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1 # Cellular innate and adaptive immunity are affected by chronic exercise:
2 # implication of the cannabinergic system

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21 # Abstract

22 The impact of performing exercise on the immune system presents contrasting effects on health when
23 performed at different intensities. In addition, the consequences of performing chronic exercise have
24 not been sufficiently studied in contrast to the effects of acute bouts of exercise. Our findings shed
25 light on the effects that chronic exercise elicits on several immune cell subpopulations, from the
26 innate to the adaptive immunity. For this study male Wistar rats performed treadmill running 5 times
27 a week for a period of 10 weeks, speed and duration in each exercise bout was gradually increased
28 until reaching 40 minutes at 15 m/min. Our results showed a significant decrease in lymphocyte
29 subpopulations (CD4+, Tγδ, and CD45 RA+ cells) and also indicate an alteration in the cannabinoid
30 receptors expression in some of these cells subsets. Although functional assays did not reveal any
31 variation in total immunoglobulin production or NK cells cytotoxic activity, proliferative capability
32 of total splenocytes increased in trained rats. Our results further support the notion that exercise
33 affects the immunological system and extends the description of underlying mechanisms mediating
34 such effects. Altogether, our results contribute to the understanding of the benefits of exercise on the
35 practitioner´s general health.

36

37 # Introduction

38 The beneficial impact of exercise on the practitioner's general health is a well-known fact [1–3].
39 Studies suggesting a connection between physical activity and the improvement of health have
40 generated information that ranges from describing adipose tissue loss to changes in genetic
41 expression [4,5]. It is well known that the immune system (IS) is critical to maintain health, and
42 whether it is enhanced or hindered when performing exercise is still controversial. Its proper

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43 composition and its correct function will allow it to actively overcome challenges that otherwise
44 would compromise the organism's health such as infections, autoimmune diseases, cancer, etc.

45

46 Data regarding the changes induced by exercise in cell subpopulations of the IS and their function
47 seems to be controversial [6–8]. This may be partly explained by the use of different exercise
48 paradigms. Along with this idea, many studies document the immediate changes on the IS induced by
49 a single bout of exercise, as opposed to the effects of its chronic performance, which suggest
50 different outcomes and in some cases opposite effects over the IS [8–10]. Less attention has been
51 paid to such studies and to the long-term alterations that it may produce on the IS. For instance,
52 macrophages extracted from mice trained for 12 weeks exhibited increased phagocytic activity,
53 superoxide anion production and glucose consumption when compared to macrophages obtained
54 from sedentary mice [8]. Consistently other studies have shown that chronic exercise alters the
55 function of T cells, affecting their production of pro- and anti-inflammatory cytokines, including the
56 up-regulation of IL-2, an important cytokine related to proliferation and activation [10]. Likewise,
57 trained rats presented increased glucose consumption, IL-2 production and IL-2R expression by their
58 lymphocytic subpopulations. Furthermore, those changes obtained in trained animals seem to last
59 days after the last exercise bout [10].

60

61 Many molecular pathways that are affected by exercise possess an immunoregulatory potential,
62 ranging from variations in the energy substrates [11,12] to the activation of signaling pathways with
63 direct immune-regulatory relevance, such as: the release of IL 6 by skeletal muscle [4,9], release of
64 stress hormones, catecholamines [4,13] and neurotransmitters by the sympathetic and para-
65 sympathetic system, among others. Hence, in order to contribute to the further understanding of these

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66 effects we decided to evaluate the cannabinergic system (CBS). Regarding this system, some studies
67 have reported a subtle increase of anandamide, a widely studied molecule that acts as a CB1 and CB2
68 receptor agonist, after short bouts of aerobic exercise. Such increase was sustained up to several
69 minutes after the conclusion of the physycal activity [14–16]. Furthermore, both receptors are widely
70 distributed in the immune cells and IS structures [17–19] and when activated, together or
71 independently, produce changes in the function of several immune cells, suggesting that their
72 activation is able to modulate the IS function. These modulatory actions have been explored *in vitro*
73 [20,21] and *in vivo* [19]. Likewise, the expression of cannabinergic receptors (CBR) on the surface of
74 immune cells, varies according to their activation and inflammatory status. Given that new data
75 suggests its relevance as an immune-modulatory system, the expression of these receptors provides
76 us with interesting and relevant information about the IS status.

77 Altogether, our study is focused on exploring the long-term changes that chronic exercise (CE)
78 produces in the proportion of splenocytes from the adaptive and innate immunity, assessing the
79 effects of CE on the function of splenocytes (by performing proliferation tests and cytotoxicity test
80 with total splenocytes *in vitro*), and finally on determining if the expression of CBR in immune
81 cells is affected by chronic exercise. Our findings suggest a wide variety of effects induced by CE on
82 several immune cell subpopulations, from the innate and adaptive immunity. The latter being the
83 most affected by CE, with significant decreases in some lymphocyte subpopulations (CD4+ and
84 CD45 RA+ cells). Our results also suggest an alteration in the expression of CBR as a consequence
85 of training in natural killer cells (NK), T helpers, Tγδ lymphocytes and B lymphocytes. Surprisingly,
86 functional assays did not reflect any impairment in total immunoglobulin production or cytotoxic
87 activity from splenocytes derived from trained rats, which is contrasting with the decrease in
88 lymphocytic subpopulations. Furthermore, results from the proliferation assay showed a significant

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89 enhancement in the proliferative capabilities of splenocytes from trained rats, opening a new
90 perspective on the boosted efficiency in immune cell subpopulations promoted by CE.

91

92 Materials and Methods

93 Ethic statement

94 Animal care and experimental practices were conducted at the Animal Facilities of the Instituto de
95 Fisiología Celular (IFC), Universidad Nacional Autónoma de México (UNAM). All procedures in
96 the experimental animals were approved by the Institutional Care and Animal Use Committee
97 (CICUAL), adhering to Mexican regulation (NOM-062-ZOO-1999), in accordance with the
98 recommendations from the National Institute of Health (NIH) of the United States of America (Guide
99 for the Care and Use of Laboratory Animals). Euthanasia of experimental animals was performed in
100 a humanitarian way.

101

102 Animals

103 For this study, male Wistar rats ranging between 250 to 300 g were used, proceeding from our own
104 breeding at the animal facilities of IFC, UNAM. The animals were housed at IFC with controlled
105 temperature (22°C) and 12-hr light-dark cycles, with water and Purina LabDiet 5015 chow *ad libitum*
106 (Purina, St. Louis MO). Rats were sacrificed by cervical dislocation after pentobarbital sodium
107 (Pisabental®, México) anaesthesia.

108

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109 ## Exercise Protocol

110 Animals were set in one of three experimental groups: Exercised (EXE), Treadmill control (TC) and
111 sedentary group (SED). Animals in the exercised group performed treadmill running 5 times a week
112 for a period of 10 weeks, for which a previous habituation of one week was completed. During the
113 habituation week, animals were placed inside the treadmill and then it was turned on at minimum
114 capacity (4m/min) for 5 minutes per day. Once the habituation period was completed, animals started
115 training. On the first day of training, rats ran at 7.5 m/min for 10 minutes, then speed and duration of
116 exercise was escalated gradually each consecutive day, in order to achieve a daily exercise bout of 40
117 minutes at 15 m/min by the fourth week. Remaining weeks of training were kept constant in speed
118 and duration until the sacrifice of the animals.

119 Animals sited in the TC group were placed inside the treadmill at minimum capacity (4m/min) for 10
120 minutes, 5 times per week, for the same period of time than the exercised group (10 weeks). While
121 being inside the treadmill, animals from TC group were exposed to the same context than animals
122 from the EXE group without being exercised, reflecting any effect in the results prompted by
123 sources other than exercise itself. Animals conforming the SED group were kept alive in standard
124 conditions for the same amount of time than the other two groups.

125 At the end of the training period animals from every group were allowed to rest for two days in order
126 to eliminate any possible effect of acute exercising. Afterwards, animals were sacrificed and samples
127 taken.

128 ## Flow Cytometry

129 Spleens were manually disaggregated using a 50µm nylon mesh, and cells resuspended in PBS.
130 Erythrocytes in the solution were lysed using ACK buffer (150 mM NH_4Cl, 10mM $KHCO_3$, 0.1mM

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131 Na_2EDTA, pH 7.3) for 10 minutes and washed three times with PBS, then cells were resuspended in
132 FACS buffer (PBS, FBS, 0.02% NaN_3).

