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CD4$^+$ T cells from chronic Chagas disease patients with different degrees of cardiac compromise exhibit distinct expression patterns of inhibitory receptors TIGIT, Tim-3 and Lag-3.

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Abstract

T cells are central to adaptive immune response against *T. cruzi* infection. In the chronic stage of Chagas disease, circulating parasite-specific memory T cells show reduced functionality and increased expression of inhibitory receptors, possibly as a result of persistent antigenic stimulation. This exhausted phenotype has been linked to progression of cardiac pathology while, contrariwise, the presence of polyfunctional T cells shows association with therapeutic success and more efficient control of infection. Given this, we hypothesized that inhibitory receptors TIGIT, Tim-3 and Lag-3 may be involved in immune modulation of anti-*T. cruzi* T cell response, and therefore may play a role in the containment or the unleashing of inflammatory phenomena that ultimately lead to tissue damage and pathology. In this preliminary study, we assess the frequency of $CD4^+$ T cells expressing each of these receptors and their relation to cellular activation. Samples from chronic Chagas disease patients with different degrees of cardiac compromise, and non-infected donors were analyzed under different stimulation conditions. Our results show that the frequency of $TIGIT^+$ $CD4^+$ T cells is increased in Chagas patients, while Tim-3^+ cells are more abundant in patients with signs of cardiac alterations. In addition, the frequency of Lag-3^+ cells increases in non-activated $CD4^+$ T cells from Chagas patients without demonstrable cardiopathy upon pathogen-specific *in vitro* antigenic stimulation.

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Introduction

Chronic infections are one of the circumstances under which T cells encounter the challenge of appropriately regulating the immune response in persistent presence of antigenic stimulus. In such settings, a process known as T cell exhaustion takes place, by which these cells acquire features believed to hamper sterile immunity, such as hierarchical loss of effector functions, altered gene expression regulation and metabolic disarrangements[1–3]. The increased and/or sustained expression of inhibitory receptors on the cellular surface of T cells is another of these characteristics, and is often regarded as the hallmark of cell exhaustion[3,4].

In chronic infection with *Trypanosoma cruzi*, i.e. chronic Chagas disease, evidence of exhausted T cells in circulation has accumulated during the last decades[5]. In fact, the frequency of cells with reduced effector capabilities has been directly correlated to cardiac compromise[6,7]. The association of therapeutic success of benznidazole with a reduction of T cells with an exhausted profile in the circulation of treated subjects[8,9] and the observation of a richer response profile in serodiscordant subjects[10] further support the relevance of persistent antigen-specific activation for ineffective adaptive immunity against the parasite.

Although PD-1 and CTLA-4 were the pioneer inhibitory receptors to be studied in relation to T cell exhaustion, other molecules were demonstrated to complement their function in a lower hierarchical level, apparently enabling the fine-tuning of T cell inhibition[2,4,11]. Among these molecules, TIGIT, Tim-3 and Lag-3 have been found to be implicated in chronic viral infections, and arise as promising targets for immune-enhancing therapies[4,11]. Their role in chronic Chagas disease is yet to be investigated.

The study of inhibitory receptors and their relationship with T cell exhaustion requires the analysis of T cell activation upon antigen specific stimulation, but the heterogeneity of response profiles displayed by the $CD4^+$ T cell subset is a hurdle to the measurement of overall activation. This has been addressed by several research groups, resulting in the development of flow cytometry techniques based on the detection of molecules, globally referred to as activation induced markers (AIM)[12–14]. Here, we take advantage of this methodology to characterize the expression of TIGIT, Tim-3 and Lag-3 in peripheral blood $CD4^+$ T cells from chronic Chagas disease patients and their relation to *T. cruzi*-specific and non-antigen-specific activation.

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33 **Donors, materials and methods**

34 ***Subjects inclusion and blood sample collection***

35 Blood samples were collected from non-infected donors and patients with chronic
36 Chagas disease after the nature of this study was explained to them and written consent
37 was given, in accordance with the guidelines of the protocol approved by the Medical
38 Ethics Committee of the and the Hospital General de Agudos "Dr.Ignacio Pirovano" and
39 the Instituto Nacional de Parasitologia "Dr.M.Fatala Chaben". The sample collection
40 protocol followed the tenets of the declaration of Helsinki. All subjects were of age at the
41 time of sample collection. The study population included 20 patients with chronic Chagas
42 disease with at least 2 reactive serological tests, who were categorized, according to
43 Kuschnir's classification[15] into groups A (without demonstrable cardiac pathology,
44 Kuschnir class 0, n=10) or C (with signs of cardiac alterations, Kuschnir class 1 or 2,
45 n=10). Ten donors were included as well, with negative serology for *T. cruzi* infection.

46 Samples consisted in 35 to 50 ml peripheral venous blood, collected in EDTA anti-
47 coagulated tubes and processed up to 4 h after collection. Peripheral blood mononuclear
48 cells (PBMC) were isolated by centrifugation (400 ×g, room temperature, 40 min) in a
49 Ficoll-Paque gradient medium (GE Healthcare Bio-Sciences, Uppsala, Sweden),
50 quantified by manual count in Neubauer chamber, and aliquoted in fetal bovine serum
51 (FBS; Natocor, Córdoba, Argentina) with 10% v/v DMSO to be cryopreserved in liquid
52 nitrogen until used.

