Tissue damage during acute *Trypanosoma cruzi* infection is associated with reduced reparative regulatory T cell response and can be attenuated by early interleukin-33 administration.

- 5 Short title: Reparative regulatory T cell response in *T. cruzi* infection.
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18 ABSTRACT

19 Tissue-repair regulatory T cells (trTregs) constitute a specialized regulatory subset renowned for 20 orchestrating tissue homeostasis and repair. While extensively investigated in sterile injury 21 models, their role in infection-induced tissue damage and the regulation of protective 22 antimicrobial immunity remains largely unexplored. This investigation examines trTregs 23 dynamics during acute Trypanosoma cruzi infection, a unique scenario combining extensive 24 tissue damage with robust antiparasitic CD8+ immunity. Contrary to conventional models of 25 sterile injury, our findings reveal a pronounced reduction of trTregs in secondary lymphoid 26 organs and tissues during acute T. cruzi infection. This unexpected decline correlates with 27 systemic as well local tissue damage, as evidenced by histological alterations and 28 downregulation of repair-associated genes in skeletal muscle. Remarkably, a parallel decrease 29 in systemic levels of IL-33, a crucial factor for trTregs survival and expansion, was detected. We 30 found that early treatment with systemic recombinant IL-33 during infection induces a notable 31 surge in trTregs, accompanied by an expansion of type 2 innate lymphoid cells and parasite-32 specific CD8+ cells. This intervention results in a mitigated tissue damage profile and reduced 33 parasite burden in infected mice. These findings shed light on trTregs biology during infection-34 induced injury and demonstrate the feasibility of enhancing a specialized Tregs response 35 without impairing the magnitude of effector immune mechanisms, ultimately benefiting the 36 host. Furthermore, this study settles groundwork of relevance for potential therapeutic 37 strategies in Chagas' disease and other infections.

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39 AUTHOR SUMMARY

Chagas' disease, caused by the protozoan *Trypanosoma cruzi*, induces severe organ damage
caused by the interplay between the parasite and the immune response. In our investigation,

42 we delved into the role of tissue-repair regulatory T cells (trTregs) during the acute phase of T. 43 cruzi infection in mice. Surprisingly, we observed a reduction in trTregs during the peak of tissue 44 damage, contrary to their usual accumulation after injury in other contexts. This decline aligned 45 with decreased levels of interleukin-33, a critical factor for trTregs survival. Administering 46 interleukin-33 at early infection times not only boosted trTregs but also expanded other 47 reparative and antiparasitic immune cells. Consequently, these treated mice exhibited reduced 48 damage and lower parasite levels in tissues. Our findings offer insights into trTregs' behavior 49 during infection-induced injury, suggesting a promising avenue for therapeutic interventions in 50 Chagas' disease and related conditions. This study lays the groundwork for potential strategies 51 that balance the immune response, supporting tissue repair without compromising the ability 52 to control the infection, which could have broader implications for infectious diseases and tissue 53 damage-related pathologies.

54

55 INTRODUCTION

56 Regulatory T cells (Tregs) are CD4+ T lymphocytes with immunoregulatory properties 57 characterized by the expression of the transcription factor Forkhead box P3 (Foxp3) [1]. The 58 immunomodulatory role of Tregs has been extensively described across diverse biological 59 processes, encompassing tolerance maintenance, autoimmunity and allergy, as well as cancer, 60 infectious and immunometabolic diseases [2]. This wide spectrum of activities underscores the 61 adaptability of Tregs in regulating various effector responses, including Th1, Th2, and Th17 62 immunity. This phenomenon, recognized as Tregs specialization, enables them to tailor their 63 regulatory capacity to distinct scenarios and the specific immune profiles requiring modulation 64 [3].

65 Recently, it has been identified a separate subset of Tregs that goes beyond their classic 66 suppressive function to specialize in maintaining tissue homeostasis and promoting repair

67 following damage [4]. These cells, referred to as tissue repair Tregs (trTregs), were initially 68 believed to be confined to non-immune tissues, but have subsequently been found in lymphoid 69 organs as well [5]. Tissue repair Tregs exhibit a high level of activation and share a phenotypic 70 and transcriptomic core signature across different tissues. Nevertheless, they also possess 71 unique characteristics and functions shaped by the microenvironment of their respective 72 residing sites. The trTregs program is established through a stepwise process, beginning with an 73 initial commitment in peripheral lymphoid organs and culminating in final differentiation within 74 tissues [6–8]. Regarding function, trTregs located in adipose tissue adapt to regulate metabolism 75 and restrain obesity [9]. This cell subset also orchestrates tissue regeneration and homeostasis 76 in the colon [10], participates in skeletal muscle (SM) regeneration and control of fibrosis 77 [11,12], facilitates wound healing and hair growth in the skin [13,14] and promotes myelin 78 regeneration in the central nervous system (CNS) [15]. Throughout these contexts, the survival, 79 expansion, and acquisition of tissue-repair properties by trTregs have been demonstrated to rely 80 on IL-33, an alarmin belonging to the IL-1 family, released upon cellular damage [16]. 81 Correspondingly, trTregs express the specific IL-33 receptor subunit, ST2 [5].

82 The majority of studies on trTregs have focused on their behavior under homeostatic conditions 83 or in models of sterile injury, where they accumulate locally to facilitate tissue healing [17]. 84 Nonetheless, limited information exists concerning trTregs behavior and their dependence on 85 the IL-33/ST2 axis for controlling tissue damage induced by infections, which exposes these cells 86 to a distinct environment. Currently, only a handful of studies have demonstrated local trTregs 87 accumulation and their role in promoting tissue repair during acute infections caused by 88 influenza virus [18], herpes simplex virus [19], and cytomegalovirus [20]. Intriguingly, trTregs 89 accumulation in the lung and cornea appeared to rely more on IL-18, another IL-1 family 90 cytokine, than on IL-33 signaling. Additionally, trTregs increased in the liver during S. japonicum 91 helminth infection, where they significantly ameliorated tissue pathology [21]. In contrast, ST2+ 92 Tregs that accumulated in the intestinal lamina propria of chronically HIV-infected patients had

93 a limited role in tissue repair, as these individuals exhibited heightened epithelial permeability 94 and tissue fibrosis [22]. In protozoan infections, different roles for trTregs have been reported, 95 with their protective function noted in cerebral malaria [23], while their relevance in SM 96 pathology during toxoplasmosis appeared negligible [24]. Despite these emerging reports, the 97 role of trTregs and the IL-33/ST2 axis in tissue repair and homeostasis during infections remains 98 poorly delineated. Few studies have examined the impact of manipulating the abundance of 99 trTregs on antimicrobial immunity and pathogen load control. Given that trTregs are recognized 100 for their strong suppressive potential [4,25], further exploration of their influence on effector 101 immune responses is necessary. This exploration will contribute to a better understanding of 102 the interplay between microbial persistence, tissue damage and repair, and the underlying 103 immunopathology that characterizes certain chronic infections.

104 Chagas' Disease (American Trypanosomiasis) is a chronic infection caused by the protozoan 105 parasite Trypanosoma cruzi. It is endemic in Latin America, but cases are also reported in non-106 endemic regions, with approximately 6-7 million people estimated to be infected worldwide 107 [26]. During the acute phase, the parasite invades cells from tissues such as muscle, liver, gut, 108 lymph nodes, spleen, and the CNS, where it actively replicates, inducing cell death and tissue 109 damage. Consequently, the acute phase of this infection is associated with high parasitemia and 110 nonspecific symptoms. Type 1 immunity, characterized by elevated levels of proinflammatory 111 cytokines like IFN-y [27–29], along with innate and adaptive immune cells like NK cells, inflammatory macrophages, and CD8+ T lymphocytes [28,30,31], work together to minimize 112 113 parasite replication and burden. However, this immune response is insufficient to completely 114 eradicate the parasite from the host, leading to chronic infection. During the chronic phase, around 30% of infected individuals will develop specific symptoms related to digestive or cardiac 115 116 pathology after 10-30 years [32]. Presently, evidence suggests that both parasite persistence 117 and a sustained inflammatory environment mediate the characteristic tissue damage observed 118 in this disease during the chronic phase [33]. In addition to the classical pathology, muscular

pain and weakness are frequently observed in both acute and chronic Chagas' patients [34–36].
Furthermore, an association between SM parasitism and myositis, with structural alterations of
muscular fibers, has been demonstrated in chronically infected humans [37,38], as well as in
mouse models of acute and chronic infection [39–43].

123 In this study, we used a mouse model of acute *T. cruzi* infection to investigate trTregs and their 124 association with tissue damage and protective immunity. We examined trTregs dynamics in this 125 infection scenario, characterized by a unique combination of elevated systemic tissue injury and 126 a limited Tregs response [44]. We found reduced trTregs numbers in target tissues that 127 correlated with reduced systemic IL-33 levels. By supplementation with recombinant IL-33, we 128 dissected the impact of trTregs and other IL-33-responsive immune subsets on tissue damage, 129 parasite control, and infection progression. Through this work, we shed light on the intricate 130 balance between microbicidal and regenerative responses driven by trTregs and the IL-33/ST2 131 axis, an area still inadequately defined in infections and of particular relevance to the 132 progression of acute Chagas' Disease.

