated genes in red and downregulated genes in blue. **(D)** Representative histograms of Blimp-1 expression (right) and mean frequency of Blimp-1⁺ splenocytes (left) among total gated CD4⁺ T cells from non-infected mice (ctr, gray shaded area and bar) or among CD4⁺GzB⁺ (red line and bar) or CD4⁺GzB⁻ (black line and bar) from WT infected mice at day 14 pi. Individually analyzed mice, n=4. Error bars=SEM, data are representative of 2 independent experiments. **(E)** Representative histograms (left and central panel) and mean MFI values of CD39 expression (right panel) on gated FoxP3⁻ (gray shaded area

and bar) or FoxP3⁺ (blue line and bar) CD4⁺ T cells from non-infected (ctr) mice (left); GzB⁻FoxP3⁻ (black line and bar), GzB⁻FoxP3⁺ (purple line and bar) or GzB⁺FoxP3⁻ (red line and bar) CD4⁺ T cells from infected WT mice (center), at day 14 pi; individually analyzed mice (n=4), error bars =SEM; data are representative of 4 independent experiments. **(F)** Mean frequency (left) and absolute numbers (right) of GzB-expressing cells among CD45.1⁺ (B6.SJL WT) or CD45.2⁺ (B6 WT or KO) CD4⁺ T splenocytes in mix-BM chimeric mice, at day 14 pi; WT:WT control chimera group, n=3; other chimera groups, n=6 or 7. Error bars= SEM, *ns*= non-significant, ****p*≤ 0.001, *****p*≤ 0.0001 (Student t-test). Gating strategy and representative dot plots are shown in Figure 5-figure supplement 1. Data are representative of 3 independent experiments.

Figure 6: CD4CTLs are severely reduced in *Il18ra*^{-/-} mice. (A and B) Frequencies and absolute numbers of PRF⁺ cells among CD4⁺ T cells **(A)** and of PRF⁺ cells among GzB⁺CD4⁺ T cells **(B)** in WT (blue bars) and *Il18ra*^{-/-} (red bars) spleens. Data are compiled from two independent experiments with n= 4-5 animals in each group, each symbol represents an individual mouse. **(C)** Representative dot plots of GzB, CRTAM, NKG2D and NKG2A/C/E staining in WT and *Il18ra*^{-/-} mice. **(D)** Mean frequencies and **(E)** absolute numbers of GzB⁺ or CRTAM⁺, NKG2D⁺ and NKG2A/C/E⁺ cells among CD4⁺ and CD4⁺GzB⁺ T cells, respectively. **(A-E)** CD4⁺ T cells gated as in Figs. 1A-1D; n=4 individually analyzed mice in each group; error bars= SEM, **p*≤ 0.05, ***p*≤ 0.01, ****p*≤ 0.001, *ns*= non-significant (Student t-test). Data are representative of 2 independent experiments. **(F)** Frequency of dead target cells (B cell blasts) in cytotoxic assay, after 14 h of co-culture with CD4⁺CD44^{hi} T cells sorted from WT (blue line) or *Il18ra*^{-/-} (red line) infected mice at day 13 pi. B cells were coated with anti-CD3 (left) or not (right). Mean of triplicates +/- SEM for each E:T ratio point are shown. **(G)**

Survival curve (left) and parasite load in the heart (right) of infected *Il18ra*^{-/-} mice, adoptively transferred with CD4⁺GzB⁺ cells at day 7 pi (red curve), or not (black curve); time-line in Figure 6-figure supplement 1; n=10 mice in each group; *p* = 0.0277; survival data are compiled from two independent experiments.

Figure 7: Cells with CD4CTL phenotype predominate in the cardiac tissue of infected mice and are at higher frequencies in patients with Chagas cardiomyopathy. (A) Representative dot plots (gated as in Figure 7-figure supplement 1A), and mean frequency of CD8⁺ and CD4⁺ T cells infiltrating the heart of B6 infected mice at day 14 pi. (B and C) Representative dot plot and mean frequency of (B) GzB⁺ and (C) LAMP-1⁺ (CD107a⁺) cells, among gated CD4⁺ T cells infiltrating the infected heart; ctr= non-infected, ND= non-detected. (D) Mean parasite load in the cardiac tissue at different time points pi, obtained by real-time PCR, of individually analyzed mice as described in Methods; Data are representative of at least 8 (A and B) or 2 (C and D) independent experiments. (E) Representative dot plots and mean frequency of CRTAM⁺ and NKG2D⁺ cells among gated intracardiac CD4⁺GzB⁺ T cells. DP= CRTAM⁺ NKG2D⁺ double positive cells. Individually analyzed mice, n= 4; error bars= SEM; data are representative of 4 independent experiments. (F) Representative dot plots and mean frequency of PFR⁺ and GzB⁺PFR⁺ cells among intracardiac CD4⁺GzB⁺ T cells; data are compiled from two independent experiments with n= 4 or 5 animals in each group, each symbol represents an individual analyzed mice. (G) Intensity of PRF staining (MFI) in and (H) Frequency of PRF⁺ cells among CD4⁺ T cells in PBMCs from patients with chronic Chagas disease cardiomyopathy (CC; n=10) and healthy donors (HD; n=4) cultured overnight in medium alone (ctr) or in the presence of trypomastigote antigens (+trypo); **p*≤ 0.05, ***p*≤ 0.01, *ns*= non-significant; data