53 ***Parasite lysate***

54 *T. cruzi* trypomastigote/amastigote lysate was prepared from VERO cells infected with
55 Sylvio strain (Discrete Typing Unit TcI[16]) parasites (MOI 3:1) supernatants, as described
56 elsewhere[17]. After lysis, the suspension was filter-sterilized through a 0.2 µm pore-size
57 membrane, aliquoted, and stored at -80 °C until use.

58 ***Cell culture and stimulation***

59 For antigen stimulation, 3×10^6 PBMC from each subject were seeded in 6 wells from a
60 96-well U-bottom plates and cultured in assay medium alone or with 10 µg/ml *T. cruzi*
61 lysate, or 5 µg/ml phytohaemagglutinin (PHA, Sigma, St Louis, MO, USA). Cells were
62 incubated at 37°C in a humidified, 5% CO_2 atmosphere for 18h.

63 ***Flow cytometry analysis of T cells***

64 After culture, cells were centrifuged at 400 ×g for 10 min at room temperature (RT) and
65 supernatants were discarded. Next, they were washed with PBS by centrifugation at 700
66 ×g during 3 min at RT, transferred into a 96-well V-bottom plate and resuspended in 25
67 µl of staining solution, containing the antibodies detailed in Table 1, diluted in 1X

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68 live/dead fixable viability dye (Zombie-Aqua, Biolegend, San Diego, CA, USA). After
69 staining for 30 min at RT, cells were washed with PBS, fixed with Fixation Buffer
70 (Biolegend) for 20 min at RT in the dark and washed with PBS. Isotype control stained
71 samples were used to set the cut point values for each marker. A minimum of 5×10^5
72 events within the lymphocyte population were acquired in a FACSCanto II (BD
73 Biosciences, San Diego, CA, USA) flow cytometer using FACS Diva Software (BD
74 Biosciences). Flow cytometry analysis was carried out with the program FlowJo (FlowJo
75 LLC, Ashland, OR, USA). All antibodies were used at optimal concentrations determined
76 by previous titration experiments.

77 **Table 1.** Fluorescent-labeled antibodies and isotype controls used for flow cytometry
78 experiments.

Antibody	Clone	Vendor
FITC-conjugated anti-Lag3	11C3C65	Biolegend
PE-conjugated anti-TIGIT	A15153G	Biolegend
PE-Cy7-conjugated anti-OX40	ACT35	Biolegend
APC-conjugated anti-CD4	RPA-T4	BD Biosciences
APC-Cy7-conjugated anti-CD25	BC96	Biolegend
BV421-conjugated anti-CD3	UCHT1	Biolegend
PerCP-Cy5.5-conjugated anti-Tim-3	F38-2E2	Biolegend
FITC-mouse IgG1 k	11C3C65	Biolegend
PE-mouse IgG 2ak	A15153G	Biolegend
PerCP-Cy5.5 -mouse IgG1 k	F38-2E2	Biolegend
PE-Cy7 –mouse IgG1 k	Mouse IgG1, κ	Biolegend
APC-mouse IgG1 k	RPA-T4	BD Biosciences
APC-Cy7-mouse IgG1 k	BC96	Biolegend
BV421-mouse IgG1 k	UCHT1	Biolegend

79

80 ***Statistical analysis***

81 The effect of the stimulation condition within each group was analyzed by Friedman's
82 test followed by multiple comparisons by pairwise Wilcoxon's test, with data points paired
83 by patient. The effect of groups within each stimulation condition was evaluated by
84 Kruskal-Wallis's test, followed by multiple comparisons by Dunn's test. Significance was
85 considered using $\alpha=0.05$. For both multiple comparisons methods, p values were
86 adjusted using the Benjamini-Yekutieli method. All statistical analysis methods were
87 implemented using open source R packages stats[18] and dunn.test[19].

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88 **Results**

89 ***Ox40 and CD25 are useful as activation markers in T. cruzi specific CD4+ T cell***
90 ***response***

91 Seeking to determine whether the AIM assay proposed by Reiss et al.[13] is useful for the
92 measurement of $CD4^+$ T cell activation against *T. cruzi* antigens, PBMC from non-
93 infected individuals (group NI), and chronic Chagas disease patients with (group C) and
94 without (group A) cardiac compromise were stimulated *in vitro* with parasite lysate to
95 evaluate pathogen-specific response, and with PHA to assess overall, non-specific
96 activation. Cells were stained and analyzed by flow cytometry. The gating strategy used
97 for this analysis is represented in **Figure 1A** and **B**.

