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The β 1-Adrenergic Receptor Contributes to Sepsis-Induced Immunosuppression Through Modulation of Regulatory T-Cell Inhibitory Function*

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1 **The β_1 -adrenergic receptor contributes to sepsis-induced immunosuppression**
2 **through modulation of regulatory T cell inhibitory function**

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42
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54 **Abstract**

1
2 55 *Objective:* Although cardiovascular benefits of β_1 -adrenoreceptor blockade have been
3
4 56 described in sepsis, little is known about its impact on the adaptive immune response,
5
6
7 57 specifically CD4 T cells. Herein we study the effects of β_1 -adrenoreceptor modulation
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9
10 58 on CD4 T cell function in a murine model of sepsis.

11
12 59 *Design:* Experimental study.

13
14 60 *Setting:* University laboratory

15
16
17 61 *Subjects:* C57BL/6 mice

18
19 62 *Interventions:* High-grade sepsis was induced by cecal ligation and puncture (CLP) in
20
21
22 63 wild-type mice ($\beta_1^{+/+}$) with or without esmolol (a selective β_1 -adrenoreceptor blocker),
23
24 64 or in β_1 -adrenoreceptor knockout mice ($\beta_1^{-/-}$). At 18 hours after surgery
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26
27 65 echocardiography was performed with blood and spleen collected to analyze
28
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30 66 lymphocyte function.

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32 67 *Measurements and Main Results:* At 18 hours, $\beta_1^{+/+}$ CLP mice exhibited characteristics
33
34 68 of high-grade sepsis and three surrogate markers of immunosuppression, namely
35
36
37 69 decreased splenic CD4 T cells, reduced CD4 T cell proliferation and increased Treg
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40 70 cell proportions. Pharmacologic and genetic β_1 -adrenoreceptor blockade reversed the
41
42 71 impact of sepsis on CD4 T and Treg proportions and maintained CD4 T cell
43
44 72 proliferative capacity. β_1 -adrenoreceptor blocked CLP mice also exhibited a global
45
46
47 73 decrease in both pro- and anti-inflammatory mediators and improved *in vivo*
48
49 74 cardiovascular efficiency with maintained cardiac power index despite the expected
50
51
52 75 decrease in heart rate.

53
54 76 *Conclusion:* β_1 -adrenoreceptor activation enhances Treg inhibitory function and thus
55
56 77 contributes to sepsis-induced immunosuppression. This can be attenuated by β_1 -
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78 adrenoreceptor blockade, suggesting a potential immunoregulatory role for this
79 therapy in the management of sepsis.

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81 **Abbreviations:**

82 AR: adrenergic receptor

83 CD: cluster of differentiation

84 CI: cardiac index

85 CLP: cecal ligation and puncture

86 CO: cardiac output

87 CPI: cardiac power index

88 ELISA: enzyme-linked immunosorbent assay

89 FBS: fetal bovine serum

90 IL: interleukin

91 MAP: mean arterial pressure

92 PBS: phosphate-buffered saline

93 PHA: phytohemagglutinin

94 PMA: phorbol myristate acetate

95 RPMI: Roswell Park Memorial Institute

96 SNS: sympathetic nervous system

97 SV: stroke volume

98 Th: T helper cell

99 TNF: tumor necrosis factor

100 Treg: regulatory T lymphocyte

101

102 **Introduction**

103

104 Sepsis is a leading cause of intensive care unit admission and recognized by the World
105 Health Organization as a major healthcare issue (1). Unfortunately, despite significant
106 efforts, all research conducted over the last four decades has systematically failed to
107 yield new therapies (2).

108

109 Sepsis is the consequence of a highly complex and dysregulated inflammatory host
110 response to infection leading to life-threatening organ dysfunction (3). **After initial
111 activation, the immune system is downregulated, leading to immunosuppression. This
112 is characterized in part by an increase in regulatory T cells (Treg cells) and a decreased
113 ability to respond to subsequent infections (4). A** disproportionate and prolonged
114 activation of the sympathetic nervous system (dysautonomia) also occurs,
115 characterized by loss of cardiovascular variability, and excessive release of
116 endogenous catecholamines (5). Guidelines however continue to recommend
117 norepinephrine and epinephrine as **first-line vasopressor or inotropic support therapies
118 to achieve circulatory stabilization in septic shock (6). This advice is proffered
119 notwithstanding an extensive literature highlighting the potent immunomodulatory
120 properties of these catecholaminergic agents (7). Faced with this paradox, the concept
121 of “decatecholaminization” has emerged over the last decade to minimize, where
122 possible, the use of catecholamines during sepsis (3, 7).**