133

134 RESULTS

Systemic tissue damage during acute *T. cruzi* infection associates with tissue parasitism and immune infiltrate

As a first step to dissect the ability of trTregs to control damage, we initially assessed the extent and progression of tissue injury in our experimental model of acute *T. cruzi* infection. As established in our lab [45], intraperitoneal injection of 5,000 trypomastigotes (Tulahuen strain) induces a peak of parasitemia at 21 days post-infection (dpi) (Fig 1A). This is accompanied by the maximum parasite load in tissues such as SM, heart, spleen, and liver (Fig 1B), as well as the peak of immune cell expansion in the spleen (Fig 1C). In agreement with previous findings [39,46], histological examination of SM at the infection peak revealed the presence of parasite
nests and diffuse mononuclear infiltrate associated with necrosis and calcification of muscular
fibers, absent in samples from non-infected (NI) mice (Fig 1D). Quantification of the immune
infiltrate in SM showed that leucocyte counts were also at their maximum at 21 dpi (Fig 1E).

147 To further elucidate the impact of T. cruzi infection on muscle physiology, we conducted a 148 whole-tissue RNAseq comparing infected (INF) versus NI quadriceps. The transcriptome analysis 149 identified 1621 differentially expressed genes (DEGs) between both conditions. Non-supervised 150 pathway analysis of the DEGs using EnrichR revealed that, in addition to pathways associated 151 with immune responses such as interferon-gamma, interferon-alpha and complement 152 responses, several pathways related to muscle physiology such as oxidative phosphorylation, 153 myogenesis and adipogenesis were among the most significantly enriched pathways (Fig 1F). As 154 expected, volcano plots revealed that most genes associated with the pathways linked to 155 immune responses were upregulated by the infection (S1A Fig and S1 Table). In contrast, the 156 majority of genes linked to muscle physiology pathways were downregulated (Fig 1G and S1 157 Table), supporting the notion that acute infection disrupted SM homeostasis. Consistent with 158 histological and transcriptomic evidences of SM damage, we found increased plasma activity of 159 creatine phosphokinase (CPK) and creatine phosphokinase of muscle and brain (CPK-MB) at 21 160 dpi compared to NI mice (Fig 1H). The alteration of additional markers of systemic damage such 161 as increased activity of lactate dehydrogenase (LDH), glutamic oxaloacetic transaminase (GOT) 162 and glutamic pyruvic transaminase (GPT), along with hypoglycemia, indicated affection of 163 various target tissues beyond SM, as previously reported by our group [44,47]. As expected, the 164 greatest tissue alteration coincides with highest parasitemia counts and tissue parasitism (Figs 165 1A and 1B) as well as with the peak of immune cells expansion in the spleen (Fig 1C) and 166 maximum immune infiltration in SM (Fig 1E), and other target tissues such as heart and liver 167 (S1B Fig). Given these features, the acute phase of *T. cruzi* infection emerged as an instrumental 168 setting to study trTregs roles during the infection.

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170 Bona fide trTregs are reduced in target tissues and lymphoid organs at the peak of

171 infection

172 Given that the greatest alteration of biochemical markers of tissue damage was observed at the 173 peak of infection, we selected 21 dpi as the initial time point to evaluate by flow cytometry the 174 presence of total Tregs as well as of trTregs in different T. cruzi target sites. As previously 175 documented [44], T. cruzi infected mice exhibited reduced frequencies of total Tregs in spleen 176 and liver around the peak of the infection (S2A and S2B Figs). An even more pronounced 177 decrease was observed when analyzing other peripheral tissues known to be common parasite targets, such as SM and the heart. We then quantified trTregs that were identified within the 178 179 Foxp3+ Tregs population according to their co-expression of ST2 and KLRG-1, as proposed by 180 Delacher et al. [5]. We detected a notable reduction in the presence of trTregs, both in terms of 181 frequency within total Tregs and in absolute numbers, in SM, liver and spleen from INF animals 182 compared to NI controls (Figs 2A and 2B). This subset was not evaluated in the heart due to the 183 remarkably low infiltration of total Tregs, which impeded a more in-depth examination of this 184 organ.

185 Afterwards, we determined whether trTregs identified through the co-expression of ST2 and 186 KLRG-1 in the context of the infection possessed distinctive phenotype of *bona fide* tissue repair 187 cells, setting them apart from classic ST2- KLRG-1- lymphoid-like Tregs as previously described 188 in other experimental settings [5,7,48–52]. Thus, we compared the expression of transcription 189 factors (BATF, IRF4and Ki-67) and surface molecules (CD44, CD62L, TIGIT, ICOS, GITR, PD-1 and 190 CTLA-4), as well as amphiregulin (Areg) production by flow cytometry in trTregs and ST2- KLRG-191 1- Tregs from spleen of INF and NI mice identified as depicted in Fig 2A. As shown in the 192 representative histograms from S3 Fig and summarized in the heatmap in Fig 2C, the expression 193 levels of most evaluated markers, with the exception of CD62L, were higher in trTregs compared 194 to ST2- KLRG-1- Tregs obtained from the spleen of NI mice. Notably, trTregs from the spleen of 195 INF mice also exhibited a bona fide trTregs phenotype, displaying even stronger phenotypic 196 features in comparison to their ST2- KLRG-1- Tregs counterparts, with the highest expression of 197 certain markers as BATF, IRF4, ICOS and GITR along with reduced CD44, TIGIT and PD1 levels. A 198 principal component analysis (PCA) of the phenotypic data demonstrated that while ST2- KLRG-199 1- Tregs from spleens of NI and INF mice clustered closely, trTregs segregated apart along PC1, 200 that explains around 70% of the variance among the samples (Fig 2D). Furthermore, trTregs from 201 NI and INF mice showed some differences between them at expense of PC2, which explains 202 around 20% of the variance mainly driven by CD62L expression.

As trTregs have been shown to increase once the tissue damage is well established [11,12,18– 204 21], we evaluated trTregs response along the acute phase of the infection (Fig 2E). Despite 205 observing a transient increase in trTregs numbers in the liver at 10 dpi, we found a significant 206 reduction in the frequency and absolute number of this cell subset at 21 dpi in all evaluated 207 tissues. It is noteworthy that while trTregs absolute numbers recover at later time points, they 208 do not substantially increase as observed in other injury models.

In summary, our results indicate that during acute *T. cruzi* infection, despite the severe systemic
tissue damage, trTregs are particularly reduced within an already restricted total Treg pool. The
few remaining trTregs found at the peak of infection share several characteristics with *bona fide*trTregs, presenting particularities likely as a consequence of the infection.

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214 Systemic IL-33 levels are reduced during acute *T. cruzi* infection.

215 It is established that trTregs development is a multi-step process that initiates with a 216 specialization commitment in secondary lymphoid organs and culminates after migration into 217 residence tissues [6–8]. Remarkably, IL-33 has been shown to play a role in all these different 218 stages [53]. In light of the general decline in trTregs numbers despite the elevated tissue damage 219 around the peak of acute infection, we performed a kinetic study to assess IL-33 concentrations 220 at both systemic and peripheral sites. Our analysis revealed that plasma IL-33 concentration was 221 detectable in NI mice and diminished during the course of acute T. cruzi infection, showing a 222 statistically significant decrease at 21 dpi, followed by a return to baseline levels at 35 dpi (Fig 223 3A). A similar trend was observed when quantifying IL-33 in the spleen homogenates (S4A Fig). 224 In contrast, the quantification of total IL-33 in homogenates from target tissues showed an 225 increased concentration at 21 and 35 dpi in SM (Fig 3A) while it remained relatively constant 226 throughout the tested period in the liver (S4A Fig). These results indicate that, despite a 227 conservation or increase in IL-33 levels in peripheral non-lymphoid tissues, its concentration is 228 reduced at systemic level and in secondary lymphoid tissues, suggesting that the initial steps of 229 trTregs development may be affected during *T. cruzi* infection.

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IL-33 supplementation expands trTregs from acutely infected animals *in vitro* but fails to prevent trTregs reduction in established infection.

233 To investigate the possible role of trTregs in managing damage and the progression of pathology 234 in *T. cruzi* infection, we aimed to increase trTregs numbers by administrating recombinant IL-33 (rIL-33) in our experimental setting. As a proof of principle, we first evaluated the responsiveness 235 236 of trTregs from INF mice to IL-33. To this end, splenic Tregs (CD4+ Foxp3-GFP+) sorted from INF 237 mice and NI mice were stimulated with anti-CD3, anti-CD28 and rIL-2 in the presence or absence 238 of rIL-33 (Fig 3B). The addition of IL-33 to cultures containing Tregs from INF mice resulted in an 239 expansion of trTregs, leading to a final percentage of ST2+ KLRG-1+ Tregs comparable to that 240 obtained in IL-33-supplemented NI Tregs cultures. Notably, IL-33 supplementation failed to 241 induce ST2 and KLRG-1 expression in conventional T cells (CD4+ Foxp3-GFP-) obtained from the 242 spleens of either INF or NI animals (S4B Fig). These results indicate that rIL-33 specifically acts 243 on the Tregs pool by expanding trTregs even when they are isolated from the *T. cruzi* infection

244 environment, supporting its potential for treating INF animals.