98 As shown in **Figure 1C-E**, while the frequency of activated cells was similar between
99 groups both upon non-antigen specific stimulation with PHA (p=0.9) or without
100 stimulation (p=0.5), statistically significant differences were observed in the frequency of
101 $Ox40^+CD25^+$ $CD4^+$ T cells between each of the chronic Chagas patients groups and the
102 non-infected subjects when cells were stimulated with parasitic antigens (p=0.003 for A
103 vs. NI, p=0.01 for C vs. NI, **Figure 1C**). All groups responded significantly to PHA
104 stimulation in comparison with the unstimulated condition (**Figure 1E**). No difference was
105 observed between groups A and C in response to parasite antigens. In addition, the fold
106 change in the frequency of $CD4^+Ox40^+CD25^+$ T cells upon stimulation with *T. cruzi*
107 antigens, but not with PHA, was greater for both groups of infected patients than for the
108 NI control group (**Figure 1F, G**).

109 In conclusion, the AIM method using the marker combination Ox40/CD25 can be used
110 to detect anti-*T. cruzi* response in $CD4^+$ T cells from chronic Chagas disease patients,
111 upon *in vitro* stimulation with parasite lysate.

112 ***Chronic Chagas disease patients have increased frequencies of circulating***
113 ***$CD4^+TIGIT^+$ T cells***

114 Next, we assessed the expression of the inhibitory receptor TIGIT on circulating $CD4^+$ T
115 cells from Chagas disease patients and control donors. **Figure 2A** represents the
116 expression profile of this receptor in representative donors from each group.

117 As depicted on **Figure 2B** and **C**, the frequency of $TIGIT^+$ cells was not affected in each
118 case by stimulation with *T. cruzi* antigens, nor with PHA. Nonetheless, a striking
119 difference was observed between chronic Chagas disease patients and non-infected
120 individuals. Although statistical significance was not reached when each of the groups
121 were considered separately, the frequency of $CD4^+TIGIT^+$ T cells was significantly higher
122 in chronic Chagas disease patients than in the control group subjects upon stimulation

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123 with *T. cruzi* lysate or PHA (p=0.047 and p=0.021 respectively, **Figure 2D**). In the
124 unstimulated condition, although there is a visible trend with a low p-value (p=0.059), the
125 difference is, strictly speaking, non-statistically significant.

126 The expression of TIGIT was also analyzed separately within activated ($Ox40^+CD25^+$) or
127 non-activated ($Ox40^-$ or $CD25^-$) $CD4^+$ T cells. Upon non-specific stimulation with PHA,
128 the frequency of TIGIT-expressing activated $CD4^+$ T cells was significantly reduced
129 (p>0.01) in comparison with the unstimulated and the lysate conditions, but this was not
130 observed for chronic Chagas patients with cardiac manifestation (**Figure 2E**). When the
131 non-activated cells were analyzed, only subjects from the chronic Chagas patients
132 groups showed an increased frequency of $TIGIT^+$ events upon stimulation with PHA as
133 compared with the non-stimulated condition. No other differences were observed
134 regarding the effect of the stimuli on the cells of each of the groups.

135 ***Tim-3-expressing CD4⁺ T cells are more abundant in the circulation of chronic***
136 ***Chagas patients with compromised cardiac function***

137 Since Tim-3 is another inhibitory receptor related to T cell exhaustion, we decided to
138 investigate its possible implications for chronic Chagas disease. The frequency of $CD4^+$
139 T cells expressing Tim-3 was assessed using the same experimental approach
140 described above. The expression profiles for these cells in a representative subject from
141 each group are shown in **Figure 3A**.

142 The results depicted on **Figure 3B** revealed that the frequency of $CD4^+Tim\text{-}3^+$ T cells
143 were statistically undistinguishable between groups in absence of antigenic stimulation,
144 or upon non-specific stimulation with PHA. However, exposure to *T. cruzi* lysate unveiled
145 differences in the frequency of such cells, which is higher in subjects from the C group
146 compared to the NI group (p=0.04). In fact, the fold increase in the expression of this
147 receptor is greater for chronic Chagas patients in group C than for the other two groups
148 (**Figure 3C**), with the difference being statistically significant only for the comparison
149 between C and NI subjects (p~0.008, medians: 0.91, 1.03 and 1.28 for NI, A and C
150 respectively). This difference was observed separately in both the activated and the non-
151 activated subsets upon encounter with parasite antigens (**Figure 3D**).

152 Within each group, the abundance of $Tim\text{-}3^+$ cells was affected by stimulation with PHA,
153 being significantly higher compared to the non-stimulated and the parasite lysate
154 stimulation conditions, in non-infected subjects and patients from group A, but not in
155 patients from group C (**Figure 3D**). Of note, when activated and non-activated $CD4^+$ T
156 cells were analyzed separately, the same difference was observed in the non-activated

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157 subset for both the A and NI groups, but the A group showed a statistically significant
158 increase in activated Tim-3$^+$ CD4$^+$ T cells as well (**Figure 3E, F**).

159 ***Lag-3 expression may withdraw CD4+ T cells from activation against T. cruzi***
160 ***antigenic stimulus in chronic Chagas patients without cardiac compromise***

161 The third and last inhibitory receptor we looked into in the context of chronic Chagas
162 disease was Lag-3. The expression profile of this receptor under different stimulation
163 conditions is shown for a representative subject in **Figure 4A**.