123

124 Beta-1 adrenoreceptor (β_1 -AR) blockade is a therapeutic approach aimed at
125 downregulating the excessive adrenergic stimulation induced by sepsis. Initial single-
126 center studies in human septic shock have mainly reported hemodynamic benefits with

127 improvements in cardiac efficiency and, in one study, a survival benefit (8-10).

128 Premorbid β_1 -blocker exposure is also associated with a decrease in ICU mortality (11-
129 13) which may relate to their immunomodulatory actions (14-16).

130

131 Relatively few experimental and, in particular, clinical studies have investigated the
132 inflammatory effects of β_1 -AR blockade in sepsis. These studies report a global
133 decrease in both pro- and anti-inflammatory circulating cytokines though have not
134 delved into underlying mechanisms (14-17). Little is also known about the effects of
135 β_1 -AR blockade on the early adaptive immune response (18, 19). We hypothesized
136 that β_1 -AR blockade plays a fundamental role, during sepsis in the early adaptive
137 immune response, enabling a reduction of sepsis-induced immunosuppression. The
138 present study was thus designed to assess the effects of β_1 -AR blockade on CD4 T
139 cells in a murine model of sepsis.

140

141 **Materials and Methods**

142

143 *Sepsis model*

144 A full description is provided in **Supplemental Digital Content – Methods**. β_1 -AR
145 (C57BL/6J, DBA/2, 129Sv) knockout mice ($\beta_1^{-/-}$) and wild type littermates ($\beta_1^{+/+}$),
146 obtained free of charge from the Institute of Pharmacology and Toxicology of Munich,
147 Germany, were used for this study. The French Animal Care Committee, in accordance
148 with European regulations, approved all protocols (n°APAFIS#4636). Sepsis was
149 induced by cecal ligation and puncture (CLP) in both male and female mice aged 12-
150 16 weeks, weighing 22-32 grams. **For fluid resuscitation, all mice received an**
151 **isovolemic subcutaneous fluid bolus of 5 ml physiological saline solution (0.9% NaCl;**
152 **Baxter, Guyancourt, France) per 100g body weight.**

153

154 An incision was performed between the scapulae in all mice and a mini-osmotic pump
155 (2001D, Alzet, Cupertino, CA) implanted subcutaneously in animals to be treated with
156 the selective β_1 -AR blocker, esmolol (Esmocard®, Amomed, Pantin, France). In non-
157 treated groups, animals only underwent a skin incision without implantation of the mini-
158 pump. The esmolol dosage was chosen according to both the literature and preliminary
159 experiments aiming to reduce heart rate (HR) by 20% (**Supplemental Digital Content**
160 **– Figure 1).**

161

162 *Experimental design*

163 $\beta_1^{+/+}$ mice were randomly assigned to four groups: sham, sham + esmolol, CLP or CLP
164 + esmolol. Esmolol was infused at 18 mg/kg/h (8 μ l/h, 120 μ l over the 15 hours of
165 intervention) via a subcutaneous mini-osmotic pump which was activated 3 hours post-

166 insertion. $\beta_1^{-/-}$ mice were randomly assigned to two groups: sham or CLP. The number
167 of mice used and reasons for failed measurements and for exclusion are provided in
Supplemental Digital Content – Methods and Table 1.