245 For the *in vivo* treatment, with the aim of preventing the infection-induced reduction of trTregs, 246 we administered rIL-33 (or PBS as control) intraperitoneally at 12, 15 and 18 dpi, as schematized 247 in Fig 3C. In an alternative approach, mice were co-administered with rIL-2 and rIL-33 248 considering the relevance of IL-2 for Tregs survival [54] and taking into account that systemic 249 levels of this cytokine remain unchanged even after the increased cell demand resulting from T 250 cell expansion during acute T. cruzi infection [44]. As a positive control of its biological activity, 251 we observed that in vivo intraperitoneal (i.p.) administration of IL-33 to NI animals led to trTregs 252 expansion (Fig 3D), as previously reported [5]. Remarkably, the treatment with rIL-33 or rIL-33 253 plus rIL-2 in INF mice failed to increase trTregs frequency or absolute numbers in SM and spleen 254 when compared to PBS-treated controls (Fig 3E). The injection of rIL-33 alone had effect on the 255 liver, resulting in an increase in trTregs frequency and a subsequent recovery in trTregs absolute 256 numbers, which reached levels similar to those observed in the livers of untreated NI mice 257 (represented by grey dashed lines) (Fig 3E). To further address whether IL-33 supplementation 258 had an impact on tissue damage or infection progression, despite the limited effect on trTregs, 259 we initially examined the plasma levels of biochemical markers indicative of tissue damage. The 260 treatment with rIL-33 did not improve these markers, while the injection of rIL-2 plus rIL-33 261 appeared to worsen them compared to PBS-treated controls (S4C Fig). Moreover, global 262 indicators of disease progression, such as total weight loss at the peak of infection and overall 263 survival, also showed no differences between treated and control mice (S4D and S4E Figs).

Since systemic (i.p.) treatment with IL-33 in INF mice did not yield any significant effects on the evaluated parameters, we speculated that local administration in peripheral tissue may better target trTregs. Therefore, we tested the effect of intramuscular (i.m.) rIL-33 injections. In this approach, INF mice were injected at 12, 15 and 18 dpi with rIL-33 (0.3µg per muscle) in one hind

limb and with PBS in the other as control, according to the procedure described by Kuswanto et
al., 2016 [12]. Similar to systemic treatment, i.m. rIL-33 injection in NI animals resulted in an
accumulation of Tregs in SM, which contained a high proportion of trTregs (S4F Fig), while it had
no effect on INF animals (S4G Fig).
Overall, these results indicate that, although trTregs from INF mice are intrinsically able to
respond to IL-33, the reduction of trTregs that occurs during acute *T. cruzi* infection cannot be

274 rescued by rIL-33 administration, likely due to particular signals generated in the context of an
275 established infection.

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277 Inflammatory and microbial-derived signals are unable to restrict IL-33 mediated

278 expansion of trTregs in vitro.

279 To comprehend the mechanisms contributing to the limited impact of rIL-33 on inducing trTregs 280 during T. cruzi infection, we directed our attention towards molecules known to counteract IL-281 33's biological effects, potentially activated by this parasitic infection. Specifically, we examined 282 soluble ST2 (sST2), a spliced variant of ST2 lacking the cytosolic and transmembrane domains, 283 which acts as a decoy receptor to neutralize IL-33 activity under various inflammatory conditions 284 [55]. Our findings revealed undetectable levels of sST2 in the serum from both NI and INF mice 285 (Fig 4A). Within tissues, sST2 levels were shown to be similar between INF mice and NI 286 counterparts in spleen and SM lysates while in the liver, sST2 levels could not be quantified as 287 they were remarkably high, surpassing the methodological higher detection point even in 288 diluted lysates.

Beyond sST2, several pro-inflammatory cytokines such as IL-1β, IL-27, IFN-γ and TNF have been
demonstrated to prevent IL-33 effects on target cells [56–58]. Indeed, the latter two can block *in vivo* the expansion of trTregs induced by rIL-33. Given the significant increase in some of these

292 and other inflammatory signals like IL-6, IL-12 and IL-18 during acute T. cruzi infection [44,59,60], 293 we explored their potential role in preventing the IL-33-mediated trTregs expansion in our 294 experimental setting. To this end, we took advantage of the *in vitro* approach described in Fig. 295 3B. Sorted splenic Tregs from NI mice were cultured in the presence of the trTregs expansion 296 cocktail (anti-CD3, anti-CD28 plus rIL-2 and rIL-33) along with other recombinant cytokines, 297 conditioned media or other stimuli (Fig 4B). None of the individual cytokines tested nor a Th1 298 differentiation cocktail (IL-12 plus anti-IL-4) were capable of preventing the expansion of trTregs 299 induced by IL-33. We also considered that, in the context of T. cruzi infection, multiple 300 inflammatory signals may concurrently exist and synergize to block IL-33's effect. Therefore, we 301 simulated these conditions using conditioned media obtained after 24-hour of polyclonal 302 stimulation of splenocytes obtained from NI or INF (10 and 21 dpi) mice. None of these 303 conditioned media were able to abrogate the IL-33-induced trTregs expansion. Then, a second 304 approach involving transwell cultures was designed to emulate the early stages of infection. In 305 this assay, where Tregs were co-cultured in the upper transwell chamber with splenocytes of NI 306 mice either alone or in the presence of trypomastigotes, IL-2 and IL-33 also retained their 307 capacity to expand trTregs.

Finally, considering that the maximal trTregs reduction correlates with the highest parasitemia, and taking into account previous results indicating that Treg differentiation is affected by the presence of parasites [44], we further evaluated a possible inhibitory effect from microbial ligands. To this end, heat-killed or lysed trypomastigotes were added to the cultures, however, even in the presence of these sources of microbial ligands, trTregs cell expanded normally in the presence of IL-2 + IL-33 (Fig 4C).

Collectively, these findings suggest that the ineffectiveness of IL-33 treatment in promoting trTregs cells is not likely due to increased levels of a well-known IL-33 modulator such as sST2 or the influence of prominent inflammatory cytokines or microbial ligands heightened in the

317	context of <i>T. cruzi</i> infection. Instead, a complex combination of different signals, an unidentified
318	modulator, or other mechanisms may underlie the absence of effect after IL-33 injection in
319	established <i>T. cruzi</i> infection.

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321 Early rIL-33 administration expands trTregs and improve disease outcome in infected

322 mice.

In a subsequent effort to modulate the trTregs response *in vivo* during acute infection, and considering that results from the previous section suggested that IL-33 injection expands trTregs *in vivo* in NI mice but its effects are restrained by an unidentified signal(s) emerging along the infection, we opted to treat INF mice with rIL-33 on 0, 3, and 6 dpi (Fig 5A).

327 Early IL-33 injection expanded trTregs in infected (INF) mice, as evidenced by the tendency for 328 trTreg frequency and absolute numbers to increase in SM, alongside significant increases 329 observed in the liver and spleen by 21 dpi (Figs 5B, 5C and S5A Fig). Of note, trTregs numbers 330 from IL-33 treated INF animals exceeded those observed in untreated NI mice (S5A Fig. 331 represented by grey dashed lines). These results illustrated the efficiency of early IL-33 332 treatment in sustaining elevated trTregs throughout at least the two-week period up to the peak 333 of the infection, raising the possibility it may impact on tissue repair mechanisms and infection 334 progression. To assess this, we measured several parameters such as biochemical markers of 335 tissue damage, total body weight loss and SM strength (S5B-D Figs). The analysis of the outcome 336 of these parameter evaluations revealed an overall improvement in the health state of the IL-337 33-treated group at 21 dpi, as summarized in the heatmap in Fig 5D. To characterize changes 338 occurring in target tissues with IL-33 treatment, we performed histological analysis on SM. The 339 results shown similar alterations in SM architecture between both experimental groups, 340 characterized by the presence of a diffuse mononuclear infiltrate, necrosis and calcification of

341 muscular fibers. Additionally, all mice showed scarce number of centrally nucleated muscle cells,

342 which are associated with tissue regeneration [61] (S5E Fig).

The responsiveness to IL-33 is mediated by ST2, expressed in different immune cell types [16,55]. Therefore, it is expected that our early IL-33 treatment during *T. cruzi* infection may affect other ST2-expressing cell subsets involved in tissue damage control such as type 2 Innate lymphoid cells (ILC2) [49,62]. Accordingly, we assessed ILC2 infiltrate in different tissues using the gating strategy shown in S5F Fig, adapted from the protocol reported by Tait Wojno and Beamer [63]. As anticipated, ILC2 frequency and absolute numbers were also increased after rIL-33 injection during early *T. cruzi* infection (Figs 5E and 5F, and S5G Fig).

350 In addition to trTregs and ILC2, IL-33 can activate effector immune cells either directly through 351 ST2 signaling on the target cell or indirectly by inducing the production of intermediate cues, 352 such as IFN-y [10,55,64,65]. We, therefore, evaluated if early IL-33 administration affected 353 parasite specific CD8+ T cells, an effector response critical for T. cruzi control [66]. Interestingly, 354 IL-33 treated animals showed increased frequencies of this cell subset in SM, liver as well as in 355 spleen (Figs 5G and 5H). These changes in frequency corresponded with a tendency toward 356 higher counts of parasite-specific CD8+ T cells in SM, conserved counts in the liver, and increased 357 counts in the spleen (S5H Fig). In correlation with these results, INF mice that received IL-33 358 injection showed significantly decreased parasitism in non-lymphoid tissues such as SM, heart 359 and liver (Fig 5I).

Altogether, these findings demonstrate that early rIL-33 administration can improve the course
of acute infection, not only by reducing tissue damage, but also by increasing parasite control.
These results may be attributed to the combined effect of IL-33 on different immune cell subsets
such as trTregs, ILC2 and effector CD8+ T cells.