1220 analyzed by paired Student t test. **(I)** Frequency of PRF⁺ cells among CD4⁺ T cells from
 1221 PBMCs of patients with chronic Chagas disease cardiomyopathy (CC; n=10) and
 1222 healthy donors (HD; n=4) cultured ON in the presence of trypomastigote antigens.
 1223 Gating strategy and representative dot plots are shown in Figure 7-figure supplement 2.
 1224 **(J)** Correlation analysis between the percentage of CD4⁺PRF⁺ T cells among CD4⁺ T
 1225 cells in PBMCs from CC patients and left ventricular ejection fraction (LVEF) (left
 1226 panel) or left atrium diameter (right panel); indicated *p* and *r* values were calculated
 1227 using Pearson correlation test.

A **B** **C** **D** **E** **F** **G** **H**

A **B** **C** **D** **E** **F** **G** **H**

A

B

CD4⁺GzB⁺ (CD4CTL) signature genes

D

E

F

C

A **B** **C** **D** **E** **F** **G** **H** **I** **J**

SUPPLEMENTAL MATERIAL

Cytotoxic CD4⁺ T cells driven by T-cell intrinsic IL-18R/MyD88 signaling predominantly infiltrate *Trypanosoma cruzi*-infected hearts

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Supplemental Figures.

Figure 1–figure supplement 1: Comparative percentages and absolute numbers of GzB⁺ and PRF⁺ T cell subsets. (A) Isotype control staining of CD4⁺ T cells, gated as in Fig. 1A, from infected mice. (B) Gating strategy for C and D plots. (C) Representative dot plots of GzB staining and mean frequency of GzB⁺CD8⁺T cells (on the right). (D) Representative dot plots of PRF and GzB staining in CD8⁺ T cells. (E) Mean frequency and (F) absolute numbers of CD4⁺ and CD8⁺ T cells in the spleen of non-infected (ctr) and infected mice. (G) Mean frequency and (H) absolute numbers of GzB⁺CD4⁺ and GzB⁺CD8⁺ T cells. Mice individually analyzed at day 14 pi (n=4); error bars= SEM. Data are representative of 6 independent experiments.

Figure 2-figure supplement 1: Gating strategy of cytotoxic assays. (A) Gating strategies and PI staining of IC-21 macrophages loaded with total amastigote protein extract (CFSE^{lo}) or not (CFSE^{hi}) and cocultured with 4 x 10⁵ purified CD4⁺ T cells (right), or not (left); specific cytotoxicity values are shown in **Figure 2B**; **(B)** Gating strategies for CTV-stained target splenocytes, loaded with the indicated antigenic peptides or not (ctr), 20 h after i.v. injection in non-infected or Y strain-infected mice. The *T. cruzi*-derived PA8 (class I-restricted) and SA85 (class II-restricted) peptides were employed. Specific cytotoxicity values for the *in vivo* assay are shown in **Figure 2C**.

Figure 2-figure supplement 2: The majority of GzB⁺CD4⁺ and GzB⁻CD4⁺ T cells is composed of non-naive (activated effectors and memory cells). Representative dot plots and gating strategy of staining for GzB, CD44 and CD62L (on the left) and mean frequency of naive (CD44^{lo}) and non-naive (CD44^{hi}) CD4⁺ T cells (on the right) in: **(A)** non-infected mice and gated on total CD4⁺TCRβ⁺ and **(B)** infected mice and gated on GzB⁺ (top) or GzB⁻ (bottom) CD4⁺TCRβ⁺ cells, at day 14 pi. Bars represent mean values of cell frequency of individually analyzed mice; n=4; Error bars = SEM. Data are representative of 3 independent experiments.

Figure 2-figure supplement 3: Expression of *Tbx21* and *Cd8a* in sorted GzB⁺CD4⁺ T cells. (A) Sorting gate and frequency of CD4⁺GzB⁺ (EYFP)⁺ T cells obtained from infected and tamoxifen-treated GzmbCreER^{T2}/ROSA26EYFP mice. CD4⁺ cells were enriched by negative selection with magnetic beads, as described in Methods, and then submitted to FACS-sorting. Pre-sorting CD4⁺ T cell frequency is shown on the left panel and post-sorting on the right. (B) Sorted cells had RNA extracted and *Tbx21*, *Runx3d*, *Zbtb7b* and *Cd8a* expression tested by qRT-PCR, as described in Methods. Results for *Runx3d* and *Zbtb7b* expression are shown in Fig. 2H. ND = not detected.