169

170 *Immunophenotyping*

171 Splenocytes were isolated according to standard methods. Splenocyte Fc receptors
172 were blocked (CD16/CD32-purified antibodies, eBioscience™, ThermoFisher
173 Scientific, San Diego, CA) and cell surface markers used to identify total T cells
174 (CD3+), CD4 T cells (CD3+ CD4+), CD8 T cells (CD3+ CD4-) and Treg cells (CD3+
175 CD4+ CD25^{hi} CD127^{low}, Foxp3) in viable cells. Th1 (INF- γ +), Th2 (IL-4+) and Th17 (IL-
176 17+) status was also characterized by intracellular staining of CD4 T cells. A full
177 description is provided in **Supplemental Digital Content – Methods, and the Treg
178 cell gating strategy in Supplemental Digital Content – Figure 2.**

179

180 *Proliferation assay*

181 Splenocytes were stained with Tag-it Violet™ Proliferation and Cell Tracking Dye (5
182 μ M/10⁶ cells; Biolegend, San Diego, CA) according to the manufacturer's instructions
183 and stimulated with phytohemagglutinin (PHA; 4 μ g/10⁶ cells; Remel, ThermoFisher
184 Scientific, San Diego, CA) for 72 hours at 37°C. Tag-it Violet signal was assessed in
185 live CD4 T cells by flow cytometry. A full description is provided in **Supplemental
186 Digital Content – Methods.**

187

188 *Purification of CD4+ CD25- T cells and CD4+ CD25+ Treg cells*

189 CD4 T and Treg cells were isolated in a two-step procedure following the
190 manufacturer's instructions (CD4+ CD25+ Regulatory T Cell Isolation Kit,

191 MiltenyiBiotec, Bergisch Gladbach, Germany). A full description is provided in

192 **Supplemental Digital Content – Methods and Supplemental Digital Content –**

193 **Figure 3 panels A and B.**

194

195 *Co-culture of CD4 T cells with catecholamine-stimulated Treg cells*

196 Treg cells were stimulated with 1 μ M dobutamine (β_1 -AR agonist), norepinephrine (α_1 -

197 and β_1 -AR agonist), phenylephrine (α_1 -AR agonist); (all from Sigma-Aldrich) or PBS

198 for 30 minutes, then washed twice to remove the catecholamines. Pre-treated Treg

199 cells were co-cultured with CD4 T cells at a 1:4 ratio. Cells were activated with anti-

200 CD3/CD28 beads and proliferation assessed by taking pictures every 2 hours for 5

201 days (37 °C, 5% CO₂) using the 20x objective of the IncuCyte® S3 Live-Cell Analysis

202 System (Sartorius, Goettingen, Germany). Analysis was performed using IncuCyte™

203 Basic Software. A full description is provided in **Supplemental Digital Content –**

204 **Methods and Supplemental Digital Content Figure 3 panel C.**

205

206 *Echocardiography*

207 Transthoracic echocardiography was performed 18 hours after CLP with a VEVO770

208 (FUJIFILM VisualSonics, Toronto, Canada) and a 30 MHz mechanical probe. A full

209 description is provided in **Supplemental Digital Content – Methods.** Mean arterial

210 pressure (MAP) was measured by a blood pressure transducer (Emka, Paris, France)

211 inserted into the left carotid artery.

212

212 *Blood counts*

213

213 In 5–6 mice from each group, complete blood counts were measured (Micros 60

214 ABX, Radeberg, Germany).

215 *Quantification of inflammatory mediators*

1
2
3 216 In 5–6 mice from each group, the plasma levels of 46 inflammatory mediators were
4
5
6 217 measured in duplicate using the Magnetic Luminex® Performance Assay – Mouse XL
7
8 218 Discovery Premixed kit (Bio-Techne, Minneapolis, MN) according to the
9
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11 219 manufacturer’s instructions.

12
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14 220 *Immunohistochemistry*

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18 221 A full description is provided in **Supplemental Digital Content – Methods**. Hearts
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20 222 were sectioned into transverse slices from apex to base and embedded in optimal
21
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23 223 cutting temperature compound (CellPath, Newtown, Powys, UK) for
24
25 224 immunohistochemistry. Leukocytes were stained on 7- μ m thick sections with rabbit
26
27
28 225 polyclonal antibody to CD45. Sections were counterstained with hematoxylin. For each
29
30 226 heart section CD45 positive cells were counted using a 40x objective lens in 10 fields
31
32
33 227 in a blinded manner by three different researchers independently.

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36 228 *Survival study*

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40 229 Survival was studied in $\beta_1^{+/+}$ CLP mice, $\beta_1^{+/+}$ CLP + esmolol (18 mg/kg/h) mice, and $\beta_1^{-/-}$
41
42 230 $^{-/-}$ CLP mice. Census of animals was undertaken twice a day for 5 days. Administration
43
44
45 231 of esmolol (Esmocard®, Amomed, Pantin, France) was started 3 hours post-CLP and
46
47
48 232 continued for a total infusion period of 24 hours.