364

365 DISCUSSION

366 Previous reports indicate that tissue injury, whether sterile or infection-derived, is associated 367 with trTregs accumulation due to increased IL-33 release after cell destruction [16]. This 368 reparative mechanism is well described in sterile damage, particularly within SM [4,11,12]. In 369 the context of acute *T. cruzi* infection—a model for Chagas' disease with a profound compromise 370 of various target tissues, including SM—our results unveil a novel scenario marked by a 371 diminished trTregs response and reduced plasmatic IL-33 concentration despite extensive tissue 372 damage. This suggests a specific impairment in the regulatory fate associated with tissue repair, 373 complementing our earlier work which reported limited Tregs responses but with the acquisition 374 of a type 1 specialization profile during *T. cruzi* infection [44]. Altogether, our findings delineate 375 a scenario where the reduction of a reparative/regulatory cell subset during the acute phase 376 may potentially facilitate tissue damage by compromising physiological repair mechanisms. 377 Indeed, a detailed characterization of tissue damage associated with T. cruzi infection, 378 particularly in SM, revealed a clear modification in the SM transcriptome with activation of 379 immune-related pathways and a general downregulation of genes associated with myogenesis, 380 adipogenesis, and oxidative phosphorylation pathways, linked to normal muscle physiology and 381 repair processes after injury [67–69]. Therefore, our results provide evidence that during acute 382 T. cruzi infection, the transcriptional landscape in SM denotes damage and correlates with a 383 reduced frequency of trTregs. This association may potentially connect deficient repair 384 processes with long-term consequences in chronic immunopathology during Chagas' disease. 385 Further studies may be required to deeply assess these possible connections.

The attenuation of the reparative response during acute *T. cruzi* infection was systemic, manifested by reduced frequency and absolute numbers of trTregs across evaluated tissues, including SM, liver, and spleen. Unexpectedly, IL-33 levels exhibited both consistency and discrepancy: plasma IL-33 concentration diminished, correlating with the limited trTregs

390 responses, while muscle IL-33 increased, yet failed to prevent the reduction of trTregs in that 391 tissue. Two aspects of these findings were puzzling: the unexpected reduction in systemic IL-33 392 levels despite documented tissue damage in *T. cruzi* infection and the lack of an accompanying 393 increase in trTregs in muscle despite an elevated local IL-33 concentration. The mechanisms 394 regulating IL-33, which are complex and varied, include its rapid degradation after tissue injury 395 [16,70,71]. For example, caspases 1 and 7, produced in inflammatory contexts and involved in 396 the death of infected cells, fragment IL-33 to inactivate it [72]. Additionally, IL-33 is easily 397 oxidizable in the extracellular space, limiting ST2-dependent immunological responses [73]. 398 Accordingly, the inflammatory milieu during acute *T. cruzi* infection could rapidly oxidize and/or 399 degrade IL-33, diminishing its availability, especially in plasma. In contrast, elevated IL-33 levels 400 were detected in muscle. However, this quantification was conducted in tissue lysates, 401 presenting technical limitations that impede confirmation of the extracellular presence and, 402 consequently, the availability of IL-33 for ST2+ cells. Discrepancies between IL-33 concentration 403 and trTregs numbers in SM suggest potential unavailability or counteraction by inflammatory 404 signals. Alternatively, trTregs cells may face high mortality or reduced generation during the 405 acute stage. Considering the multi-step development of trTregs beginning in the spleen [8,74], 406 the reduction in IL-33 levels in this organ during acute infection could be linked to a lower 407 generation of these cells and, consequently, their reduced arrival at target organs.

408 Given the scenario described earlier, and our aim to understand the impact of trTregs reduction 409 on disease progression, we intended to boost the numbers of this cell subset through rIL-33 410 supplementation in established T. cruzi infection. However, IL-33 treatment, administered 411 systemically or locally around the second week of infection, failed to rescue trTregs numbers 412 and had no effect on disease progression. Notably, the lack of response to IL-33 in terms of 413 trTregs expansion was specific to the infection condition, as this cytokine increased trTregs in 414 non-infected animals. The ability of trTregs from infected mice to expand upon IL-33 stimulation 415 in vitro suggests intrinsic responsiveness to this growth factor, pointing to a restrictive in vivo 416 environment imposed by the infection. As we established that inflammatory environments or 417 even the parasite itself might negatively affect Tregs differentiation [44], and considering that 418 inflammatory cytokines can impede IL-33 signaling in trTregs [57], we investigated potential 419 infection-associated cues that could counteract IL-33's effect. We found that sST2, a natural 420 decoy receptor of IL-33 signaling [55], is not increased during T. cruzi infection, suggesting that 421 it is unlikely involved in the lack of response to IL-33 in our infection setting. Further evaluation 422 through in vitro approaches, combining rIL-33 with various inflammatory or parasite signals, 423 failed to identify any molecule, cocktail, or conditioned media capable of blocking IL-33-424 mediated expansion of trTregs. These results emphasize that the intricate combination of signals 425 present in a living host undergoing acute T. cruzi infection is challenging to replicate with in vitro 426 approaches, making it difficult to identify critical determinants of an environment particularly 427 restrictive for regulatory pathways.

428 As an alternative strategy to assess IL-33 effects while avoiding the restrictive environment 429 around the peak of T. cruzi infection, we implemented early treatment during the first week of 430 infection. This approach resulted in a less severe acute infection progression along with better 431 parasite control. Given that IL-33 is able to modulate several immune cell subsets [70], we 432 focused not only on trTregs but also on ILC2 and CD8+ T cells. Early IL-33 supplementation during 433 T. cruzi infection led to a significant expansion of trTregs, consistent with previous reports 434 [12,20,24]. Notably, the magnitude of the trTregs response increased not only in secondary 435 lymphoid organs such as the spleen but also in target organs like the liver and SM, remaining 436 evident even at the infection's peak. Interestingly, early IL-33 treatment also elevated ILC2 and 437 parasite-specific CD8+ T cell frequencies. ILC2 play crucial roles in combating certain infectious 438 agents and, similar to trTregs, promote tissue reparative processes [62]. Indeed, IL-33-mediated 439 ILC2 expansion has been linked to infection resistance against cerebral malaria and various 440 intestinal infections [23,75–77]. Moreover, our comprehensive evaluation of immune cell 441 subsets highlighted that IL-33 potentiated antiparasitic CD8+ T cell immunity, aligning with

previous research that suggested a role for this alarmin in inducing robust antiviral responses
[78,79]. In particular, IL-33 has been recently shown to preserve CD8+ T cell stemness and reexpansion capacity in the context of a chronic viral infection [80].

445 In summary, our findings underscore the positive impact of IL-33 supplementation during early 446 T. cruzi infection, contributing to reduced tissue damage and improved parasite control. Building 447 upon existing research, we propose that this beneficial effect arises from the concerted action 448 of expanding cell subsets involved in tissue repair, such as trTregs and ILC2, alongside those 449 engaged in microbial control, particularly CD8+ T cells. Future studies are warranted to 450 meticulously dissect the specific roles of each of these immune cell subsets in mediating the 451 effects of IL-33 on T. cruzi infection outcome. Interestingly, the simultaneous expansion of trTregs and parasite-specific CD8+ T cells might seem counterintuitive, given numerous reports 452 453 linking regulatory responses with diminished antimicrobial immunity [81]. Our previous study in 454 the context of T. cruzi infection demonstrated that adoptive Tregs transfer represses parasite-455 specific CD8+ T cell responses [44]. Furthermore, it has been shown that tissue resident Tregs 456 exhibit heightened regulatory features [25]. Significantly, our results challenge this notion by 457 revealing that boosting specialized regulatory T cell responses does not necessarily attenuate effector responses. This simultaneous enhancement of both regulatory/reparative and 458 459 antiparasitic cell subsets, likely a result of the individual effects of IL-33 on each ST2-expressing 460 subset, proves to be beneficial for the progression of acute T. cruzi infection. This discovery 461 establishes a precedent, suggesting the potential for rational novel therapies in Chagas' Disease 462 or other infectious diseases. To date, only one study has evaluated IL-33 expression in chronic 463 Chagas disease patients showing no correlation with disease severity [82]. Therapies involving 464 IL-33 could be designed to modulate the immune system, favoring a specific combination of 465 regulatory and effector responses. The goal would be to enable effective pathogen clearance 466 while minimizing collateral damage, thereby preventing clinical pathology.

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467

468 MATERIALS AND METHODS

469 Ethics statement

- 470 Mouse handling followed international ethical guidelines. All experimental procedures were
- 471 conducted in compliance with the ethical standards set by the Institutional Animal Care and Use
- 472 Committee of Facultad de Ciencias Químicas Universidad Nacional de Córdoba, and were
- 473 approved under protocol numbers RD-733-2018.

474

475 **Mice.**

Age-matched (8 to 12 week-old) mice of both sexes were used. Foxp3-GFP reporter mice (B6.Cg-Foxp3tm2Tch/J) were obtained from The Jackson Laboratories (USA). BALB/c mice were obtained from School of Veterinary, La Plata National University (La Plata, Argentina). Animals were bred in the animal facility of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, and housed under a 12:12 h light-dark cycle with food and water ad libitum. The institutional animal facility follows the recommendations of the Guide for the Care and Use of Experimental Animals, published by the Canadian Council for the Protection of Animals.

483

484 **Parasites and experimental infection.**

For all experiments, *T. cruzi* Tulahuén strain was used. Bloodstream trypomastigotes were maintained in male BALB/c mice by serial passages every 10-11 days. For *in vivo* assays, Foxp3-GFP reporter mice were inoculated intraperitoneally with 0.2 mL PBS containing 5000 trypomastigotes.

489 For in vitro assays, trypomastigotes were obtained from the extracellular medium of infected 490 monolayers of Vero cells cultured in RPMI 1640 medium (Gibco, Invitrogen) containing 10% heat 491 inactivated fetal bovine serum (FBS, Natocor), 2 mM glutamine (Gibco), 10 mM HEPES (Gibco) 492 and 40 ug/mL gentamicin (Veinfar Laboratories). After 7 days of infection, extracellular medium 493 was collected, centrifuged at 1800 g for 30 min at room temperature and incubated for 2 h at 494 37 °C. Trypomastigotes were recovered from the supernatant and counted using a Neubauer 495 chamber. Heat-killed trypomastigotes were obtained after incubation at 56 °C for 10 min 496 (adapted from [83]), while lysed trypomastigotes were obtained after 3 cycles of freeze/thaw 497 and 5 minutes of sonication (adapted from [84]).