133 Approximately $1x10^6$ cells were incubated with the following antibodies in order to characterize
134 spleen immune subpopulations: Alexa Fluor® 488-conjugated- anti rat CD3 (IF4, Biolegend), PE
135 Cy5-conjugated- anti rat CD4 (biolegend), PE-conjugated anti rat CD8a (Biolegend), PE-conjugated
136 -anti rat CD45RA (Biolegend), Alexa Fluor® 647-conjugated- anti rat CD161 (biolegend), biotin-
137 conjugated- anti rat CD11b (OX-42, Bioloegend), PE-conjugated anti rat TCR (V65, Biolengend).

138 For the staining of cannabinoid receptors, the polyclonal primary antibodies used were: rabbit anti
139 Cannabinoid receptor I (abcam®) and rabbit anti Cannabinoid receptor II (abcam®), followed by the
140 Secondary antibodies: AlexaFluor® 488- conjugated goat anti rabbit IgG (ThermoFisher Scientific)
141 and DyLight® 649- conjugated- anti rabbit IgG (Vector laboratories).

142 In order to assess proliferation, a Cell Trace™ CFSE cell proliferation kit was used (Invitrogen™).
143 Propidium iodide was used to determine viability of cells during cytotoxic assays. Attune Cytometer
144 (life technologies) was used to obtain data which was further analyzed with FlowJo software
145 (Treestar Inc.).

146

147 Proliferation assays

148 Total splenocytes were obtained as previously described and quantified on a Neubauer chamber.
149 Subsequently, splenocytes were marked with CFSE cell tracer, , which was used according to the
150 manufacturer's protocol. Finally, cells were resuspended in RPMI-1640 medium (ATCC® 30
151 2001™). Splenocytes were cultivated in RPMI-1640 medium plus Ionomicyn (SIGMA-ALDRICH®

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152 10634™) and PMA (SIGMA-ALDRICH® P8139-1MG™) at concentrations100 nm and and 25ng/ml
153 for 72 hours. Proliferation was assessed using an Attune cytometer (Life Technologies) with blue and
154 red lasers, obtained data was further analyzed with FlowJo software (Treestar Inc.).

155

156 Cell Culture

157 Yac 1 cell line (ATCC® - TIB 160™) was cultivated in RPMI 1640 medium (ATCC® 30-2001™)
158 supplemented with 10% Fetal bovine serum (FBS, ATCC® 30-2020™) and kept with air, 95%;
159 carbon dioxide (CO^2), 5% at 37ºC. Yac 1 cells were expanded for a week then counted on a
160 Neubauer chamber and finally stained with CFSE kit for further use in cytotoxic assays.

161

162 *In vitr*o cytotoxic assay

163 Yac 1 cells were stained with cell trace™ CFSE kit according to the manufacturer's protocol and
164 used as target cells for the assay. A single cell splenocyte suspension was obtained from rats in the
165 different experimental conditions as previously described, they were counted and used as the effector
166 cells in the assay. Finally both, effector and target cells were co-cultivated in RPMI medium
167 supplemented with 10% FBS for 4 hours, into 96 round well plates. Thereafter co-cultures were
168 removed from the incubator and stained with Propidium Iodide and washed with Facs buffer.
169 Acquisition was performed in an Attune Cytometer (life technologies) and data further analyzed
170 with FlowJo software (Treestar Inc.).

171

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172 ## Corticosterone and IgG levels assesssment

173 Animals were anesthetized and sacrificed one day after concluding their experimental condition, at
174 the same time that they had been set for exercising (14:00-16:00 hr.). Cardiac puncture was
175 performed in order to extract blood, which was immediately centrifuged at 4000rpm to collect the
176 blood serum. Subsequently, blood serum was divided into two aliquots and stored at -70ºC for later
177 use. Corticosterone levels were assessed with a Corticosterone ELISA kit (Abcam® ab-108821) and
178 procedures underwent according to manufacturer's protocol. Assessment of the levels of IgG in
179 serum were carried out on a 96 flat bottom well plate. The plate was previously sensitized with a
180 dilution of blood serum (1:1000), washed and blocked with a 1% albumin solution. Subsequently, ,
181 the plate was incubated for 2 hours antibodies α IgG rat HRP were incubated, and once incubation
182 finished, several washes were performed, chromogen was added to the wells, the reaction was
183 stopped and the reading of the plate was carried out on a Stat Fax 4200 microplate reader (Awarness
184 Technology).

185

186 ## Statistical analysis

187 For data regarding the changes of every cell subpopulation, a one-way ANOVA ($\alpha = 0.05$) was
188 performed followed by a Tukey post-hoc test. Differences were considered significant when $p < 0.05$,
189 with the actual p value and n being stated in each figure legend. Before the selection of the ANOVA
190 test the normal distribution of the data was assessed via Shapiro-Wilk test. A similar process was
191 carried out for the statistical analysis of the data regarding the proliferation and cytotoxicity tests, as
192 well as for the data concerning the levels of corticosterone and IgG in serum. For the assessment of
193 the expression of CBR, a two-way ANOVA ($\alpha = 0.05$) was performed, because of the consideration

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194 of two independent variables (group and CBR), followed by a Bonferroni post-hoc test with the same
195 significant difference criterion. Data from all the experiments were charted as mean ± standard error ,
196 and analysed with Prism 5 software for Mac (GraphPad Software Inc.)

197

198 Results

199 Chronic-moderate exercise alters the composition of splenocyte
200 subpopulations

201 Distribution of immune cells is a parameter that provides information about deficiencies or
202 alterations from the IS, therefore we decided to evaluate several cell subpopulations from the innate
203 and adaptive immune system in the spleen of rats that underwent different experimental conditions
204 (supplementary Fig 1). The immune cell subpopulations from the innate immune response that were
205 analyzed corresponded to: NK cells (CD161+) and macrophages (CD11b+) for which flow cytometry
206 analysis did not reflect a significant difference among groups (Fig 1). Analyzed cells from the
207 adaptive immune response were: total T lymphocytes (CD3+), T helper lymphocytes (CD4+),
208 cytotoxic T lymphocytes, Tγδ lymphocytes (Tγδ+) and B lymphocytes (CD45 RA+). For those cells
209 studied, flow cytometry analysis reflected a decrease in the proportion of T helper lymphocytes and
210 in B lymphocytes from the EXE group when compared to both control groups, SED and TC (Figs 2
211 and 3), therefore considering such changes an effect of CE. On the other hand Tγδ lymphocytes
212 showed an increase in the EXE group when compared to SED and TC groups (Fig 2), once more
213 reflecting a change attributable to training. On the other hand, T cytotoxic cells decreased in TC and
214 EXE groups in contrast to SED control group, reflecting an effect non attributable to exercise, but to

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215 the exposure to the treadmill (Fig 2). Total T lymphocytes (CD3+), did not show changes among the
216 experimental groups (Fig 2).

217

218 **Fig 1. Changes in the composition of splenocyte populations related to the innate immune**
219 **response.**

220 (A) Representative dot plot of the cytometric analysis of the subpopulation percentages. (B)
221 Determination of splenocyte subpopulations from the innate immune response one day after being
222 exposed to each condition in the different groups: SED, TC and EXE; data from 4 independent
223 experiments are expressed as mean ± SE. No subpopulation showed statistically significant changes:
224 Natural killer cells (ANOVA, p=0.0683, n=10), and Macrophages (ANOVA, p=0.0273, n=10).

225

226 **Fig 2. Changes in the composition of splenocyte populations related to the adaptive immune**
227 **response.**

228 (A) Representative dot plot of the cytometric analysis of the subpopulation percentages. (B)
229 Determination of splenocyte populations from the adaptive immune response in the different groups:
230 SED, TC and EXE; data from 4 independent experiments are expressed as mean ± SE. Subpopulation
231 that showed statistically significant changes were: T helper lymphocytes (ANOVA, p=0.0008, n=5)
232 and Tγδ lymphocytes (ANOVA, p=0.0002, n=10). T lymphocytes (ANOVA, p=0.3739, n=12),
233 cytotoxic T lymphocytes (ANOVA, p=0.0016, n=12). * Means statistically different from the two
234 other groups, P<0.05.