49
50 233
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53 234 *Statistical analysis*

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57 235 Results are expressed as mean and standard deviation (mean \pm SD). Results in $\beta_1^{-/-}$
58
59
60 236 mice were compared using a Mann-Whitney test while the Kruskal-Wallis test was

237 used to compare $\beta_1^{+/+}$ groups. When the Kruskal-Wallis test was significant, *post hoc*
238 comparisons were performed between sham, CLP, and CLP + esmolol groups using
239 Dunn's multiple comparisons and a Bonferroni correction. Variations were calculated
240 as follows: $100 - ((\text{mean group 1} / \text{mean group 2}) \times 100)$.

241
242 To analyze the effect of catecholamines on Treg cells co-cultured with CD4 T cells, we
243 firstly assessed the interaction between groups at each timepoint. Considering the
244 mouse as a random effect, the p-value of the test for the treatment effect was
245 calculated. Only the last p-value (obtained at 132 hours) is shown. For measurement
246 of plasma inflammatory mediators, groups were compared using the Mann-Whitney
247 test. Benjamini-Hochberg adjustment was performed for multiple comparisons.

248
249 Kaplan–Meier curves were drawn. Significance was assessed using the log-rank test.
250 P values <0.05 were considered statistically significant. Statistical analysis was
251 performed using R, version 3.6.2 (R Foundation for Statistical Computing, Vienna,
252 Austria), using Graph-Pad Software 6.0, (San Diego, CA) and NCSS Statistical
253 Software 9 (East Kaysville, UT).

254 **Results**

255

256 **β_1 -AR stimulation contributes to a post-septic immunosuppressive phenotype.**

257 Male and female mice were used in equal numbers for all experiments (sex ratio 1.02).

258 Eighteen hours after surgery, septic mice displayed falls in white blood cells and in

259 circulating lymphocytes (-72% vs. sham, $p=0.05$ and -79% vs. sham, **Figure 1, panel**

260 **A and B**). In spleens taken from $\beta_1^{+/+}$ mice, sepsis induced a significant decrease in

261 CD4 T cell proportions (-34% vs. sham, $p<0.001$) which was counteracted by β_1 -AR

262 blockade (+37% vs. CLP, $p=0.03$) (**Figure 1, panel C**). In $\beta_1^{-/-}$ mice, sepsis significantly

263 decreased the proportion of CD4 T cells (-17% vs. sham, $p=0.02$), however no change

264 in CD8 T cells proportion was observed between groups (**Figure 1, panel C and D**).

265

266 With respect to Treg cells (CD25^{high} CD127^{low} Foxp3+), in $\beta_1^{+/+}$ mice, CLP induced an

267 increase in the Treg cell proportion (+65% vs. sham, $p <0.001$ **Figure 1, panel E**) and

268 a trend to an increase in Treg cell count in the spleen which was inhibited by β_1 -AR

269 blockade (-19% vs. CLP, $p=0.04$) (**Supplemental Digital Content – Figure 4**).

270

271 In $\beta_1^{-/-}$ septic mice, the proportion of Treg cells was unchanged (+7% vs. sham,

272 $p=0.68$). Of note, β_1 -AR blockade in $\beta_1^{+/+}$ sham mice had no impact on CD4 T cell and

273 Treg cell proportions (**Supplemental Digital Content – Figure 5**). No modification

274 was observed in other CD4 T cells subsets (Th1 (INF- γ +), Th2 (IL-4+) and Th17 (IL-

275 17+)) in these experimental groups (**Supplemental Digital Content – Figure 6**).

276 These findings were not caused by differences in cell viability between groups

277 (**Supplemental Digital Content – Figure 7**).