164 While no statistically significant differences were found in the frequency of CD4$^+$Lag-3$^+$
165 T cells between groups in any of the stimulation conditions, this parameter was
166 significantly affected by stimulation with PHA for subjects from groups A (p=0.005) and
167 NI (p=0.01). The patients in group C showed an increase of these cells, but the difference
168 did not meet the significance cutoff (p=0.054). Interestingly, only patients in group A
169 showed an increase in representation of Lag-3$^+$ cells upon stimulation with parasite
170 lysate (p=0.017), which was also seen as a significantly higher fold change value for this
171 subset upon *T. cruzi* antigens stimulation, compared to that shown by NI subjects
172 (**Figure 4C**, p=0.023).

173 Further subsetting of the CD4$^+$ T cells population according to CD25 and Ox40 revealed,
174 remarkably, that the increase in representation of Lag3-expressing cells observed in
175 group A patients was virtually ascribed to non-activated cells (p=0.003), while no
176 significant change was observed in the frequency of Lag3$^+$ cells within the activated cells
177 gate (p=0.068, **Figure 4D, E**). The fold change in size of this population reflects this
178 conclusion (**Figure 4F**).

179 **Discussion**

180 In the light of the clear link between T cell exhaustion and chronic Chagas disease, the
181 expression profile of inhibitory receptors and their implications for anti-parasite response
182 may help shedding light on the pathogenesis of inflammatory disease associated to *T.*
183 *cruzi* infection. In this article, we report that TIGIT, Tim-3 and Lag-3 are expressed
184 differently, or they behave in diverging ways upon *in vitro* stimulation, in chronic Chagas
185 disease patients with different degrees of cardiac compromise.

186 The diversity of response profiles that CD4$^+$ T cells may display in reaction to their
187 cognate epitopes poses an obstacle for the determination of overall activation within this
188 subset. Hence, the discovery of a combination of surface markers, addressable by flow
189 cytometry, that may serve to this purpose[12–14] was a critical development for
190 experimental designs like the one presented herein. Our data showed that in

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191 experimental exposure to *T. cruzi* antigens, the combination of markers Ox40 and CD25
192 clearly signs an activation of CD4+ T cells with memory response characteristics, as it is
193 significantly different in chronic Chagas disease patients than in naïve subjects.

194 TIGIT is an immunomodulatory receptor expressed by T and NK cells, which binds to
195 CD112 and CD155, producing an inhibitory signal that negatively regulates cellular
196 response and IL-12 production from mature dendritic cells while promoting IL-10
197 secretion[11,20]. In addition, TIGIT+ Treg cells have been shown to selectively inhibit Th1
198 and Th17, but not Th2 cells[21]. In HIV infection, it is co-expressed with PD-1, and may be
199 regarded as an indicator of T cell exhaustion and severity of the infection, as TIGIT+
200 CD4+ T cells are more frequent in viral non-controllers than in elite controllers and non-
201 infected individuals[22]. Our results indicate that the frequency of circulating CD4+ T cells
202 expressing this surface marker is elevated in chronic Chagas patients from both groups
203 analyzed in this study. It is worth noting that TIGIT expression has been reported to be
204 induced at the event of TCR-signaled activation of CD4+ T cells[23,24]. In contrast, in our
205 experimental set up, the frequency of CD4+ expressing this marker showed no change
206 upon stimulation with parasite lysate, and a maigre increase with PHA. A tendency is
207 visible in samples from the A group by which the frequency of TIGIT+ cells is reduced
208 within the Ox40+CD25+ subset in the lysate activated condition. This trend, which is
209 evident in the PHA activated condition for all groups, may be associated with a decrease
210 in the representation of Treg cells inside that population in favour of non-regulatory
211 activated T cells. Of note, this was not observed in patients from the C group.

212 In order to interact with its ligand, galectin 9 (Gal-9), Tim-3 has a mucin domain that is
213 highly glycosylated[11,25]. This is of particular interest in the case of chronic Chagas
214 disease, as *T. cruzi* has been shown to tamper with T cell regulation by altering their
215 surface glycosylation pattern via enzymes of the trans-sialidase superfamily (Marques
216 da Fonseca 2019). Of note, a link between the expression of Gal-9 in myenteric ganglia
217 from chronic Chagas patients and the enteric manifestation of the disease has been
218 proposed[26]. Whether this is also the case in the cardiac forms of chronic Chagas disease,
219 and its possible implications for immune regulation in this pathology via Tim-3 is yet to
220 be investigated. Nonetheless, Lasso et al.[27] described that this receptor is expressed in
221 higher frequencies of CD8+ T cells in chronic Chagas patients than in non-*T. cruzi*
222 infected donors. Our observations indicate that this difference also applies to CD4+ T
223 cells. In fact, the frequency of Tim-3+ cells within this population increases in chronic
224 Chagas patients but not in non-infected subjects, upon exposure to parasite antigens. In
225 addition, this inhibitory receptor seems to be upregulated in non-activated cells under
226 this condition, despite this difference not being statistically significant.