Figure 3-figure supplement 1: IL-10, Lag-3 and CD49b expression. (A) Representative dot plots and gating strategy for IL-10 expression. Total spleen cells (left) and gated CD4⁺ T cells (middle and right panels) of non-infected control mice. **(B)** Representative dot plot of IL-10 staining in total splenocytes of infected mice. Dot plot and mean frequencies of IL-10⁺ cells among CD4⁺GzB⁻ and CD4⁺GzB⁺ T cells in infected mice are shown in **Fig. 3A**. **(C)** Representative dot plot of Lag-3 and CD49b expression on gated B220⁺, NK1.1⁺ or total CD4⁺ T cells from the spleen of non-infected mice (left, middle and right panels, respectively). Mean frequencies of Lag-3⁺CD49b⁺ and Lag-3⁺CD49b⁻ cells among CD4⁺GzB⁻ and CD4⁺GzB⁺ T splenocytes of infected mice are shown in **Fig. 3C**. Data are representative of 2 independent experiments.

Figure 3-figure supplement 2: Representative dot plots and gating strategies for NKG2A/C/E, NKG2D and CRTAM staining on CD4⁺ and CD8⁺ GzB⁺ T cells. Staining of spleen cells from non-infected controls (right panels) and FMO and isotype controls of spleen cells from B6 infected mice (left panels) are shown. Representative dot plots and mean frequencies of CD4⁺GzB⁺ and CD8⁺GzB⁺ T cells

expressing these cytotoxic markers are shown in **Figure 3F-H** and Figure 3-figure supplement 3, respectively.

Figure 3-figure supplement 3: Expression of NKG2A/C/E, NKG2D and CRTAM cytotoxic markers by CD8⁺GzB⁺ T cells. Representative dot plots and gating strategies of spleen cells from B6 infected mice (left panels). Graphs on the right: bars represent mean values of cell frequency of each indicated cell subpopulation in individually analyzed mice; n=4; Error bars = SEM. Data are representative of 3 independent experiments.

Figure 3-figure supplement 4: Frequency of CRTAM-expressing cells among splenic GzB⁺ and GzB⁻ CD4⁺ T cells, gating or not on CD44^{hi} cells. Bars represent mean values of the frequency of CRTAM⁺ cells among: CD4 naive cells of non-infected (ctr) mice (white bar) and CD4⁺GzB⁻ (gray bar), CD4⁺GzB⁺ (orange bar), CD4⁺CD44^{hi}GzB⁻ (black bar) or CD4⁺CD44^{hi}GzB⁺ (red bars) of infected mice at day 14 pi; Gating as shown in **Sup. Figs. 3 and 6C**. Mice were individually analyzed, n= 4; Error bars = SEM; *ns*= non-significant, Student t test.

Figure 4-figure supplement 1: Equivalent frequencies and absolute numbers of total CD4⁺ T cells in the spleens of infected *Myd88*^{-/-} and WT mice. (A) Mean frequencies and absolute numbers of total CD4⁺ and CD8⁺ T cells in the spleens of non-infected (ctr) and infected *Myd88*^{-/-} and WT (B6) mice, at day 13 pi. (B)

Representative dot plots and gating strategies for GzB, CRTAM, NKG2D and NKG2A/C/E staining of spleen cells from non-infected (ctr) WT (B6) and *Myd88*^{-/-} mice. Staining results obtained with infected mice are shown on **Figures 4C-4E**. **(C)** Mean frequencies and **(D)** absolute numbers of GzB⁺, GzB⁺CRTAM⁺, GzB⁺NKG2D⁺ and GzB⁺NKG2A/C/E⁺ cells among CD8⁺ T cells in the spleen of WT and *Myd88*^{-/-} infected mice at day 13 pi, gated as in Sup. Fig. 7. Bars represent mean values in each group; error bars = SEM; n= 5 individually analyzed mice. Results are representative of 2 independent experiments.

Figure 4-figure supplement 2: Equivalent frequency and absolute number of activated CD4⁺ T cells in WT and *Myd88*^{-/-} mice infected with *T. cruzi*. Representative dot plots (top) and mean frequencies and absolute numbers (bottom) of CD4⁺CD44^{hi} T cells in the spleens of infected WT (B6) and *Myd88*^{-/-} mice, at day 14 pi. Mice were individually analyzed; n=4; error bars = SEM. Representative of 2 independent experiments.