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279 **β_1 -AR activation induces a decrease in CD4 T cell proliferation during sepsis.**

1
2 280 After culturing splenocytes for 72 hours with PHA stimulation, the proliferative
3
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5 281 response of CD4 T cells was measured to assess sepsis-induced immune
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7 282 suppression. In $\beta_1^{+/+}$ mice, sepsis reduced the proliferative capacity of CD4 T cells (-
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10 283 15% vs. sham, $p=0.04$). However, administration of a β_1 -AR blocker limited this effect
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12 284 (-8% vs. sham, $p=0.38$). In $\beta_1^{-/-}$ mice, sepsis did not decrease the proliferative
13
14
15 285 capacities of CD4 T cells (-4% vs. sham, $p=0.71$) (**Figure 1, panel F**). These results
16
17 286 were not caused by variations in cell viability between groups (**Supplemental Digital**
18
19
20 287 **Content – Figure 8**). Of note, β_1 -AR blockade in $\beta_1^{+/+}$ sham mice had no impact on
21
22 288 CD4 T cell proliferation (**Supplemental Digital Content – Figure 5**).
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27 **β_1 -AR stimulation of Treg cells increases their immunosuppressive capacities.**

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30 291 The direct effect of β_1 -AR stimulation on the immunosuppressive function of Treg cells
31
32 292 was assessed in vitro. CD4 T cell counts tended to decrease after five days of co-
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34
35 293 culture with Treg cells pre-treated with the β_1 -AR agonist dobutamine, in comparison
36
37 294 to untreated Treg cells ($p=0.08$, **Figure 2, panel A**). This lower cell count, noted when
38
39
40 295 co-culture was performed with dobutamine pre-treated Treg cells, was not caused by
41
42 296 an increase in cell death (**Figure 2, panel B**). Similar results were found when co-
43
44
45 297 culture assays of CD4 T cells were performed in the presence of norepinephrine and
46
47 298 phenylephrine pre-treated Treg cells (**Supplemental Digital Content – Figure 9**).
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51
52 300 **β_1 -AR blockade, despite a reduced heart rate, did not alter hemodynamic**
53
54
55 301 **characteristics in sepsis.**

56
57 302 Hemodynamic parameters were measured by echocardiography (**Supplemental**
58
59
60 303 **Digital Content – Table 2**). In $\beta_1^{+/+}$ septic mice, administration of a β_1 -AR blocker
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304 induced a decrease in heart rate (-24% vs. sham, $p < 0.001$; -14% vs. CLP, $p = 0.07$). In
305 $\beta_1^{-/-}$ mice, sepsis did not affect heart rate (+9% vs. sham, $p = 0.24$).

306
307 Compared to $\beta_1^{+/+}$ sham, high-grade sepsis induced a decrease in mean arterial
308 pressure (MAP) (-27% vs. sham, $p < 0.001$), stroke volume (SV) (-40% vs. sham,
309 0.004), CI (-49% vs. sham, $p < 0.001$), and cardiac power index (CPI) (-64% vs. sham,
310 $p < 0.001$). These parameters remained unaltered by the administration of a β_1 -AR
311 blocker (MAP: +8% vs. CLP, $p = 0.51$; SV: +6% vs. CLP, $p = 0.99$; CI: +2% vs. CLP,
312 $p = 0.99$; CPI: +25% vs. CLP, $p = 0.99$). In $\beta_1^{-/-}$ mice, sepsis induced a decrease in MAP
313 (-19% vs. sham, $p = 0.04$), SV (-47% vs. sham, $p < 0.001$), CI (-37% vs. sham, $p = 0.002$)
314 and CPI (-50% vs. sham, $p < 0.001$).

316 **Sepsis does not influence leukocyte infiltration in the heart**

317 **Leukocyte infiltration into cardiac tissue was analyzed by immunohistochemistry. No**
318 **change was observed in septic compared to sham mice. Pharmacological and genetic**
319 **blockade of β_1 -AR had no influence on myocardial leukocyte infiltration (Supplemental**
320 **Digital Content – Figure 10).**

322 **β_1 -AR modulates the concentration of both pro- and anti-inflammatory** 323 **circulatory mediators.**

324 The plasma levels of 46 inflammatory mediators were measured. Briefly, all
325 inflammatory mediators were increased in $\beta_1^{+/+}$ CLP mice compared to sham controls
326 **(Supplemental Digital Content – Table 3)**. Compared to $\beta_1^{+/+}$ CLP mice, esmolol-
327 treated CLP mice showed an overall decrease in plasma concentrations of 58%
328 (18/31) and 60% (9/15) for pro- and anti-inflammatory mediators, respectively **(Figure**

329 **3).** For instance, compared to $\beta_1^{+/+}$ CLP mice, administration of a β_1 -AR blocker
1
2
3 330 reduced plasma TNF- α by 66% vs. CLP ($p=0.02$), and IL-10 by 82% vs. CLP ($p=0.01$).
4
5 331 In $\beta_1^{-/-}$ mice, sepsis induced an overall increase in both pro- and anti-inflammatory
6
7 332 mediators compared to sham animals (**Supplemental Digital Content – Table 3**).