498

499 **Parasite quantification in blood and tissues.**

500 Parasitemia was monitored by counting the number of viable trypomastigotes in blood after 501 lysis with a 0.87% ammonium chloride buffer. For tissue parasite quantification, genomic DNA 502 was purified from 50 µg of tissue (spleen, liver, SM and heart) using TRIzol Reagent (Life 503 Technologies) following manufacturer's instructions. Satellite DNA from T. cruzi (GenBank 504 AY520036) was guantified by real time PCR using specific Custom Tagman Gene Expression 505 Assay (Applied Biosystem) using the primer and probe sequences described by Piron et al. [85]. 506 The samples were subjected to 45 PCR cycles in a thermocycler StepOnePlus Real-Time PCR 507 System (Applied Biosystems). Abundance of satellite DNA from *T. cruzi* was normalized to the 508 abundance of GAPDH (Tagman Rodent GAPDH Control Reagent, Applied Biosystem), quantified 509 through the comparative CT method and expressed as arbitrary units, as previously reported 510 [44,47,86].

511

512 Cell preparation.

513 To obtain cell suspensions from solid tissues, euthanized mice were perfused with 10 mL cold 514 Hanks' Balanced Salt Solution (Gibco). Spleens and livers were obtained and mashed through a 515 tissue strainer. Liver infiltrating cells were obtained after 25 min centrifugation (600 g without 516 brake) in a 35% and 67.5% bilayer Percoll (GE Healthcare) gradient. The interphase containing 517 leukocytes was recovered and washed. Erythrocytes in spleen and liver cell suspensions were 518 lysed for 3 min in ACK Lysing Buffer (Gibco). Heart and SM (quadriceps, gastrocnemius and 519 tibialis anterior) were excised, minced and digested for 30 min in collagenase D (2 mg/mL, 520 Roche) and DNase I (100 μ g/mL, Sigma). Digested tissues were filtered through a 70 μ m filter 521 and washed. Infiltrating leucocytes were obtained after 25 min centrifugation (600 g without 522 brake) in a 40% and 75% bilayer Percoll gradient. The interphase was recovered and washed. 523 Cell numbers were counted in Turk's solution using a Neubauer chamber.

524

525 In vitro assays

526 For Tregs and Tconv culture, cells were purified from NI or 21 dpi Foxp3-GFP mice. CD4+ cells 527 were isolated from pooled splenic suspensions by magnetic negative selection using EasySep 528 Mouse CD4+T Cell Isolation Kit (StemCell Technologies) according to manufacturer's instruction. 529 Afterwards, the enriched CD4+T cell suspension was surface stained and Tregs and Tconv were 530 further purified by cell sorting with a FACSAria II (BD Biosciences) according to the following 531 phenotype: Tregs (CD4+ Foxp3-GFP+) and Tconv (CD4+ Foxp3-GFP-). Purified cells (75000 532 cells/well) were cultured for 3 days in 96-well U bottom plates coated with 2 μ g/mL anti-CD3 (eBioscience) and 1 µg/mL anti-CD28 (eBioscience) supplemented with 10ng/mL of recombinant 533 534 mIL-2 (Biolegend) to allow Treg survival. Cells were cultured in complete culture media 535 containing RPMI 1640 medium (Gibco, Invitrogen) 10% heat inactivated FBS (Natocor), 2mM 536 glutamine (Gibco, Invitrogen), 55uM 2- mercaptoethanol (Gibco, Invitrogen) and 80ug/mL 537 gentamicin (Veinfar Laboratories). When indicated, media contained 50 ng/mL recombinant 538 mIL-33 (R&D) alone or combined with the following murine recombinant cytokines: IFN-γ (50

539 ng/mL, Immunotools), TNF (50 ng/mL, Immunotools), IL-1β (2 ng/mL, R&D), IL-6 (20 ng/mL,

- 540 Shenandoah), IL-12p70 (10 ng/mL, Peprotech), IL-18 (50 ng/mL, R&D), IL-27 (20 ng/mL, R&D) as
- $\label{eq:source} 541 \qquad \mbox{well as with 2}\ \mbox{\mug/mL anti-mouse IL-4 (Peprotech). Alternatively, 50}\ \mbox{\muL of conditioned media was}$
- 542 used.
- 543 In co-culture transwell experiments, Tregs from NI mice were placed in the bottom of the culture
- 544 plate in the presence of recombinant mIL-33 and splenocytes (1:1 ratio) alone or with
- 545 trypomastigotes (1:10 ratio) that were placed in the transwell chamber.
- 546 In co-cultures with parasite ligands, Tregs from NI animals were incubated with heat-killed or
- 547 lysed trypomastigotes (ratio 1:1) in the presence of recombinant mIL-33.

548

549 **Conditioned media generation**

550 For conditioned media, total splenocytes were isolated from pooled splenic suspensions of NI, 551 10 dpi and 21 dpi Foxp3-GFP mice. Cell suspensions (5x10⁶ cells/ml) were cultured for 24 h in 552 24-well plates in complete culture media supplemented with 50 ng/mL PMA and 1 μg/mL 553 ionomycin (Sigma-Aldrich).

554

555 Biochemical determinations

Plasma was collected after blood centrifugation for 8 min at 3000rpm. Quantification of biochemical markers of tissue damage was performed at Laboratorio Biocon (Córdoba, Argentina) using a Dimension RXL Siemens analyzer. GOT, GPT, LDH and CPK activity was determined by UV kinetic method, CPK-MB activity was evaluated by enzymatic method, while glucose concentration was assessed by kinetic/colorimetric method. 561

562 IL-33 and sST2 quantification

563 IL-33 concentration was determined with an IL-33 Mouse ELISA kit (eBioscience), while sST2 was 564 quantified using a Mouse ST2/IL-33R DuoSet ELISA kit (R&D Systems) in plasma and tissue 565 lysates. Plasma samples were obtained as previously described. Tissue lysates were obtained 566 after centrifugation at 10000g during 10 min of tissue samples homogenized in PBS containing 567 0,5% BSA, 0,4 M NaCl, 1 mM EDTA, 0,05% Tween 20 and a protease inhibitor cocktail (Sigma-568 Aldrich) (adapted from [87]). GraphPad Prism 8.0.1 software was used to generate the 569 calibration curve and determine IL-33 and sST2 concentration. In tissue lysates, values were 570 normalized to total protein content determined using Bradford reagent (BioRad). Two Synergy 571 HT Multi-mode microplate reader (Biotek) was used to determine absorbances at 450 nm (ELISA) 572 and 595 nm (protein quantification).

573

574 Flow cytometry

575 Combinations of the following antibodies were used for flow cytometry: biotin polyclonal anti-576 Amphiregulin (R&D Systems), PE anti-BATF clone S39-1060 (BD Pharmingen), Super Bright 645 577 anti-CD11b clone M1/70 (eBioscience), PE-Cyanine7 anti-CD11c clone N418 (eBioscience), PE anti CD127 clone A7R34 (eBioscience), PE-Cyanine7 anti-CD19 clone eBio1D3 (eBioscience), PE-578 579 Cyanine7 anti-CD3 clone 145-2C11 (eBioscience), APC, Super Bright 645 and APC-eFluor 780 580 anti-CD4 clone GK1.5 (eBioscience), PE-Cyanine5 anti-CD44 clone IM7 (eBioscience), Alexa Fluor 581 700 and APC-Cyanine7 anti-CD45 clone 30-F11 (eBioscience and BD Pharmingen respectively), 582 APC-eFluor 780 anti-CD62L clone MEL-14 (eBioscience), PE-Cyanine5.5 anti-CD8 clone 53-6.7 583 (eBioscience), Brilliant Violet 605 anti CTLA-4 clone UC10-4B9 (Biolegend), FITC anti-Foxp3 clone 584 FJK-16s (eBioscience), Super Bright 600 anti-GITR clone DTA-1 (eBioscience), PerCp-eFluor 710 585 anti-ICOS clone 7E.17G9 (eBioscience), PerCp-eFluor 710 anti-IRF4 clone 3E4 (eBioscience),

eFluor 660 anti-Ki-67 clone SolA15 (eBioscience), PE-eFluor 610 anti-KLRG-1 clone 2F1
(eBioscience), PE-Cyanine7 anti-NK1.1 clone PK136 (Biolegend), Brilliant Violet 421 anti-PD-1
clone 29F.1A12 (Biolegend), PE and Brilliant Violet 421 anti-ST2 clone DIH9 (Biolegend) and
PerCp-eFluor 710 anti-TIGIT (eBioscience). To detect biotinylated antibodies, Streptavidin Qdot
605 (Invitrogen) was used.

For surface staining, cell suspensions were incubated with fluorochrome labeled-antibodies together with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation (Invitrogen) in PBS 2% FBS for 20 min at 4°C. To identify *T. cruzi* specific CD8+ T cells, cell suspensions were incubated with an H-2Kb *T. cruzi* trans-sialidase amino acids 569-576 ANYKFTLV (TSKB20) APCor Brilliant Violet 421- labeled Tetramer (NIH Tetramer Core Facility) for 20 min at 4 °C, in addition to the surface staining antibodies.