Figure 5-figure supplement 1: GzB expression in CD4⁺ T cells of mixed-BM chimeric mice. Gating strategy and representative dot plots of: (A) CD45.1⁺ and CD45.2⁺ cells (on the right) gated CD4⁺CD8⁻ T lymphocytes (on the left) from the spleens of mixed-BM chimeric mice. (B) GzB expression in CD4⁺CD45.1⁺ (WT) and CD4⁺CD45.2⁺ (WT or KO) T cells from the spleens of non-infected and infected chimeric mice at day 14 pi, gated as in (A). Mean frequencies and absolute numbers of CD4⁺GzB⁺ in each group are shown in **Figure 5F**.

Figure 6-figure supplement 1: Time-line of the adoptive transfer experiment shown on Figure 6G.

Figure 6-figure supplement 2: The adoptive transfer of *Ifng*^{-/-} CD4⁺ T cells increased survival to infection. Survival curve of *Il18ra*^{-/-} mice, infected with 1×10^3 trypanomastigotes of the Y strain and adoptively transferred with sorted (>98% pure) *Ifng*^{-/-} CD4⁺ T cells (green curve), or not (PBS group, black curve); n=6 mice in each group; p= 0.0295 Log-rank (Mantel-Cox) test.

Figure 7-figure supplement 1: CD4⁺ and CD8⁺ T cells in the heart of mice infected with *T. cruzi*. (A) Representative FSC x SSC dot plots and gating strategy of cells from the hearts of non-infected (left panel) and infected (right panel) WT mice, employed for data analysis shown on this figure and Figure 7. (B) Representative dot plots of blood samples from infected mice injected iv with anti-CD4-FITC mAb, 3 min before euthanasia: with no other staining (left panel) or after staining with anti-

CD4-PECy7 mAb *in vitro* (right panel). Representative dot plots of cells from the heart of non-infected and infected mice, injected *iv* with anti-CD4-FITC mAb and stained *in vitro* with anti-CD4-PECy7 (two panels on the right), and mean frequencies of intravenously stained CD4⁺ T cells in the hearts of non-infected and infected mice. Mice were individually analyzed; n=3; error bars = SEM. Representative of 2 independent experiments. **(C)** Representative dot plots and gating strategy of isotype and GzB (left panels) or LAMP1 (right panels) staining and corresponding mean frequencies of GzB⁺CD8⁺ and LAMP1⁺CD8⁺ cells among gated CD8⁺ T cells infiltrating the infected myocardium at day 14 pi. **(D)** Representative dot plots of isotype and NKG2A/C/E staining on gated CD4⁺GzB⁺ (left panels) or GzB⁺CD8⁺ (right panels) T cells and corresponding mean frequencies of NKG2A/C/E⁺ cells among GzB⁺CD4⁺ and GzB⁺CD8⁺ T cells infiltrating the infected myocardium at day 14 pi. Individually analyzed mice, n= 4; error bars= SEM; ND= not detected. Data are representative of 2 independent experiments.

Figure 7-figure supplement 2: CD4⁺ T cells expressing PRF are found in the circulation of patients suffering from chronic Chagas cardiomyopathy (CC). (A) Dot plot showing the gating strategy for CD4⁺ T cell staining on PBMC. (B) Gating strategy and representative dot plots of PRF staining in gated CD4⁺ T cells from CC patient incubated with medium (on the left), or with trypomastigote antigens (trypan) (on the right). Frequencies of PRF⁺CD4⁺ T cells and MFI of PRF staining for CC patients and HD controls are shown on **Figures 7G-7I**.

T. cruzi-induced CD4CTLs

Figure 7-figure supplement 3: Predicted model for the generation of CD4CTLs cells in response to *T. cruzi* infection. This cartoon summarizes our results and also contains information of previous published studies, cited in this work. IL-12 produced during infection acts on TCR-triggered CD4⁺ T cells, leading to T-bet expression, which promotes IFN-γ upregulation and induces *Runx3d* and *Eomes* gene expression. In turn, IFN-γ- and IL-12-signaling induce IL-18R expression. T-bet, Eomes and Runx3d drive the expression of cytotoxic effector molecules, including GzB, PRF and FasL. IL-10 expression can be induced by Blimp-1 and Eomes, although few IL-10⁺ CD4⁺GzB⁺ T cells were found in our infection model. As opposed to intraepithelial CD4CTLs of the gut, ThPOK expression is not downmodulated in splenic CD4⁺GzB⁺ T cells, until day 14 pi. IFN-γ is produced by around 18% of these cells at the peak of the CD4⁺ T cell response (14 dpi), and is necessary for CD4⁺GzB⁺ T cell proliferation and/or survival. Most CD4⁺GzB⁺ T cells express Lag-3, Tim-3 and CD39 immunoregulatory molecules, while CD27 is downmodulated. Notably, the expansion and/or survival of CD4⁺GzB⁺ T cells expressing CRTAM, NKG2D, and NKG2A/C/E cytotoxic markers depend on T-cell intrinsic IL-18R/MyD88 signaling.