235 **Fig 3. Changes in the composition of B-lymphocytes from spleen.**

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236 (A) Representative dot plot of the cytometric analysis of the subpopulation percentages. (B)
237 Determination of B-lymphocytes of spleen of rats in the different groups: SED, TC and EXE; data
238 from 4 independent experiments are expressed as mean ± SE. B lymphocytes showed a statistically
239 significant change (ANOVA, p=0.3601, n=10). * Means statistically different from the two other
240 groups, P<0.05.

241

242

243 Modulation of CBR Expression in splenocytes after chronic exercise

244 CBR are widely distributed among immune cell subpopulations and structures from the IS. The
245 expression of CBR on immune cells has been demonstrated to vary depending on activation or
246 inflammatory profile, among other parameters. Thus, we decided to evaluate if CE would promote
247 changes in the expression of CBR on splenocytes.

248 From the innate immune system, NK cells (CD161+) and macrophages were analyzed. NK cells from
249 EXE and TC groups showed a decrease in CB2 expression, compared to SED, while no change was
250 observed in CB1 expression. Macrophages (CD11b+) did not present changes in the expression of
251 any CBR among groups (Fig 4). From the adaptive immune response, T helper lymphocytes (CD4+
252 cells) presented an increase in the expression of CB1 in animals from EXE group when compared to
253 both control groups SED and TC, while no statistically significant difference was observed regarding
254 CB2 expression. A similar phenomenon was observed in Tγδ subpopulation from EXE animals,
255 which showed a higher expression of CB1 when compared to SED and TC groups (Fig 5), with no
256 change in the expression of CB2. The expression of CBR did not vary in the subpopulations of T

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257 lymphocytes (CD3+) and cytotoxic T lymphocytes when experimental groups were compared (Fig
258 5).

259 **Fig 4. Expression of CBR in splenocyte populations related to the innate immune response.**

260 Analysis of the expression of CB1 and CB2 CBR in splenocyte populations from the innate immune
261 response (NK's and macrophages) among experimental groups: SED (shaded bar), TC (white bar)
262 and EXE (Solid bar); data from 4 independent experiments are expressed as mean ± SE. Lines
263 connecting bars represent comparison among groups, * $p<0.05$. Two way ANOVA and Bonferroni
264 post-test, n=9.

265 **Fig 5. Expression of CBR in splenocyte populations related to the adaptive immune response.**

266 Analysis of the expression of CB1 and CB2 CBR in splenocyte populations from the adaptive
267 immune response (T lymphocytes; n=10, T helper lymphocytes; n=8, cytotoxic T Lymphocytes;
268 n=10 and Tγδ n=8) among experimental groups: SED (shaded bar), TC (white bar) and EXE (Solid
269 bar); data from 4 independent experiments are expressed as mean ± SE. Lines connecting bars
270 represent comparison among groups, * $p<0.05$. Two way ANOVA and Bonferroni post-test.

271

272 On the other hand, B lymphocytes from the spleen of EXE animals showed a significant increase in
273 CB2 expression, compared to those from SED and TC groups, whilst no change was reflected
274 between groups in the expression of CB1 (Fig 6).

275 **Fig 6. Expression of CBR in B lymphocytes from spleen.**

276 Analysis of the expression of CB1 and CB2 CBR in B lymphocytes from spleen among experimental
277 groups: SED (shaded bar), TC (white bar) and EXE (Solid bar); data from 4 independent experiments

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278 are expressed as mean ± SE. Lines connecting bars represent comparison among groups, * $p<0.05$.
279 Two way ANOVA and Bonferroni post-test, n=10.

280

281 Immunoglobulin G levels are not altered by chronic exercising

282 Immunoglobulin G (IgG) is the most abundant type of immunoglobulins and a reliable parameter to
283 assess the function of plasmatic cells. A change in the amount of IgG could represent an ongoing
284 infectious process or an alteration on the normal function of plasmatic cells when observed in intact
285 animals. We decided to assess if the total production of IgG would vary among our experimental
286 groups. To do so, we performed a direct semi-quantitative ELISA. When data was analyzed
287 statistically (ANOVA, n=6, p=0.0676) results did not show any significant difference among the
288 experimental groups: SED (optic density 2.731),TC and EXE (OD 2.973 and 2.871 respectively,
289 supplementary Fig 2).

290

291

292 Chronic exercise enhances proliferative capacity but not cytotoxic
293 activity of total splenocytes

294 Proliferative capacity and cytotoxic activity have been tested before in order to assess the degree of
295 competence of a subject's immune system. In order to observe if chronic exercise has any effect on
296 both features we performed *in vitro* tests. For the proliferation assay we obtained total splenocytes
297 and cultivated them on complete RPMI medium plus PMA and ionomycin for 72 hours. Data
298 analyzed reflected no difference among groups in their proliferation index (Fig 7B). Nevertheless,

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299 total splenocytes from EXE group showed a higher proportion of dividing cells when compared to
300 SED and TC groups (Fig 7C). Furthermore, we decided to test the proliferative capacity of the
301 immune cell subpopulations and we found that B lymphocytes and NK cells from the EXE group
302 exhibited a higher proportion of dividing cells than both SED and TC groups (Figure 7D).

303 **Fig 7. Effect of chronic exercise on splenocyte proliferation.**

304 (A) Representative histograms of cytometric analysis of dividing cells. Two parameters were
305 considered to assess the proliferative capacity of splenocytes: (B) the proliferation index, which did
306 not change due to chronic exercise and (C) the proportion of dividing cells, where we observed an
307 increase in the exercised group when compared to both control groups (ANOVA, p=0.0092, n=5).
308 (D) Analysis of proliferative capacity of specific splenocyte subpopulations: T helper lymphocytes
309 (ANOVA, p=0.4543, n=5) and cytotoxic T lymphocytes (ANOVA, p=0.5248, n =6) did not show
310 any change between groups; B lymphocytes (ANOVA, p=0.0006, n=6) and Natural killers (ANOVA,
311 p=0.0191, n=6) from EXE did show an in increase in the proportion of dividing cells when compared
312 to both control groups. In graphic bars SED is represented by shaded bar, TC by white bar and EXE
313 by the solid bar.

314

315 In order to measure the cytotoxic capacity of splenocytes from the different groups, we performed a
316 cytotoxicity test utilizing Yac1 cells as target cells and cultivating them with total splenocytes from
317 animals of the different groups. Yac1 cells were previously marked with CFSE, and at the end of the
318 test dead cells were dyed with propidium iodide, so double positive cells represented the target cells
319 killed. The assay was carried out at three different effector/target ratios: 10:1, 50:1 and 100:1 and
320 none showed any significant difference among treatments after the statistical analysis (Fig 8).

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321 **Fig 8. Cytotoxic activity of total splenocytes in vitro.**

322 (A) Representative dot plot of cytometric analysis of killed target cells percentage. (B) Cytotoxic
323 activity of total splenocytes was assessed in vitro at three different ratios (effector cells: target cells,
324 10:1, 50:1 and 100:1); data from 2 independent experiments are expressed as mean ± SE, with an n=6
325 for each condition. No differences were found among the groups at any of the different ratios. In bar
326 graphics SED is represented by the shaded bar, TC by the white bar and EXE by the solid bar.

327

328

329 Corticosterone level is not altered by chronic exercise

330 Corticosterone level is considered a reliable stress marker in animals. In order to further comprehend
331 the data obtained, we decided to analyze if corticosterone levels of blood serum differ among groups,
332 reflecting a possible long-term effect of stress in exercised animals. When data was statistically
333 analyzed (ANOVA, n=6, p=0.0473, Tukey's) results show a significant difference among the
334 experimental groups. Nonetheless, The post hoc analysis did not show any significant interaction
335 among the experimental groups. Means of the groups: TC and EXE (102.6 and 96.5 ng/ml
336 respectively) were notoriously higher than that of the SED group (46.8 ng/ml, supplementary Fig 3).