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227 Lag-3 is a negative regulator of T cell proliferation. It is believed to exert its inhibitory
228 function, not only by competing with the CD4 co-receptor for class II MHC binding in the
229 context of immune synapsis, but also by interacting with proteins from other signaling
230 pathways, like LSECtin[4,11]. Its immune suppressive function has been shown to be
231 subordinated to and synergistic with that of PD-1, and therefore is receiving much
232 attention from the oncotherapy research community[11]. Our results show that, in the
233 context of chronic Chagas disease, the frequency of $CD4^+$ T cells expressing Lag-3 is
234 boosted by polyclonal activation with PHA in all of the evaluated groups of donors, but
235 only in group A upon parasite-specific stimulation. An outstanding outcome of our
236 experiments is that this increased frequency of Lag-3^+ cells occurs within the non-
237 activated subpopulation. This may implicate that this receptor is preventing pathogen-
238 reactive $CD4^+$ T cells from triggering an effector response. Furthermore, as this was not
239 observed in cardiac patients, it is tempting to think on a possible participation of Lag-3^+
240 T cells-mediated modulation of the immune response in preventing inflammatory
241 pathogenesis. At this point, it should be noted that this inhibitory receptor is expressed
242 on natural and induced regulatory T cells[11]. Also, evidence of an increment on the
243 frequency of regulatory T cells induced by *in vitro* stimulation with *T. cruzi* antigens has
244 been reported previously[28]. Further analysis of our data will let us determine whether the
245 increase in the frequency of Lag-3^+ cells within the so-termed non-activated population
246 occurs within the $CD25^+$ subset, or on the contrary involves $CD25^-$ lymphocytes only,
247 which would rule out Tregs as accountable for these changes.

248 Another scope to be incorporated in future versions of this report is that of combined
249 expression of multiple inhibitory receptors. As opposed of multifunctionality and in the
250 light of the apparent regulatory hierarchy these receptors take part in[4], the conjoined
251 expression of different inhibitory receptors may imply different stages of immune
252 modulation, which may point at differences between groups of patients and shed light on
253 processes relevant for the, still elusive, pathogenesis of Chagas associated heart
254 disease. On that direction, work is underway to develop a multiparametric analytic
255 pipeline that enables, not only practical analysis of the combined expression of multiple
256 markers, but also the evaluation of changes in the levels of expression (measured as
257 mean fluorescence intensity of staining) across multiple experiments.

258 Finally, experimental *in vitro* blockade of these receptors will help us to better understand
259 the implications of the observations resulting from de data presented herein at a
260 functional level.

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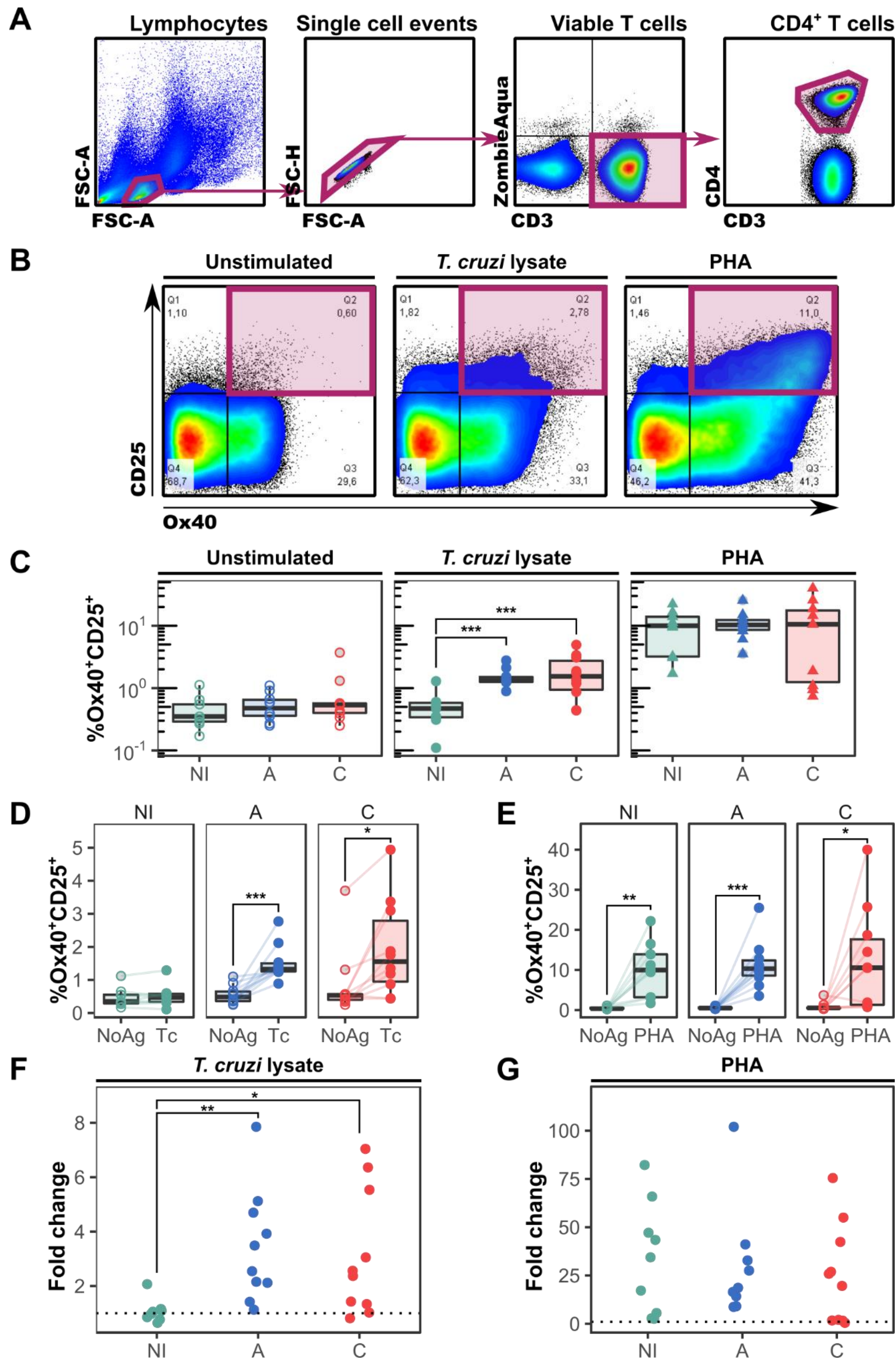
339 **Figures**