10 333
11
12 **β_1 -AR modulates survival during sepsis.**

13 334
14
15 335 The survival rate was documented in $\beta_1^{+/+}$ CLP, $\beta_1^{+/+}$ CLP + esmolol and $\beta_1^{-/-}$ CLP mice
16
17 336 (**Figure 4**). Survival at 110 hours decreased from 60% in $\beta_1^{-/-}$ CLP mice, to 37% in $\beta_1^{+/+}$
18
19 337 CLP + esmolol mice and 35% in $\beta_1^{+/+}$ CLP mice (log rank $p=0.13$).

22 338
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25 339 **Sex does not affect the β_1 -AR effect.**

26
27 340 Sensitivity analyses were performed on results obtained for major hemodynamic
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29 341 parameters (**Supplemental Digital Content – Figure 11**) and immune
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31 342 (**Supplemental Digital Content – Figure 12**) effects according to sex. No differences
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33 343 were found between male and female mice.
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344 **Discussion**

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346 In the present study, we confirm that experimental mid-grade sepsis induces three
347 hallmark features of immunosuppression as seen in human sepsis: a significant
348 decrease in splenic CD4 T cells, an increase in splenic Treg cells, and a decrease in
349 CD4 T cell proliferation capacity (4). A notable finding was the marked increase in Treg
350 cells in the spleen of septic mice, which could be counteracted by pharmacologically
351 or genetically blocking β_1 -AR signaling. This observed decrease in Treg cells in treated
352 animals was associated with a simultaneous increase in CD4 T cells and a global
353 decrease in circulating pro- and anti-inflammatory mediators, indicating the
354 involvement of β_1 -AR in the septic-immune phenotype. These results also translated
355 into an *in vivo* global improvement in cardiac efficiency.

356

357 To investigate the immune and cardiovascular roles of β_1 -AR in sepsis, we deliberately
358 chose only a fluid-resuscitated murine model of CLP to avoid any interference between
359 exogenous catecholamine administration and the immune system (20-23). This model
360 depicts all hemodynamic and survival characteristics of a typical mid-grade sepsis,
361 with an increase in both plasma pro- and anti-inflammatory mediators and a decrease
362 in CD4 T cell proportion (24-28). Analysis of CD4 T subtypes revealed that early after
363 onset of sepsis, the percentages of Th1, Th2 and Th17 cells were unchanged. Many
364 studies usually report a switch from Th1 to Th2 phenotypes, and a decrease in Th17
365 cell percentages (29-32). However, other studies found that sepsis could also disrupt
366 in a time-dependent manner both the representation and the function of CD4 T cell
367 subsets, including Th1, Th2, Th17 (20-23). Unlike other lymphocyte populations, Treg
368 cells are increased during trauma in both animals and humans, as well as during sepsis

369 and septic shock (as seen in our study) (21, 22). This increase in Treg cells contributes
1
2 370 to lymphocyte paralysis and, more broadly, enhances the post-septic
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5 371 immunosuppressive process (33).
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10 373 *β-adrenergic stimulation contributes to CD4 T cell dysfunction*