597 For transcription factors detection, cells were initially stained for surface markers, washed, fixed, 598 permeabilized and stained with Foxp3/Transcription Factor Staining Buffers (eBioscience) 599 according to eBioscience One-step protocol for intracellular (nuclear) proteins. For intracellular cytokine detection, 2×10^6 cells per well were cultured in 200 µL supplemented RPMI 1640 600 601 medium and stimulated during 2 h at 37 °C with 50 ng/mL PMA and 1 µg/mL ionomycin (Sigma-602 Aldrich) in the presence of Brefeldin A and Monensin (eBioscience). Then, stimulated cells were 603 surface-stained as indicated above, fixed and permeabilized with Intracellular Fixation & 604 Permeabilization Buffer Set (eBioscience) or IC Fixation Buffer and permeabilization Buffer 605 (eBioscience) following manufacturers' indications. In all cases, intracellular staining was 606 performed by a 30 min incubation at room temperature.

All samples were acquired on FACSCanto II (BD Biosciences), LSRFortessa (BD Biosciences) or
 Attune-NxT (Life Technologies) and data were analyzed with FlowJo software version X.0.7. For
 cell sorting, FACS Aria II (BD Biosciences) was used.

610

611 RNA sequencing

612 Perfused mouse quadriceps were obtained and stored in RNAlater Stabilization Solution 613 (Invitrogen) at -80°C. Then, 25mg of tissue was dissociated using Bead Ruptor Elite (Omni 614 International) for 45 seconds at 4.85m/s and RNA was isolated using RNeasy Fibrous Tissue Mini 615 Kit 50 (Qiagen) following manufacturer's indications. RNA concentration was determined with a 616 Qubit 2.0 Fluorometer (Invitrogen), while a 2100 Bioanalyzer (Agilent) was used for quality 617 evaluation. Poly(A) mRNA Magnetic Isolation Module (New England BioLabs) was used for cDNA 618 library preparation according to manufacturer's protocol. Quality control of libraries was 619 determined as described for RNA. Quantification of cDNA libraries was performed with PerfeCTa 620 NGS Quantification Kit for Illumina Sequencing Platforms (QuantaBio). For each experimental 621 group, 3 biological replicates were sequenced with NextsSeq 550 (Illumina). For data analysis, a 622 Salmon index was built from Gencode [88] Mouse release M27 (GRCm39) [GENCODE - Mouse 623 Release M27 (gencodegenes.org)] using Salmon [89] v1.5.1 [Release Salmon 1.5.1 · COMBINE-624 lab/salmon · GitHub] with default k-mer size (31) and the --gencode flag. FASTQ sequence reads 625 (SRA accession PRJNA941341) were mapped to the M27 index and transcript abundances were 626 estimated using salmon quant on 8 threads. Salmon quant files were subsequently loaded into 627 R v4.1.0 using tximeta v1.10.0, and differentially expressed genes were called using default 628 parameters in DESeq2 v1.32.0 per the Bioconductor vignette [Analyzing RNA-seq data with 629 DESeq2 (bioconductor.org)]. Genes with adjusted p-values ≤ 0.05 and $|\log_2 \text{ fold-change}| \geq 1$ 630 were considered differentially-expressed and included in downstream analysis. For gene set 631 enrichment analysis, EnrichR tool [90–92] was used an d MSigDB Hallmark 2020 gene sets were 632 interrogated. Volcano plots were generated using "ggplot2" package in R. The datasets 633 generated for this study can be found in the NIH repository under accession number 634 PRJNA941341 (https://www.ncbi.nlm.nih.gov/sra/PRJNA941341).

635

636 In vivo rIL-33 and rIL-2 treatment

637	Recombinant mIL-33 (Shenandoah) was administered via i.p. (2 μ g in a total volume of 200 μ L)
638	or i.m. (0.3 $\mu g/muscle$ in a total volume of 30-50 $\mu L)$ at the specified time points. Dose and
639	frequency of injections were adapted from reported protocols [12,20,24]. For i.m. treatment,
640	quadriceps, gastrocnemius and tibialis anterior from the same hindlimb received each the dose
641	detailed above. When indicated, i.p. injections also contained 1 μg of recombinant murine IL-2
642	(Gibco). PBS was used as vehicle.

643

644 Muscle strength and body weight evaluation

Muscle strength was assessed by Kondziela's inverted screen test (hang test) [93]. Mice were kept in the experimental room for 20 minutes before the test to ensure proper adaptation to the environment. Each mouse was placed in the center of a 43 cm² wire mesh consisting of 12 mm squares of 1 mm diameter wire and surrounded by a 4 cm deep wooden frame. Screen was inverted and time was measured until the mouse fell off. Maximum test duration was 2 minutes. Total body weight was determined using a precision laboratory balance.

651

652 Histological analysis

Perfused mouse quadriceps were fixed in formaldehyde solution and embedded in paraffin. Five
µm thick sections were stained with activated hematoxylin followed by eosin alcoholic solution.
Histopathological evaluation was performed by a pathologist under light microscopy.
Photographs were taken using a Nikon Eclipse TE 2000 U equipped with a digital video camera.

658 Statistics and graph creation

659 Unless otherwise indicated, both statistics calculation and graphs creation were performed with 660 GraphPad Prism 8.0.1 software. The normality of data distribution was assessed using Shapiro-661 Wilk normality test. Statistical significance of mean value comparisons was determined using t-662 test or One-way ANOVA for normally distributed data, and Mann-Whitney test or Kruskal-Wallis 663 test for non-normally distributed data, as appropriate. P values \leq 0.05 were considered 664 statistically significant and are indicated in the graphs. Outliers were identified using the ROUT 665 method. Data are presented as mean ± SEM and the number of animals of each experimental 666 group is indicated in the figure legends or shown in the plots. Principal Component Analysis graph and volcano plots were generated using "ggplot2" package in R software. Flow cytometry 667 668 plots were exported from FlowJo software version X.0.7 after data analysis.

669

670 Al Language Model Assistance

671 We used ChatGPT (developed by OpenAI) to assist in refining the written content of this study.

672 ChatGPT provided suggestions and corrections based on the input provided by the user,

- 673 enhancing the clarity and grammar of the text. ChatGPT output was critically revised by the user
- 674 to ensure it conveys the desired message

675

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- 705

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986 FIGURE CAPTIONS

987 Fig 1: Characterization of muscular and systemic tissue damage during acute T. cruzi infection. 988 Foxp3-GFP mice were infected with T. cruzi and tissue damage and infection progression were 989 evaluated at different days post infection (dpi). (A) Kinetics of parasite counts in blood. (B) 990 Kinetics of *T. cruzi* satellite DNA quantification in skeletal muscle (SM), heart, liver and spleen. 991 (C) Kinetics of total spleen CD45+ cell count. (D) Representative Hematoxylin-Eosin stain of 992 quadriceps muscle from non-infected (NI) and infected (INF) (21 dpi) mice (N = 4-7). Black arrow: 993 parasites nest, blue arrows: necrotic muscle fibers, orange arrows: calcified muscle fibers. 994 Magnification = 10X. INF images represent different areas from the same sample. (E) Kinetics of 995 total SM CD45+ cell count. (F-G) Whole guadriceps SM RNAseg data analysis from NI and INF 996 animals; N = 3 per group. (F) Non-supervised pathway analysis of the differentially expressed 997 genes between INF and NI SM. Bars show the top-ten pathways upregulated in INF SM with red 998 arrows highlighting pathways related to SM physiology. (G) Volcano plots display differentially 999 expressed genes between INF and NI SM. According to (F), genes associated with oxidative 1000 phosphorylation (left), myogenesis (center) and adipogenesis (right) pathways are highlighted 1001 in red. (H) Kinetics of plasma CPK, CPK-MB, LDH, GOT and GPT activities, and glucose 1002 concentration. (A, C, E and H) Data is presented as mean ± SEM; N = 4-15 per dpi. Statistical 1003 significance was determined by one-way ANOVA. P values are relative to 0 dpi: *p < 0.05; **p < 1004 0.01; ***p < 0.001; ****p < 0.0001. (B) Data are presented as mean and values are normalized 1005 to spleen tissue parasitism at 10 dpi; N = 4-5 per dpi. (A-E and H) Data were collected from 2-3 1006 independent experiments.

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Fig 2: Tissue repair Tregs are reduced in target tissues and lymphoid organs during acute *T*.
 cruzi infection. Tissue repair Tregs (trTregs) were studied by flow cytometry in tissues from *T*.
 cruzi infected Foxp3-GFP mice at different days post infection (dpi). (A) Representative dot plots

1011 showing ST2 and KLRG-1 staining in total Tregs present in skeletal muscle (SM), liver and spleen 1012 obtained from non-infected (NI) and infected (INF) (21 dpi) mice. ST2+ KLRG-1+ cells (pink gate) 1013 were defined as trTregs. (B) Graphs displaying trTregs frequency within total Tregs (upper row) 1014 and absolute number (bottom row) in different tissues from NI and INF animals. Bars indicate 1015 the mean ± SEM. For SM, squares represent pools with N=3 mice. For spleen and liver, circles 1016 represent individual mice. (C) Heatmap displaying the relative expression or frequency of the 1017 indicated markers in splenic trTregs (pink gate) or ST2- KLRG-1- Tregs (golden gate) as defined 1018 in (A), evaluated in NI and INF animals (N=5-6). (D) Principal component analysis of data 1019 presented in (C). (E) Kinetics of trTregs frequency within total Tregs (upper row) and absolute 1020 number (bottom row) in SM, liver and spleen. Data are presented as mean \pm SEM; N = 6-21 per 1021 dpi. (B, E) For SM, cell counts are normalized to tissue weight. Statistical significance was 1022 determined by Unpaired t test (B) and Kruskal-Wallis test (E). P values in (B) represent pairwise 1023 comparisons, while in (E) are relative to 0 dpi: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.001; ***p < 0.001; **p < 0.001; 1024 0.0001. (A-E) Data were collected from 2-3 independent experiments.