340

341 **Figure 1.** AIM assay with markers Ox40 and CD25 is useful to assess CD4+ T cell
342 activation against *T. cruzi* antigens. **A.** Schematic representation of the gating strategy
343 used to analyze CD4+ T cell activation. **B.** Representative cytograms from a chronic

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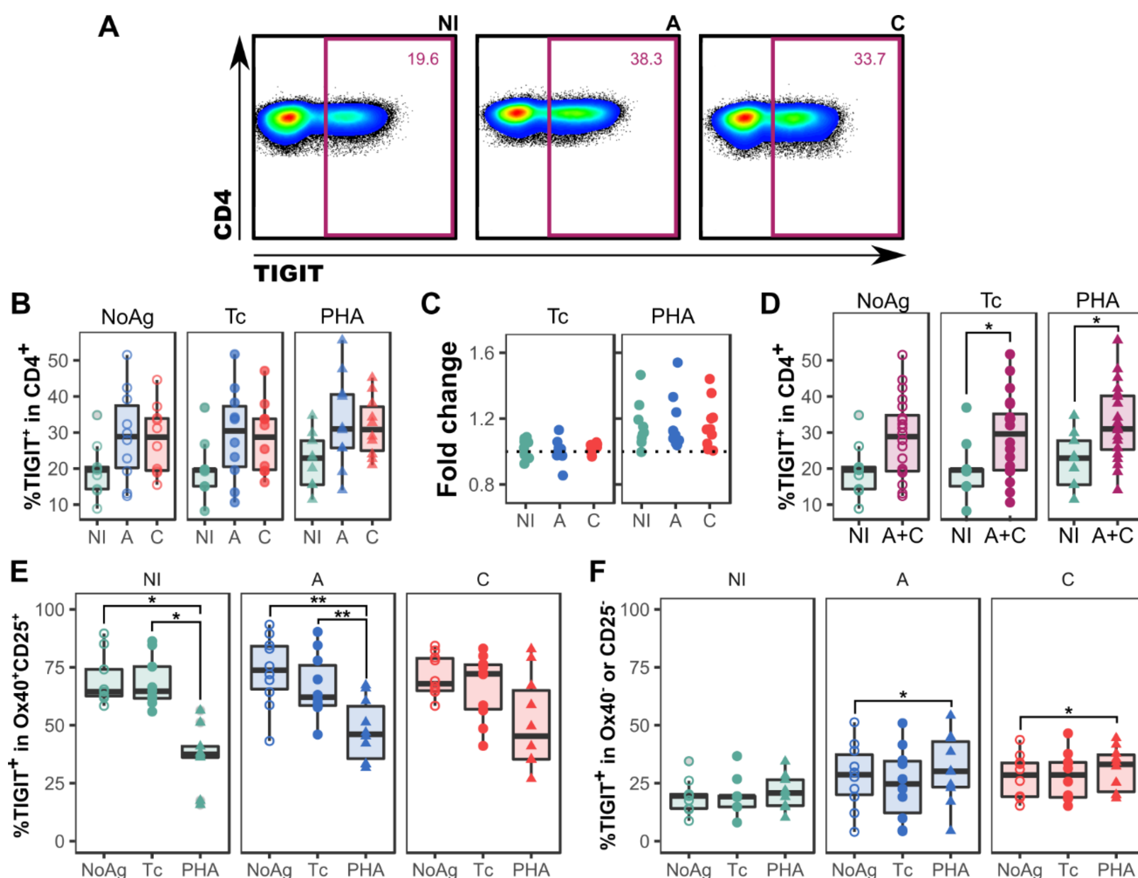
344 Chagas patient from group A, under different stimulation conditions. **C.** Differences
345 between groups in the frequency of Ox40⁺CD25⁺ CD4⁺ T cells under different stimulation
346 conditions. **D, E.** Frequency of Ox40⁺CD25⁺ CD4⁺ T cells upon *T. cruzi* lysate (**D**) or PHA
347 (**E**) stimulation, paired by subject. **F, G.** Fold change in the frequency of Ox40⁺CD25⁺
348 CD4⁺ T cells upon stimulation with parasite lysate (**F**) or PHA (**G**), with respect to the
349 unstimulated condition. *: p<0.05; **: p<0.01; ***: p<0.005. NoAg: unstimulated condition;
350 Tc: *T. cruzi* lysate stimulation.