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12 374 In both innate and adaptive immune cells, adrenergic receptors are widely expressed
13
14 375 though not well characterized, particularly for the β subtype (34). Numerous studies
15
16 376 have demonstrated the presence of β-AR on almost all lymphocyte subtypes (35-37).
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19 377 However, until recently, only β₂-AR but not β₁-AR subtypes were found on T cells (38,
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21
22 378 39). As a consequence, β₁-AR stimulation was not considered to have a major role in
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24 379 inflammation (40). β₁-AR were found to be widely expressed on splenic CD4 T cells
25
26 380 only recently. In cirrhotic mice, the β₁-AR agonist dobutamine could re-activate
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28
29 381 inhibited Treg cells, suggesting the presence of β₁-AR on their surface (41).
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31 382 Accordingly, we too found that *in vitro* CD4 T cell proliferation tended to be reduced by
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34 383 Treg cells pre-treated with dobutamine. Apart from its β₁-AR activity, dobutamine also
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36 384 has activity on the β₂-AR. This is known to exert some anti-inflammatory effects when
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39 385 stimulating Treg cells and which may have mitigated the β₁ effects (36). In addition to
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41 386 CD4 T cell dysfunction, stimulation of β-ARs in innate and adaptive cells has recently
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43
44 387 been confirmed as a crucial factor involved in immunoparalysis. We have summarized
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46 388 the effects of β-AR agonists on innate and adaptive immunity in **Supplemental Digital**
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49 389 **Content – Figure 13 (7)**.
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53 391 *β-adrenoreceptor blockade reversed sepsis-induced dysautonomia*

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56 392 In numerous conditions, such as acute heart failure, burn injuries, and septic shock,
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58 393 β₁-AR blockade has been consistently associated with protective effects on
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394 inflammation and hemodynamics (15, 42, 43). As others, we too found a global
1
2 395 decrease in pro- and anti-inflammatory mediators in animals with mid-grade sepsis
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4
5 396 treated by β_1 -AR blockade (15, 44, 45). Underlying mechanisms are still not elucidated.
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7 397 In *Listeria monocytogenes* infected mice, acute cold or restraint stress (surrogates of
8
9 398 sympathetic adrenergic stimulation) inhibited the host T cell response and increased
10
11 399 cytokine production (46). In this model, pharmacological blockade of the β_1 -AR but not
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13
14 400 the β_2 -AR could downregulate this pro-inflammatory state and shorten time to bacterial
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16
17 401 clearance (47).

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19 402
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21 403 In β_1 -AR blocked animals with mid-grade sepsis, we found the decrease in Treg cells
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23
24 404 in the spleen was associated with a concomitant increase in CD4 T cells, as reported
25
26 405 previously (48). In addition, we found that the proliferative capacity of splenic CD4 T
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28
29 406 cells in septic animals was maintained when the β_1 -AR was blocked either
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31 407 pharmacologically or genetically. This suggests that β_1 -AR activation contributes to
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34 408 sepsis-induced immunosuppression, which could be reversed by β_1 -blockade. By
35
36 409 comparison, volunteers challenged with lipopolysaccharide, and then being infused
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38
39 410 with norepinephrine for 5 hours showed a shift to an anti-inflammatory state
40
41 411 (decreased TNF- α /IL-10 ratio) which could be counterbalanced by β -AR blockade
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43
44 412 (increased TNF- α /IL-10 ratio) (49). β_1 -AR blockade in our septic animals reduced the
45
46 413 inflammatory burden and, taken as a whole, helped to shift from an
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48
49 414 immunosuppressive state to a state of immune homeostasis.

50
51 415 Beside this immune rebalancing in experimental mid-grade sepsis, it is important to
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53 416 reiterate that β_1 -AR blockade also has major cardiovascular effects. In agreement with
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56 417 most other experimental or clinical studies, septic mice receiving β -AR blockade
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58 418 showed no alteration in cardiac power, despite a decrease in HR, suggesting improved
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419 cardiac efficiency (10, 15, 44, 50). However, it should be borne in mind that animals
420 only received an initial subcutaneous bolus of fluid resuscitation, and no fluids nor
421 catecholamines thereafter. While the association between an increased Treg cell
422 percentage during sepsis and an immunosuppressive state is well proven, effects on
423 survival remain inconclusive (51, 52). The benefits of β_1 -AR blockade on immune and
424 cardiovascular function should improve survival. However, in the present study, and
425 without any treatment (antibiotics, fluid or vasopressors), no improvement was seen in
426 survival. In a similar CLP surgery model, Ackland *et al.* also found that metoprolol
427 infusion commenced 6 hours after the onset of fecal peritonitis did not confer any
428 benefit in improving survival whereas pretreatment was effective (45). A dose effect
429 may be relevant as the β_1 -AR blocker was infused only during the first 24 hours after
430 CLP surgery. In a single-center study prolonged administration of esmolol in septic
431 shock patients, titrated to maintain heart rate between 80-94 bpm, was associated with
432 increased survival (10).