337

338 Discussion

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339 Although widely studied, the consequences of physical activity over the IS remain as a promising
340 field not only to expand our comprehension on basic physiology, but also on physiopathology and the
341 different processes that take part on the orchestration of the immune response. At first instance, our
342 investigation tries to emulate in rats a popular paradigm of exercising in modern times across the
343 global population, which involves the chronic performance of medium intensity resistance exercise.
344 Such particularities in our model have led us to asses its raw impact over the IS composition and its
345 function. Notwithstanding, the vast majority of studies have focused in the effects of short bouts of
346 exercise over the immediate changes in composition and function of the IS [1,8,22,23], and
347 neglecting those focused on the effect of CE and its long lasting effects over the IS [9,24]. In this
348 study we observed that components from the innate immune response were not affected by chronic-
349 moderate exercise, while elements from the adaptive immune response did change in those animals
350 that underwent physical training. T helper lymphocytes and B lymphocytes were decreased in trained
351 animals, contrary to what would be expected according to the popular statement of moderate exercise
352 enhancing a pro-inflammatory state [25,26]. On the other hand, Tγδ lymphocytes increased in
353 animals from the EXE group, augmentation that could reflect an increased surveillance and
354 protection of the upper respiratory tracts (URT) and mucosa tissue, idea that would be in accordance
355 with the strengthened resistance against URT infections due to moderate exercising and opposite to
356 the well documented effect of higher susceptibility to these infections in high performance athletes
357 [23,27–31]. Nonetheless, the reduction of T helper and B lymphocyte populations was unexpected,
358 since these cells play a major role at recognizing antigens and therefore at orchestrating immune
359 responses against new and already known threats and once activated they can polarize towards a pro-
360 inflammatory or anti inflammatory state which modulates the activity of several other components
361 from the innate and adaptive immune response. Consequently, the decrease of T helper lymphocytes
362 as well as that of B lymphocytes made us wonder if it could be translated into a deficient immune
363 response of animals of the EXE group.

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364 Once we determined that our exercising paradigm did affect the composition of immune cells
365 subpopulations, our next goal became to assess if those changes would be translated into functional
366 alterations. To accomplish our purpose, we determined total IgG in serum among experimental
367 groups and we also performed proliferation and cytotoxicity test *in vitro*. On the proliferation test,
368 two parameters were evaluated: the proliferation index and the percentage of dividing cells. The first
369 represents the mean of divisions that dividing cells underwent during the assay, which showed no
370 difference among groups. In turn, the percentage of dividing cells among groups during the
371 experiment showed statistical differences, being higher in splenocytes from the EXE group.
372 Altogether, this data suggests that cells from EXE group are not more efficient at dividing once they
373 have been activated but that more cells in proportion from EXE group are prone to proliferate once
374 they have been exposed to PMA and ionomycine. These results made us wonder if this trend would
375 be persistent in basal conditions, so we compared the percentage of proliferation in splenocytes
376 among the experimental groups without activation by PMA and ionomycine and the statistical
377 analysis showed no difference among them, reflecting a response produced by activation and not an
378 anomaly that could reflect an inflammatory state that in turn could favor an autoimmune response.
379 Thereafter, we evaluated the percentage of dividing cells from specific subpopulations, T helper
380 cells, cytotoxic T cells, B lymphocytes and NK cells, showing an increase in the percentage of
381 dividing cells in the last two subpopulations by effect of exercise. On the other hand, statistical
382 analysis from the cytotoxicity test did not show variation among the experimental groups in any of
383 the target/effector cell ratios tested, so data provided from this experiment does not suggest a higher
384 cytotoxic activity from NK cells as a consequence from exercise. Nonetheless, exercise enhances the
385 amount of NK cells that proliferate, which may indicate a higher immune-surveillance against
386 transformed and virally infected cells in chronically exercised subjects.

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387 Even though we found a decrease in major lymphocyte subpopulations (CD4+ and CD45 RA+ cells),
388 which has been reported before for other immune cells in long term exercised individuals [26,32–34],
389 we also determined that a bigger proportion of splenocytes is prone to activate when stimulated, plus
390 other functions of splenocytes from the EXE animals were not impaired. Given the fact that long-
391 term exercised subjects do not report any kind of immune suppression, we have come to hypothesize
392 that the decrease in the composition of some cell subpopulations may represent a more efficient IS,
393 which requires less elements but presents a stronger reaction when needed. This idea is also
394 supported by the results shown by the ELISA test, which showed that levels of IgG do not change
395 among the groups, even though the subpopulation of B lymphocytes is decreased in EXE animals.

396 Finally, recent works have denominated the endo-cannabinoid system (ECS) as an
397 immunomodulatory system; inhibiting the function of highly reactive and pro-inflammatory cells
398 [21,35,36]. The ECS exerts its functions through the activation of its receptors, which expression
399 vary greatly depending on cell subpopulation, activation or inflammatory status [20,35,37,38], being
400 increased in the surface of more reactive cells. In this work we demonstrated that CE promotes
401 changes in the expression of CBR's in splenocytes, findings that concur with previous works
402 showing that during moderate exercise bouts there is an increase of circulating ECS agonists [14,15].
403 We must emphasize that changes in CBR's expression remained after one skipped day of training,
404 which suggests that subjects exercising on a daily basis or in intervals of every two days might be
405 maintaining this alterations. Our methodology allowed us to determine differences in the expression
406 of CBR among cell subpopulations even in both control groups. These differences might be
407 explained by the intrinsic variability among cell subpopulations. We also assessed an increased
408 expression of CB1 receptor in the subpopulations of T helper and Tγδ lymphocytes and an increase in
409 the expression of CB2 receptor in B lymphocytes of exercised animals. Therefore enhanced
410 expression of CB1 in T helper, Tγδ and CB2 in B lymphocytes could represent a mechanism to

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411 diminish the activity of these highly metabolic and inflammatory cells from EXE animals, as
412 reflected by the proliferation tests. Some of these data differ from anterior reports concerning
413 expression of CBR, nonetheless most of those reports used different techniques and did not measure
414 the protein conforming CBR's [17,39,40].

415 We would like to address those changes presented on this work that can not be attributable to CE,
416 like the composition of cytotoxic T cells for which TC and EXE groups differ when compared
417 against SED group, reflecting an effect relying probably on the stress produced by the placement of
418 the animals inside the treadmill, being that, the one thing that those groups had in common. The same
419 explanation seems plausible for the expression of CBR in NK and cytotoxic T cells. Nevertheless
420 analysis of corticosterone did not showed significant difference among the experimental groups,
421 different sensitivity to several molecules has been reported for the wide variety of cells from the IS,
422 leaving the possibility of other molecular interactions that escaped our control and awareness.

423

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438

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439 # Author Contributions

440 SVS performed the training of animals, the extraction of tissues, most of the experimental procedures
441 and the analysis of data, writing and edition of this manuscript.

442 KNC helped with the design of experiments regarding the proliferation assays and cytotoxicity.

443 MIP performed some of the experiments and analyzed some of the data regarding the asses of cell
444 subpopulations distributions.

445 RD collaborated in the direction of this work and the design of the experimental procedures. Also
446 contributed with reagents and the majority of expenses derived from this work.

447 OP collaborated in the direction of this work, the analysis of data and preparation of the draft. Also
448 contributed with reagents.

449 JMM participated in the direction of SVS , the design, analysis and writing of the present paper. Also
450 contributed with reagents, and all the flow cytometric analyses.

451

452 # Conflict of interest statement

453 The authors declare that they have no conflict of interest.

454

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586

587 # Supplementary figure legends

588

589 **Supplementary Fig 1. Gating strategy for the flow cytometric analysis of rat spleen**
590 **subpopulations and their expression of CB1 and CB2 receptors.**

591 Single cell suspension was prepared and stained with fluorochrome-conjugated antibodies to separate
592 splenocyte subpopulations and to mark cannabinoid receptors (CB1 and CB2). Data was analyzed
593 with FlowJo software 8.7 for Mac. Lymphocytes were identified by their scatter properties (FSC-A x
594 SSC-A plot). Splenocyte subpopulations were characterized by surface staining and gated for their
595 quantity assessment. Subsequently each cellular subpopulation was analyzed for their expression of
596 both cannabinoid receptors in their surface.

597

598

599 **Supplementary fig 2. Levels of IgG are not affected by chronic exercise.**

600 The analysis of total IgG was assessed for every experimental group with the use of a direct semi-
601 quantitative ELISA. Statistical analysis did not show any significant difference among the
602 experimental groups: SED (shaded bar), TC (white bar) and EXE (Solid bar). P>0.05. ANOVA,
603 p=0.0676, n=6.