351

352 **Figure 2.** Expression of TIGIT in CD4⁺ T cells from non-infected subjects and chronic
353 Chagas disease patients. **A.** Representative expression patterns in one subject from
354 each group. **B.** Frequency of TIGIT⁺ cells in CD4⁺ T cells for each group, under different
355 stimulation conditions. **C.** Fold change of the frequency of CD4⁺TIGIT⁺ upon parasite
356 lysate or PHA stimulation, relative to that in the unstimulated condition. **D.** Frequency of
357 TIGIT⁺ cells in CD4⁺ T cells, in non-infected subjects and both groups of chronic Chagas
358 disease patients (A+C) collapsed. **E, F.** Frequency of TIGIT⁺ cells in CD4⁺ T cells, for
359 each experimental group, under different stimulation conditions within the activated
360 (Ox40⁺CD25⁺, **E**) and non-activated (Ox40⁻ or CD25⁻, **F**) populations. *: p<0.05; **:
361 p<0.01; ***: p<0.005. NoAg: unstimulated condition; Tc: *T. cruzi* lysate stimulation.

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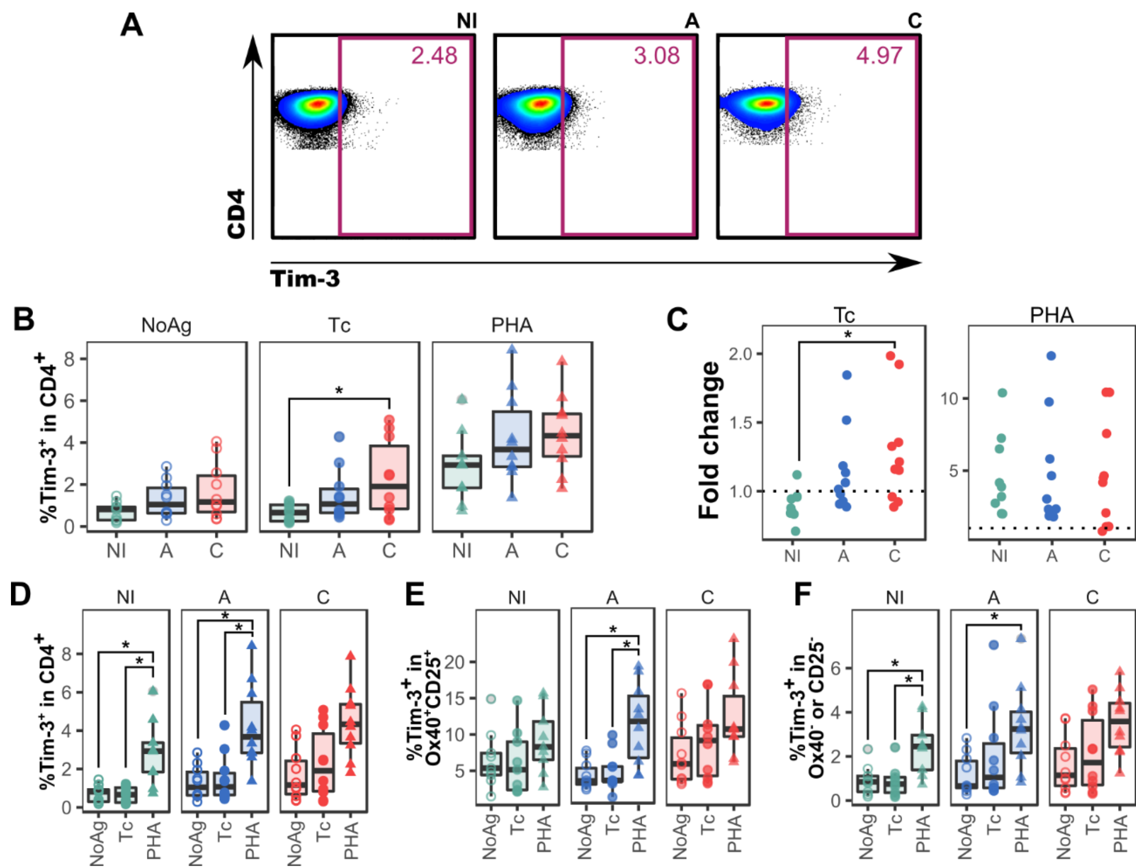

362

363 **Figure 3.** Expression of Tim-3 in CD4⁺ T cells from non-infected subjects and chronic
364 Chagas disease patients. **A.** Representative expression patterns in one subject from
365 each group under the same stimulation condition. **B.** Frequency of Tim-3⁺ cells in CD4⁺
366 T cells for each group, under different stimulation conditions. **C.** Fold change of the
367 frequency of CD4⁺Tim-3⁺ upon parasite lysate or PHA stimulation, relative to the value
368 in the unstimulated condition. **D-F.** Frequency of Tim-3⁺ cells for each stimulation
369 condition, grouped by experimental groups (NI, A and C), in total (**D**), activated (**E**) and
370 non-activated (**F**) CD4⁺ T cells. *: $p<0.05$; **: $p<0.01$; ***: $p<0.005$. NoAg: unstimulated
371 condition; Tc: *T. cruzi* lysate stimulation.