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1026 Fig 3: IL-33 supplementation fails to prevent trTregs reduction in established infection. (A) IL-1027 33 concentration in plasma and SM lysates obtained from Foxp3-GFP mice at different days post 1028 infection (dpi). Muscle values were normalized to total protein content. Data are presented as 1029 mean ± SEM; N = 3-15 per dpi. (B) Representative dot plots showing ST2+ KLRG-1+ Tregs (trTregs) 1030 frequency within total Tregs isolated from the spleen of non-infected (NI) and infected (INF) (21 1031 dpi) Foxp3-GFP mice. Left plots correspond to uncultured Tregs, while middle and right plots 1032 correspond to Tregs activated with anti-CD3+anti-CD28+IL-2 with or without rIL-33 for 72 hours. 1033 (C) Experimental scheme illustrating the treatment of Foxp3-GFP mice with an established 1034 infection with IL-33 or IL-33+IL-2. (D) Flow cytometry analysis of Tregs present in the spleen of 1035 non-infected (NI) Foxp3-GFP mice 72 h after receiving 3 doses of intraperitoneal IL-33 or PBS as

1036 described in (C). Dot plots show the frequency of trTregs within total Tregs. (E) Graphs displaying 1037 trTregs frequency within total Tregs (upper row) and absolute number (bottom row) in skeletal 1038 muscle (SM), liver and spleen from INF animals (21 dpi) receiving intraperitoneal PBS, IL-33 or 1039 IL-2/IL-33 as described in C. Bars indicate the mean ± SEM. For each tissue, gray dashed lines 1040 indicate the average of trTregs count in untreated NI mice. Statistical significance was 1041 determined by Kruskal-Wallis test (A) and Mann-Whitney test (E). P values in (A) are relative to 1042 0 dpi: p < 0.05; p < 0.01; while in (E) represent pairwise comparison. Data are representative 1043 of two (A-B) and one (D-E) independent experiments.

1044

1045 Fig 4: Inflammatory and microbial-derived signals are unable to restrict IL-33 mediated 1046 expansion of trTregs in vitro. (A) Soluble ST2 (sST2) concentration in plasma, as well as in spleen, 1047 skeletal muscle (SM) and liver lysates obtained from Foxp3-GFP mice at different days post 1048 infection (dpi). In spleen and SM, values were normalized to total protein content and are 1049 presented as mean ± SEM. In the liver, results from a 1/10 dilution of liver lysates are depicted 1050 as absorbance units due to falling outside the assay's dynamic range, as indicated by the black 1051 dashed line. Statistical significance was determined by one-way ANOVA in spleen and SM. 1052 Representative of one experiment with N = 4-7 per dpi. ND: non-detectable. (B-C) 1053 Representative dot plots showing ST2+ KLRG-1+ Tregs (trTregs) frequency within total Tregs 1054 isolated from the spleen of non-infected (NI) Foxp3-GFP mice for 72 h with anti-CD3+anti-CD28 1055 together with the addition of different cytokines as follow: IL-2; IL-2+IL-33 and IL2+IL33 plus: 1056 cytokines associated to Th1 signals, innate signals, as well as conditioned media or transwell co-1057 cultures providing soluble spleen-derived signals (B) or microbial ligands (C), as indicated above 1058 each plot. (B-C) Data were collected from 4 independent experiments. Spl: splenocytes, Tps: 1059 trypomastigotes.

1060

1061 Fig 5: Early rIL-33 administration expands trTregs and improve disease outcome in infected 1062 mice. Immune response and disease progression was evaluated in infected Foxp3-GFP mice 1063 after receiving intraperitoneal IL-33 the day of infection and on 3 and 6 days post infection (dpi). 1064 (A) Experimental scheme. (B) Representative dot plots showing ST2+ KLRG-1+ Tregs (trTregs) 1065 frequency within total Tregs isolated from the skeletal muscle (SM) of PBS or IL-33-treated 1066 infected mice. (C) Graphs displaying trTregs frequency within total Tregs in SM, liver and spleen 1067 at 21 dpi. For SM, squares represent pools with N = 4-5. For spleen and liver, circles represent 1068 individual mice. (D) Heatmap showing relative values corresponding to parameters of disease 1069 progression at 21 dpi. Each column represents one mouse. (E) Representative dot plots 1070 illustrating type 2 innate lymphoid cells (ILC2) frequency within CD45+ CD4- CD8- Lin (CD3, CD19, 1071 NK1.1, CD11c)- CD11b- cells. Graphs correspond to SM from PBS or IL-33-treated infected 1072 mouse. (F) ILC2 frequencies within Live CD45+ cells in SM, liver and spleen at 21dpi. (G) 1073 Representative dot plots showing TSKB20/K^b staining in SM CD8+ cells from PBS or IL-33-treated 1074 infected mice. (H) Percentage of parasite-specific CD8+ T cells in SM, liver and spleen at 21dpi. 1075 (I) T. cruzi satellite DNA quantification in SM, heart and liver at 21 dpi. (C, F, H and I) Bars 1076 represent the mean ± SEM. Statistical significance was determined as follow: Mann-Whitney 1077 test for SM and unpaired t test for liver and spleen (A); unpaired t test (F and H) and Mann-1078 Whitney test (I). Data are representative of two (C, D, H and I) and one (F) independent 1079 experiments.

1080

1081 SUPPORTING INFORMATION

S1 Fig: Peripheral target tissues display inflammatory responses during acute *T. cruzi* infection. Inflammatory response was evaluated in *T. cruzi* infected Foxp3-GFP mice at different days post infection (dpi). (A) Whole quadriceps muscle (SM) RNAseq data analysis from NI and INF mice as described in Fig 1F-G; N = 3 per group. Volcano plots displaying differentially expressed genes

(dots) between INF SM and NI SM. According to Figure 1F, genes associated with interferon
gamma response, interferon alpha response and complement pathways are highlighted in red.
(B) Number of CD45+ cells in heart and liver, determined by flow cytometry at different dpi. Data
are presented as mean ± SEM; N = 4-9 per group. For liver, counts correspond to total leukocyte
numbers, whereas for heart, cell numbers are normalized to tissue weight. Statistical
significance was determined by one-way ANOVA and P values relative to 0 dpi. *p < 0.05; ****p
< 0.0001.

1093

1094 S2 Fig: Tregs frequency is reduced in lymphoid and non-lymphoid target tissues during acute 1095 T. cruzi infection. Tregs response was evaluated by flow cytometry in spleen, liver, skeletal 1096 muscle (SM) and heart from non-infected (NI) and infected (INF) (21 days post infection) Foxp3-1097 GFP mice. (A) Representative dot plots showing the frequency of Tregs (CD4+ Foxp3-GFP+) 1098 within total CD4+ cells from each tissue. (B) Bars displaying Tregs frequency within total CD4+ 1099 cells as the mean ± SEM. Circles represent individual mice; squares represent pools with 3-5 1100 mice. For SM and heart, cell counts are normalized to tissue weight. Statistical significance was 1101 determined by unpaired t test for spleen, liver and SM; and by Mann-Whitney test for heart. P 1102 values are indicated in the graphs. (A-B) Data were collected from 3 independent experiments.

1103

S3 Fig: ST2+ KLRG-1+ Tregs from *T. cruzi* infected mice exhibit a phenotype compatible with bona fide trTregs. Flow cytometry phenotypic analysis of Tregs subsets present in the spleen of non-infected (NI) or infected (INF) (21 dpi) Foxp3-GFP mice. Histograms show the expression of each cell marker in ST2+ KLRG-1+ (pink) and ST2- KLRG-1- (golden) Tregs as defined in Fig 2A. Numbers on top right corner of each plot indicate either mean fluorescence intensity or frequency of positive cells for each marker.

1110

1111 S4 Fig: IL-33 supplementation fails to prevent trTregs reduction in established T. cruzi 1112 infection. (A) IL-33 concentration was evaluated in spleen and liver lysates obtained from Foxp3-1113 GFP mice at different days post infection (dpi). Values were normalized to total protein content. 1114 Data are presented as mean ± SEM; N = 4-12 per dpi. (B) GFPneg CD4+ conventional T cells 1115 (Tconv) isolated from the spleen of non-infected (NI) and infected (INF) (21 dpi) Foxp3-GFP mice 1116 were evaluated by flow cytometry. Representative dot plots showing ST2+ KLRG-1+ cells 1117 frequency within total Tconv. Left plots correspond to uncultured Tconv, while middle and right 1118 plots correspond to Tconv activated with anti-CD3+anti-CD23+IL-2 with or without rIL-33 for 72 1119 hours. (C-E) Analysis of disease progression in INF mice treated with intraperitoneal IL-33 or IL-1120 33+IL-2 as described in Fig 3C. (C) Plasma LDH, GOT, GPT, CPK and CPK-MB activities, and glucose 1121 concentration at 21 dpi. (D) Percentage of total body weight reduction at 21 dpi compared to 15 1122 dpi. (E) Survival curve in the different experimental groups. (F, G) Representative plots depicting 1123 the frequencies of GFP+ CD4+ Tregs cells (F, G) and ST2+ KLRG-1+ cells (G) in NI (F) and INF (G) 1124 mice receiving intramuscular IL-33. Statistical significance was determined by Kruskal-Wallis test 1125 (A), Mann-Whitney test (C-D), Mantel-Cox test (E) and Wilcoxon test (G). P values in (A) are 1126 relative to 0 dpi: ****p < 0.0001; while in (C-D and G) represent pairwise comparisons; ns: non-1127 significant.