604

605 **Supplementary fig 3. Serum corticosterone concentration one day after the last exercising bout.**

606 Data is shown as mean (ng/ml) +- SE for each group. There was no significant difference among
607 group values in concentration of serum corticosterone. Groups analyzed: SED (shaded bar), TC
608 (white bar) and EXE (Solid bar). When data was statistically analyzed (ANOVA, n=6, p=0.0473,
609 Tukey's) results show a significant difference among the experimental groups. Nonetheless, The post
610 hoc analysis did not show any significant interaction among the experimental groups. Means of the
611 groups: TC and EXE (102.6 and 96.5 ng/ml respectively) were notoriously higher than that of the
612 SED group (46.8 ng/ml).

613

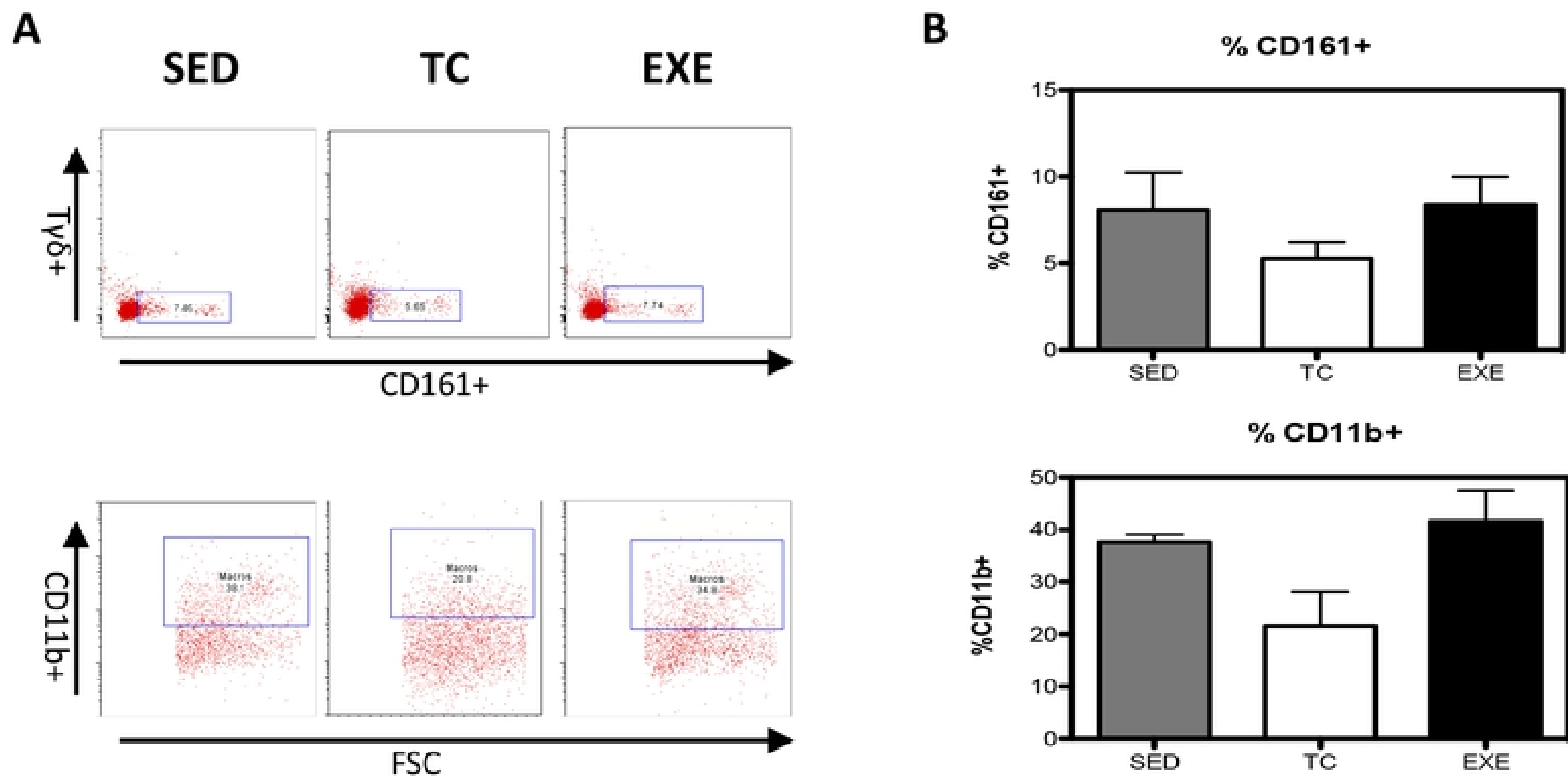

Figure 1

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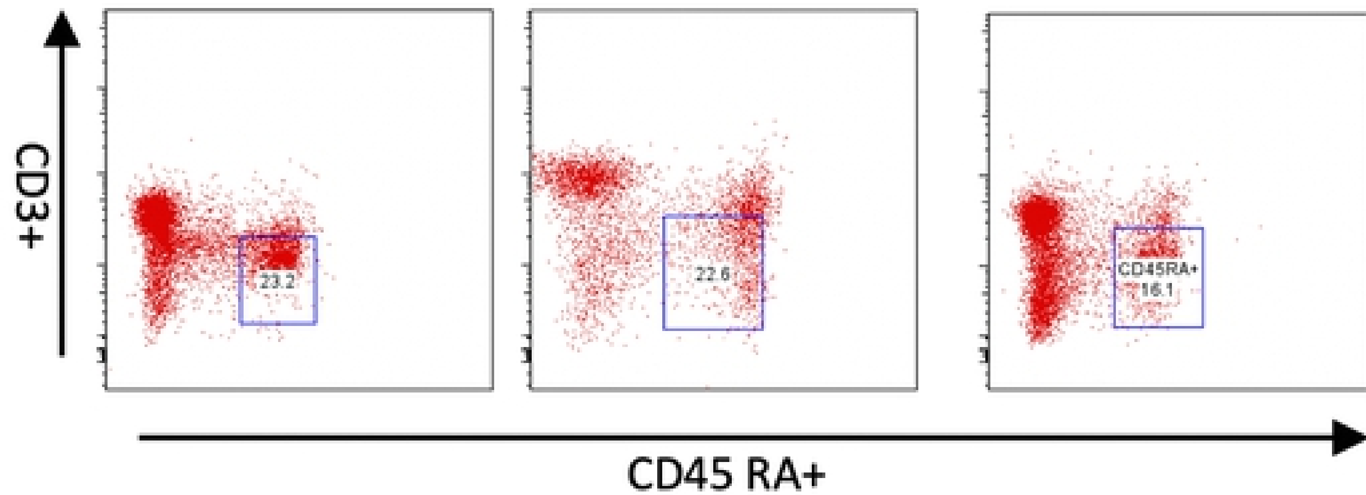
Figure 2