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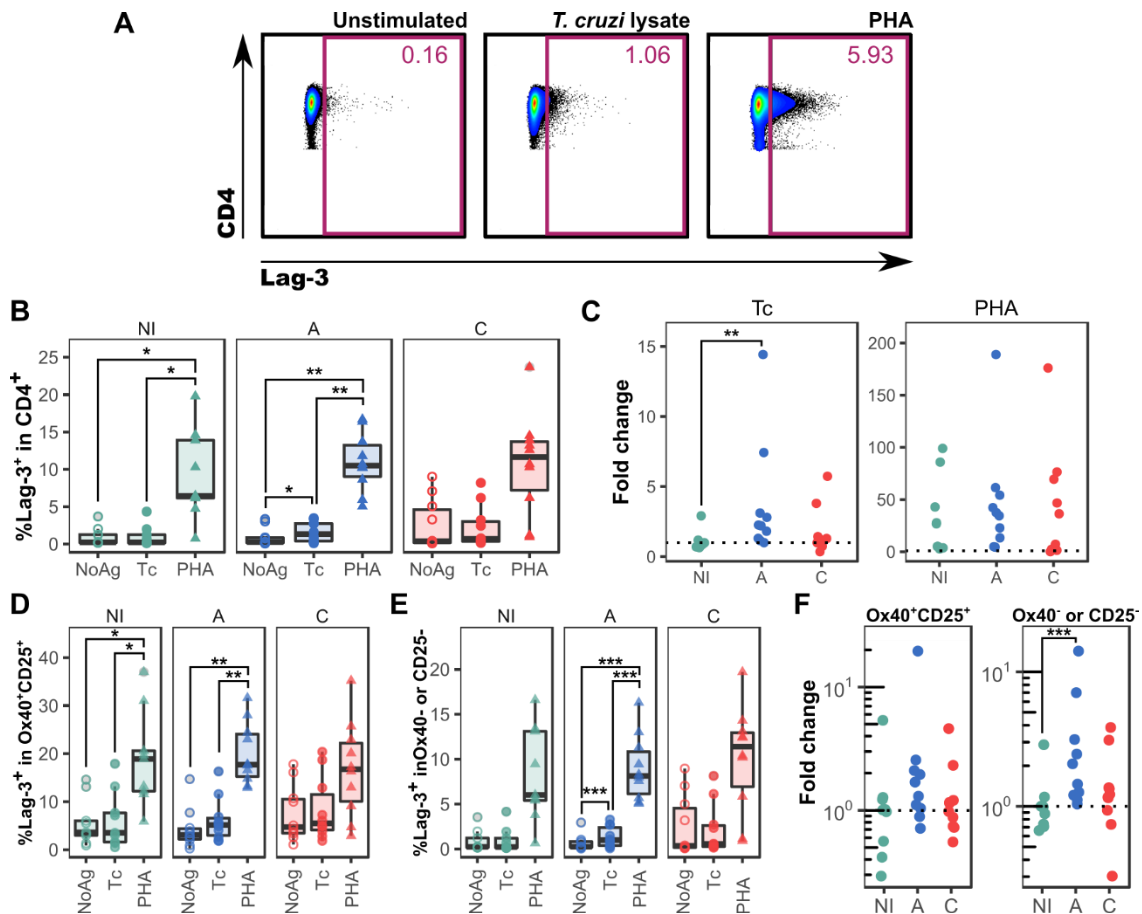

372

373 **Figure 4.** Expression of Lag-3 in CD4⁺ T cells from non-infected subjects and chronic
374 Chagas disease patients. **A.** Representative expression patterns in one subject from
375 group A, under each stimulation condition. **B.** Frequency of Lag-3⁺ cells in CD4⁺ T cells
376 under different stimulation conditions, grouped by experimental groups (NI, A and C). **C.**
377 Fold change of the frequency of CD4⁺Lag-3⁺ upon parasite lysate or PHA stimulation,
378 relative to the value in the unstimulated condition. **D,E.** Frequency of Tim-3⁺ cells for
379 each stimulation condition, grouped by experimental groups (NI, A and C), in activated
380 (**D**) and non-activated (**E**) CD4⁺ T cells. **F.** Fold change of the frequency of CD4⁺Lag-3⁺,
381 relative to the value in the unstimulated condition, upon parasite lysate stimulation in
382 activated (Ox40⁺CD25⁺) and non-activated (Ox40⁻ or CD25⁻) CD4⁺ T cells. *: $p<0.05$; **:
383 $p<0.01$; ***: $p<0.005$. NoAg: unstimulated condition; Tc: *T. cruzi* lysate stimulation.