1128

S5 Fig: Early rIL-33 administration improves the global health status without reducing SM alterations during acute infection. Immune response and disease progression was evaluated in infected Foxp3-GFP mice after receiving intraperitoneal IL-33 the day of infection and on 3 and 6 days post infection (dpi). (A) trTregs count in skeletal muscle (SM), liver and spleen at 21 dpi. For SM, squares represent pools with N=4-5. For spleen and liver, circles represent individual mice. For each tissue, gray dashed lines indicate the average of trTregs count in untreated non-infected (NI) mice. (B) Plasma LDH, GOT, CPK, GPT and CPK-MB activities, and glucose

1136	concentration at 21 dpi. (C) Total body weight loss between 15 and 21 dpi. (D) Inverted screen
1137	test (max: 120 seconds) and (E) Hematoxylin-Eosin stain of quadriceps muscle at 21 dpi. Images
1138	are representative of N = 4. Black arrow: centrally nucleated muscular fibers. Magnification = 4X
1139	(left) and 10X (right). (F) Gating strategy used to identify type 2 innate lymphoid cells (ILC2) as
1140	CD45+ CD4- CD8- Lin (CD3, CD19, NK1.1, CD11c)- CD11b- CD127+ ST2+ cells. Dot plots are
1141	representative of SM from IL-33-treated infected mouse. (G) ILC2 and (H) parasite-specific CD8+
1142	cell count in SM, liver and spleen at 21 dpi. For SM, counts are normalized to tissue weight. (A-
1143	D, G and H) Bars represent the mean \pm SEM. Statistical significance was determined as follow:
1144	Mann-Whitney test for SM and unpaired t test for liver and spleen (A); unpaired t test (B-D, G
1145	and H). (A-D, G and H) P values from pairwise comparison are indicated in the graphs. Data are
1146	representative of two (A-D and H) and one (E and G) independent experiments.
1147	
1148	S1 Table: List of genes associated with inflammatory response and skeletal muscle physiology.

- 1149 Differentially expressed genes between infected (15 days post infection) and non-infected mice
- 1150 are shown for each cellular pathway.

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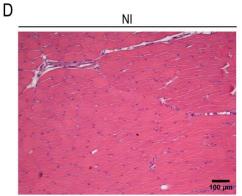


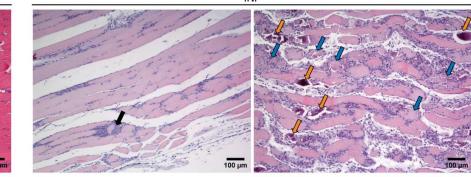
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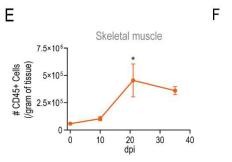
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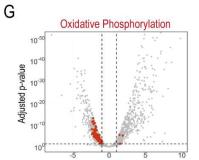


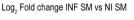


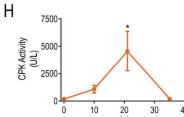




Myogenesis





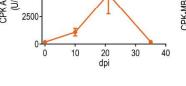


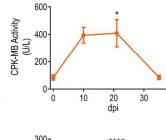
dpi

1000-

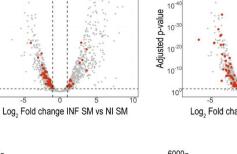
0-

GOT Activity (U/L)



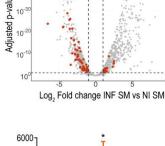


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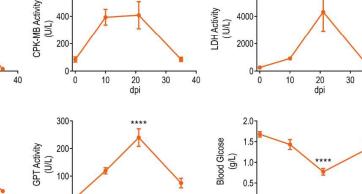


0.0

dpi



Adipogenesis





bioRxiv preprint doi: https://doi.org/10.1101/2024.02.15.580513; this version posted February 20, 2024. The copyright holder for this preprint *Fig* 2 (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission. А SM Spleen Liver SM Liver Spleen trTregs trTregs 10 10 trTregs 80-30-8p = 0.0002 p < 0.0001 p = 0.0004 17.2 23.6 4.6 %trTregs in Tregs %trTregs in Tregs %trTregs in Tregs •••• . 60-6. ... 20-10 ... NI 40-4 10-..... 20i 2-KLRG-1- ST2-103 104 0. 0 0 Ň INF Ň INF NI INF 10 trTregs 10 trTregs 10 trTregs 7.3 0.6 4.2 104 10 4×10²· 6×10³ 4×10⁵ p = 0.0033 p = 0.0050 p < 0.0001 103 103 #trTregs (/gram of tissue) INF 3×10² 3×105 . s6au Lu# 2×10^{3.} #trTregs ST2 (PE) 0 2×10² 2×105-KLRG-1- ST2-Ţ 1×10⁵ 1×10² 0 104 105 10³ 104 105 103 10 KLRG-1 (PEeF610) 0 0 0 NI NI INF INF NI INF С D NI INF trTregs (INF) 100% 1.0 BATF KLRG-1- ST2- Tregs 80% 0.8 (INF) IRF4 PC 2 (21%) 0.6 60% Á -%Ki-67+ 0 KLRG-1- ST2- Tregs 40% 0.4 CD44 (NI 20% 0.2 CD62L 0% 0 TIGIT trTregs (NI) ICOS -2

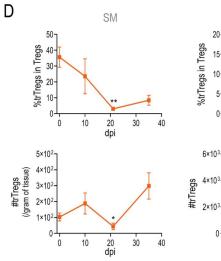
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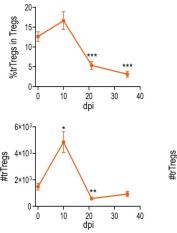
PC 1 (70.4%)

-3

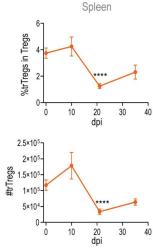


GITR

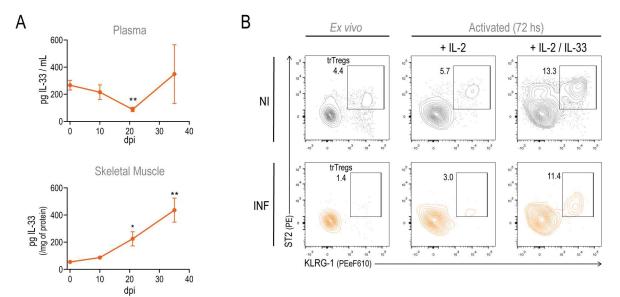
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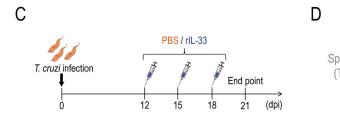


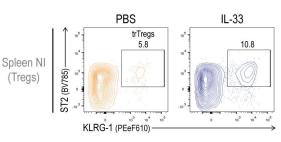
Liver



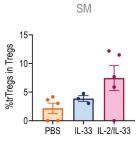
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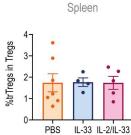


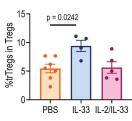


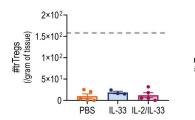


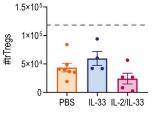


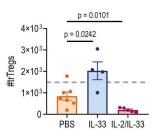






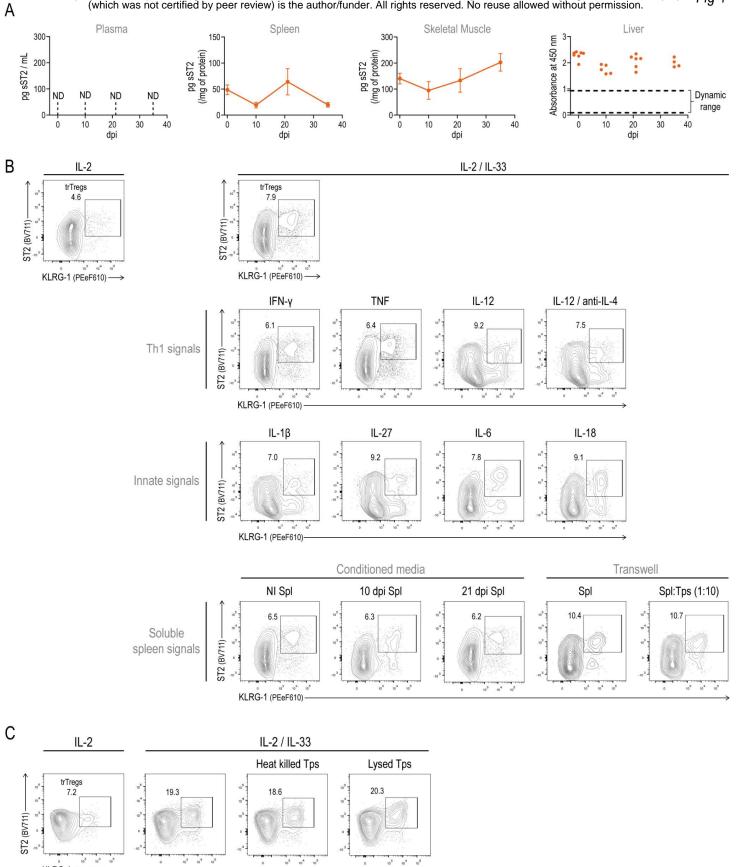






Liver

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KLRG-1 (PEeF610)-