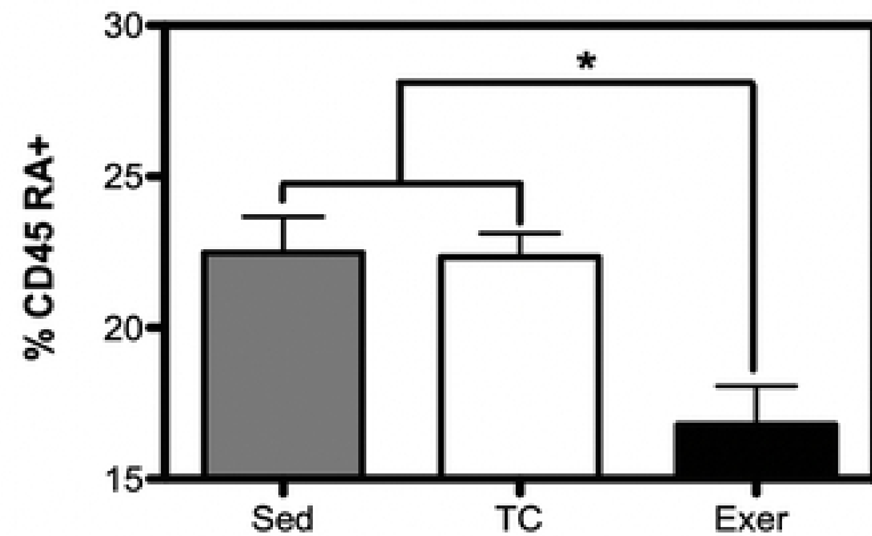
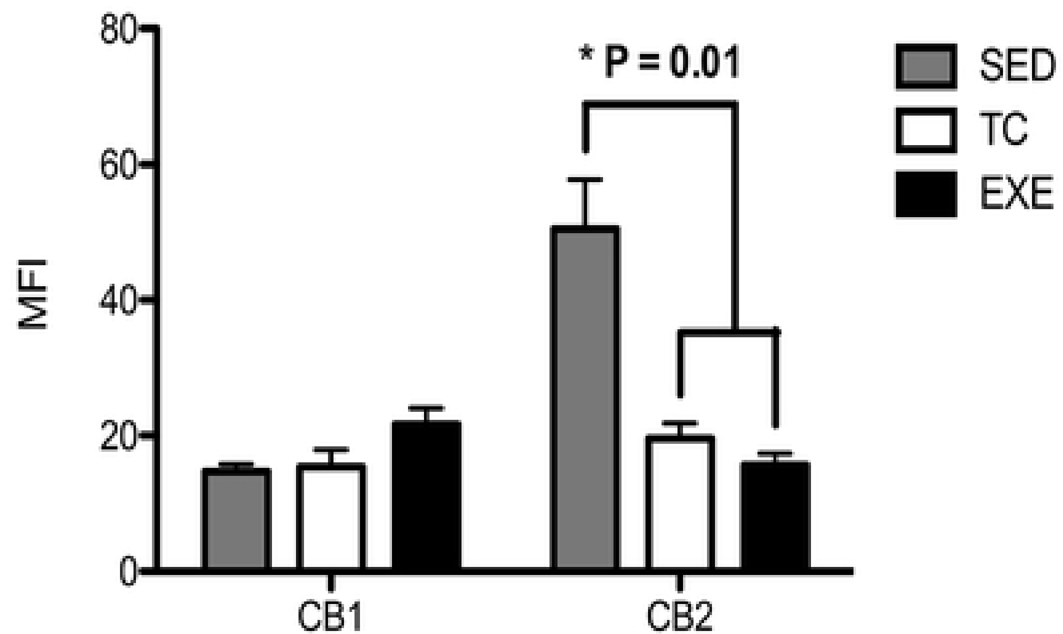


Figure 3



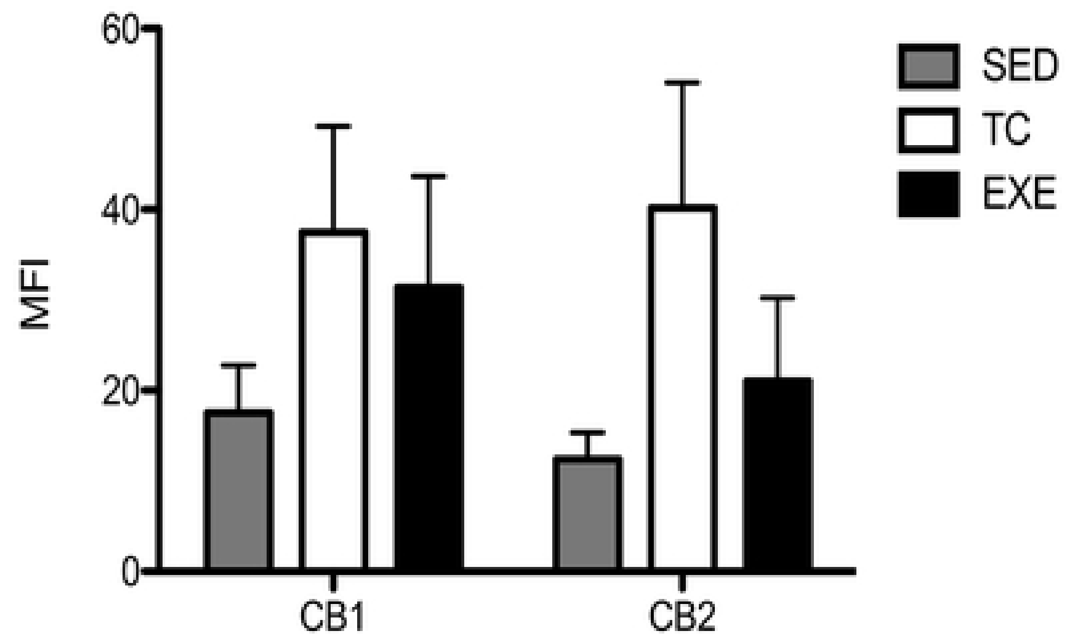
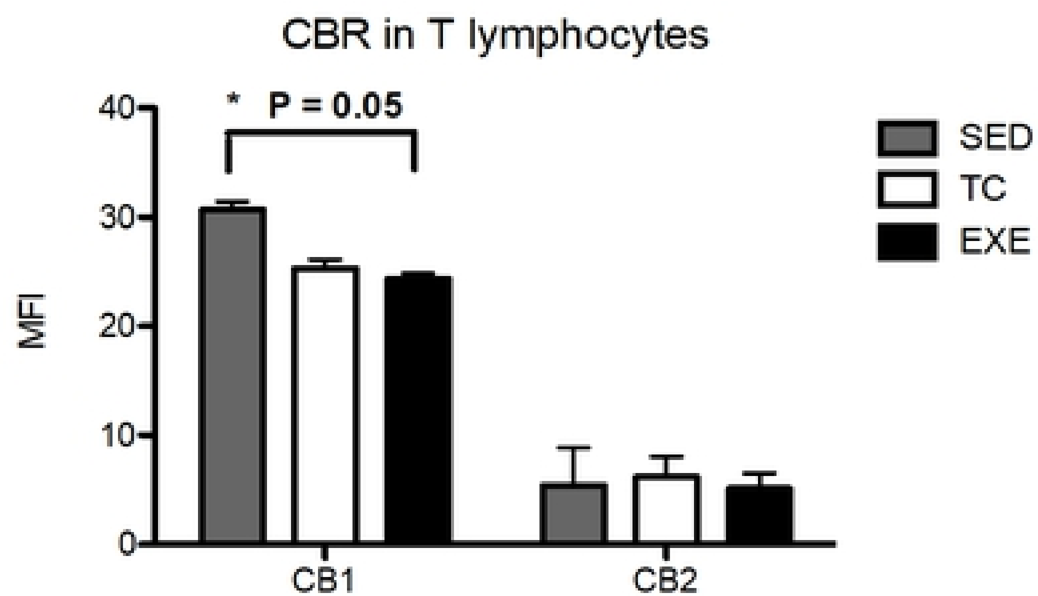
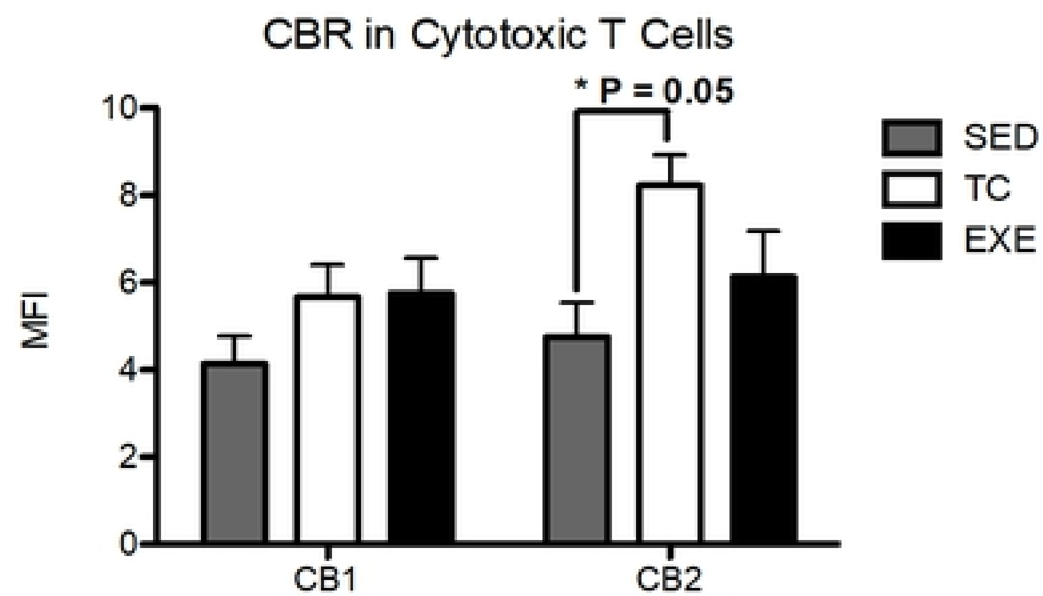
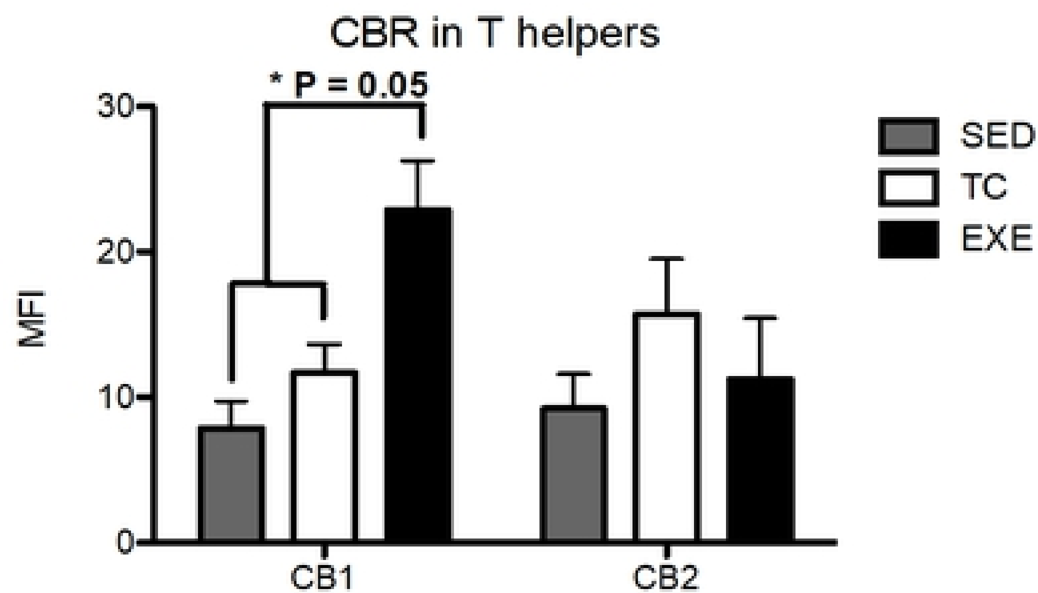
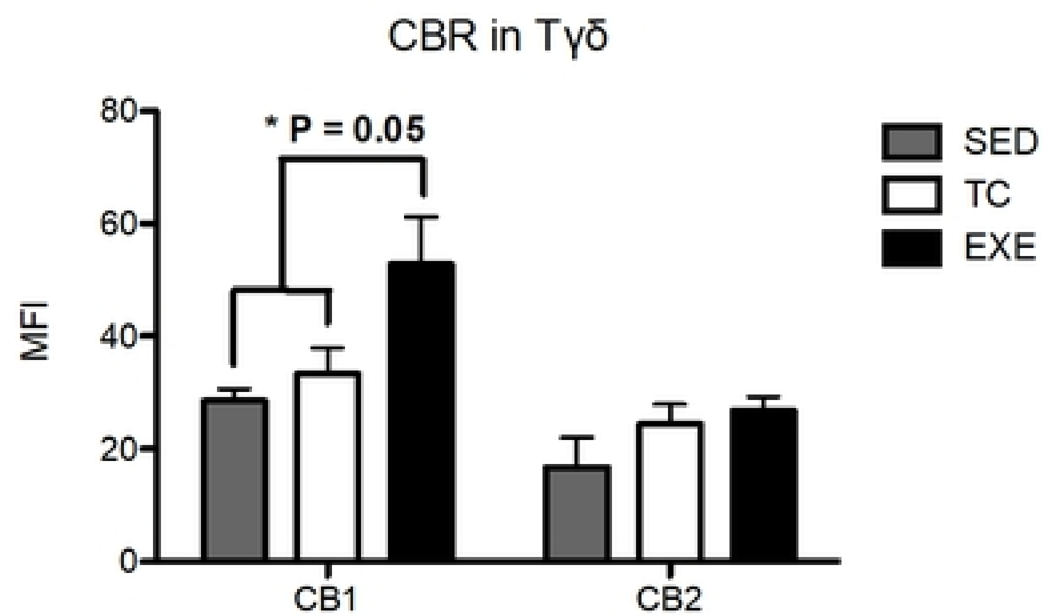


Figure 4









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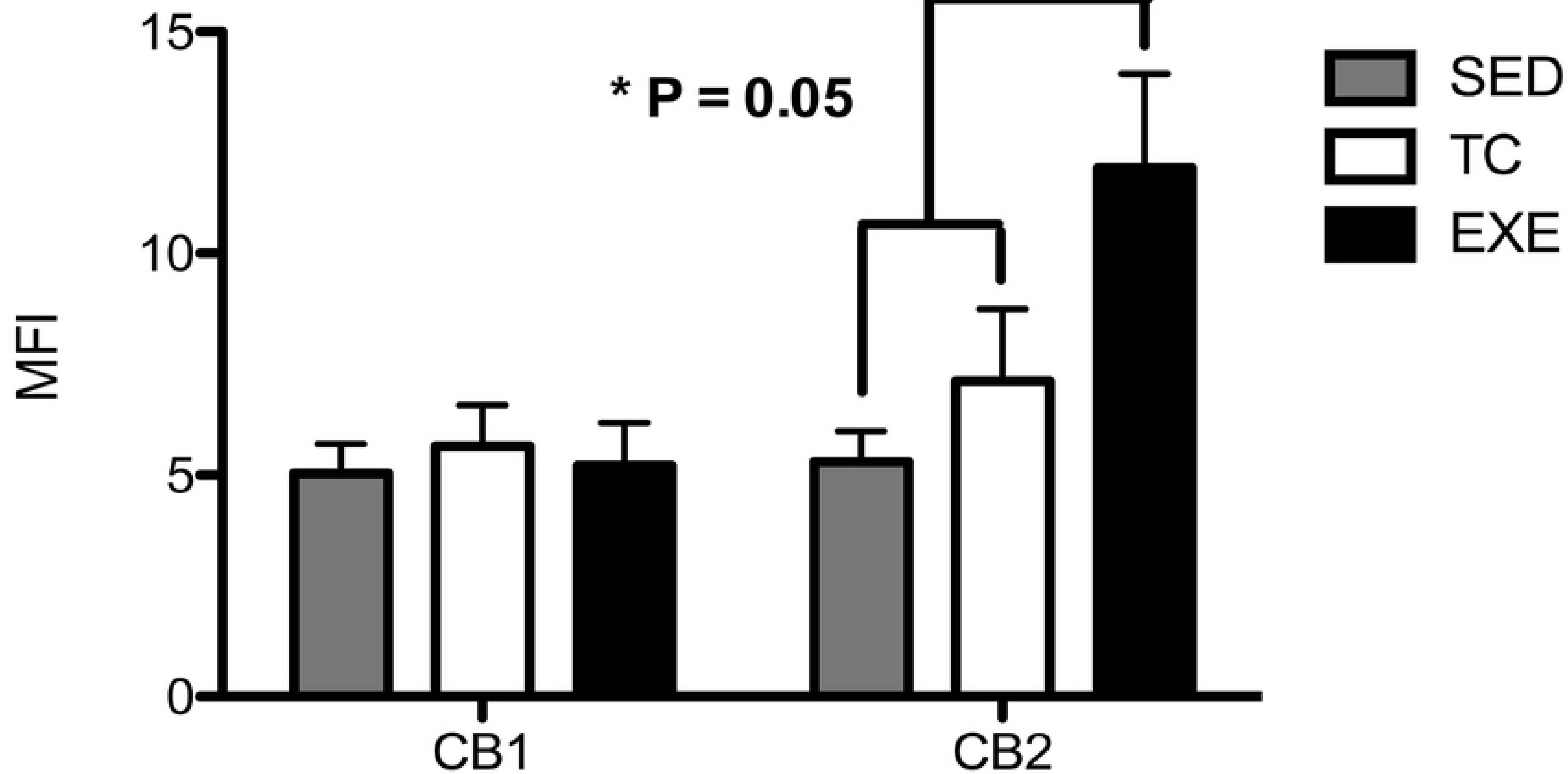

Figure 6

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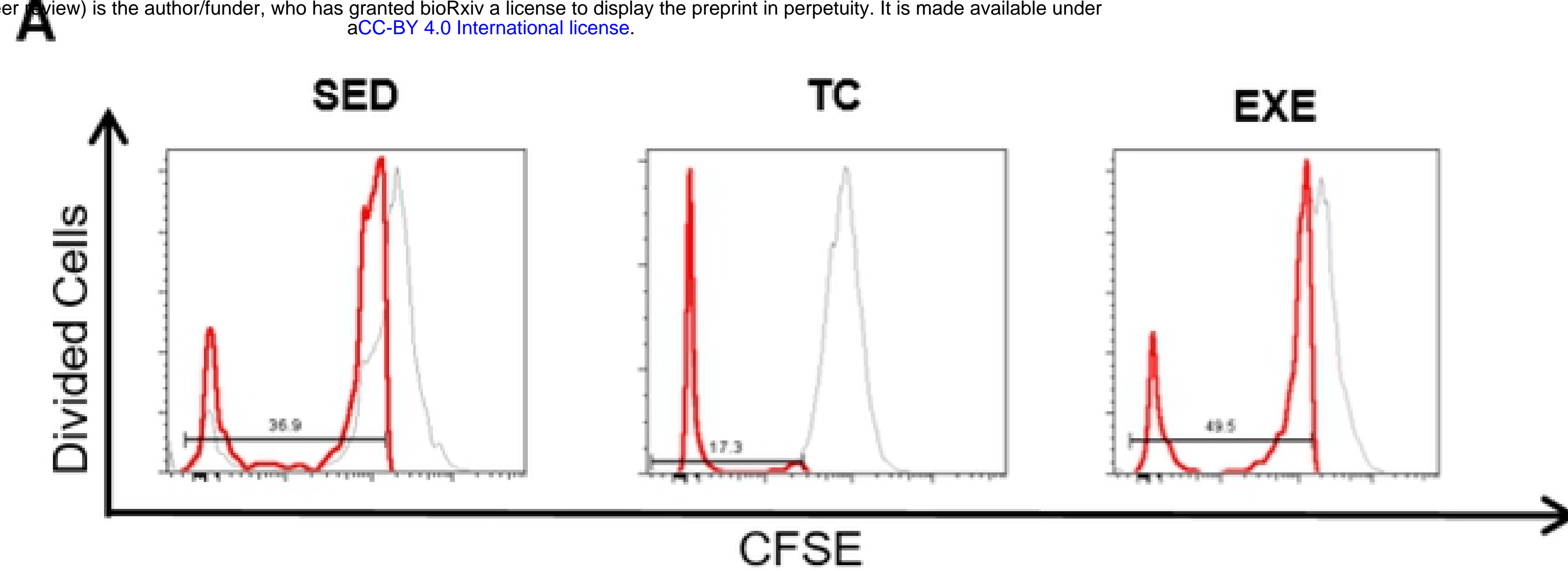

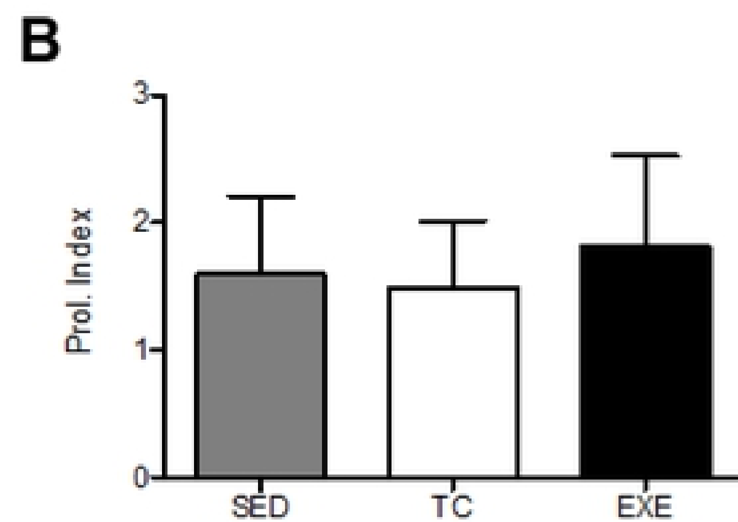

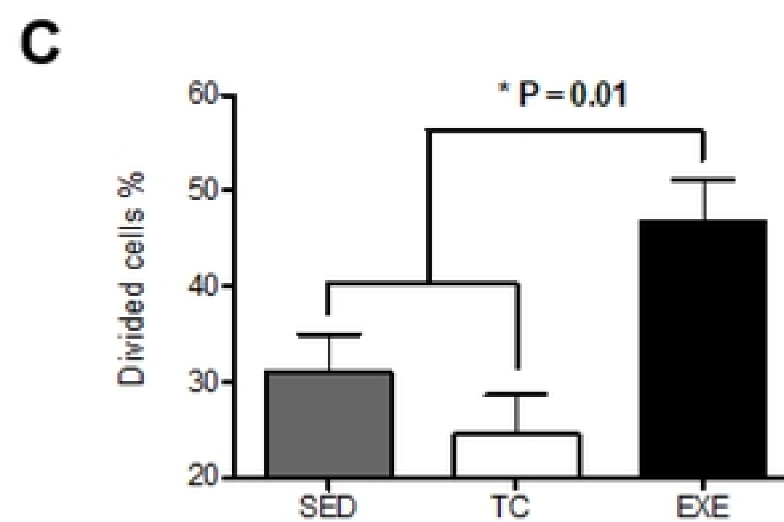

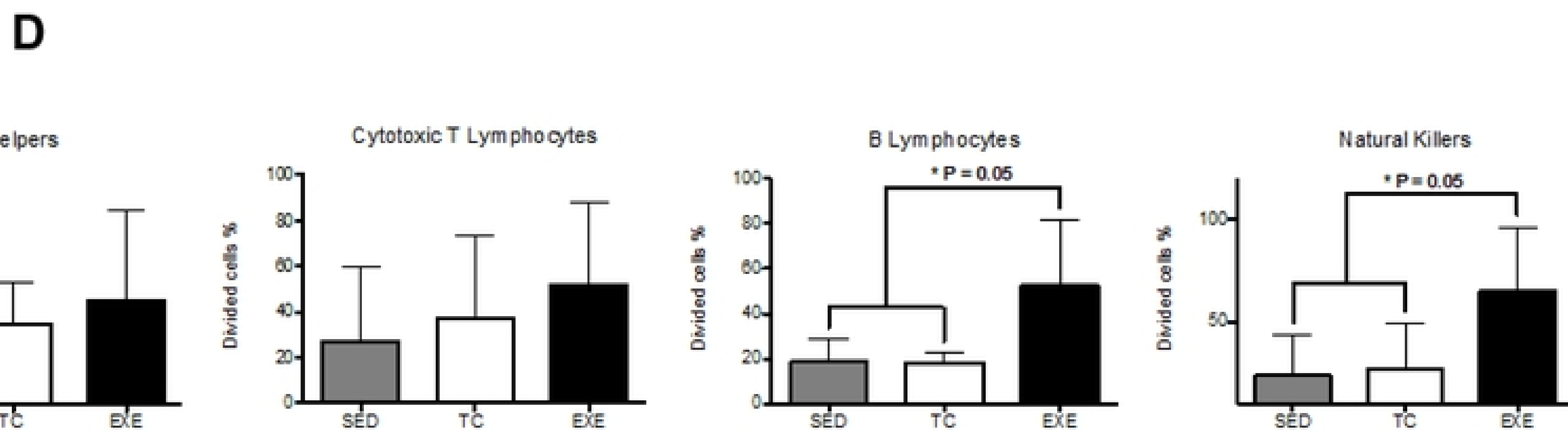

Figure 7

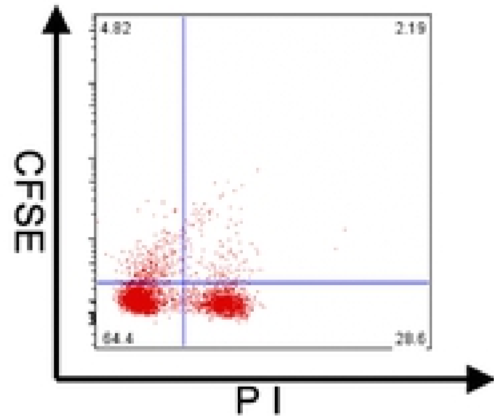

Figure 8