434 *Limitations*

435 β_1 -AR and β_2 -AR are widely expressed on both innate and adaptive immune cells.
436 Specifically, it has been reported that naïve CD4 T cell subtypes Th1, Th17 and Treg
437 cells, but not Th2, express β_2 -AR (35, 53). β_2 -AR stimulation promotes a shift from
438 Th1 to a Th2 anti-inflammatory pattern and could activate Treg suppressive functions
439 (36, 54). β_2 -AR stimulation may enhance the macrophage M2 anti-inflammatory
440 phenotype, favoring IL-10 production by CD4 T cells (37). Beneficial effects of β_2 -AR
441 modulation remain unclear during sepsis. In a murine endotoxemia model, β_2 -AR
442 blockade reduced survival while, in septic pigs, it amplified sepsis-induced hepatic
443 injury (37, 55, 56). In this study, we only investigated the impact of β_1 -AR blockade on

444 alteration of CD4 T cell patterns. The impact of selective β_2 -AR modulation as well as
445 non-specific β -AR blockade on CD4 T cells during sepsis merits investigation.

446
447 Our initial intention was to create a CLP model with an expected 48 hour-mortality rate
448 of 70% (26). However, despite using a larger 18G needle to puncture the cecum
449 (instead of the usually recommended 22G needle for mice) and without administering
450 antibiotics (avoided so as not to interfere with the infectious/inflammatory processes),
451 the 48 hour-mortality was 30% in the non-treated CLP mice control group.
452 Nonetheless, this remains a severe model. It would be worthwhile repeating the study
453 with full-code resuscitative measures including ongoing fluid administration,
454 catecholamines (if required), and antibiotics (24). Limitations of murine CLP models
455 should also be acknowledged, for example youth, underlying good health, a
456 homogenous infectious insult and severity, and a similar time-interval from CLP
457 induction to evaluation. The immune system in mice is also not identical to humans
458 and human sepsis is far more heterogeneous in nature. Nonetheless, such models
459 remain useful for mechanistic understanding and an initial assessment of the impact
460 of interventions. Measurements were only performed at 18 hours post-CLP. Serial
461 assessments in a less severe model could clarify the evolving inflammatory profile of
462 this disease.

463 464 *Perspectives and potential clinical implications*

465 The deleterious effects of a disproportionate and prolonged activation of the adrenergic
466 system are now well established (5, 7). In both clinical trials and cohort studies, short-
467 and long-term administration of β -AR blockers are associated with a mortality benefit.
468 However, there is yet no conclusive explanation for this benefit (10, 13). In this study,

469 we confirmed that our CLP sepsis model is associated with an immunosuppressive
1
2 470 phenotype characterized by an increase in Treg cells and a decrease in CD4 T cell
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5 471 proliferation capacity. These features could be reversed by a β ₁-AR blockade.

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7 472 If confirmed, our results could lead to a redesign of clinical trials with lower doses of
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10 473 β ₁-AR blocker, improving hemodynamic tolerance in the early stage of sepsis and
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12 474 septic shock. These findings have yet to be confirmed in other animal models and in
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14 475 human sepsis but supports the potential benefits of decatecholaminization during
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17 476 sepsis and, especially, septic shock.

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22 478 In summary, in the murine model tested, the β ₁-AR contributes to sepsis-induced
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24 479 immunosuppression through modulation of regulatory T cell inhibitory functions. β ₁-AR
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26 480 blockade, while restoring CD4 T cell homeostasis, also decreased global systemic
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29 481 inflammation and improved cardiac efficiency (**summary Figure 5**). Thus, instead of
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31 482 considering β ₁-AR blockade only for its hemodynamic effects, we suggest an
32
33
34 483 immunoregulatory mechanism by which β ₁-AR blockers may also be beneficial.

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1
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9
10
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12
13

14
15 489 **Authors' contributions.**

16
17
18 490 MD, AK: study concept and design. MD, HL: Experiments (MD: involved in all
19
20
21 491 experiments, HL involved in flow cytometry supervision. CL, AK with the help of
22
23 492 François Husson: statistical analysis, AK, MD, PA, JPF, MS, BGC: Drafting of the
24
25 493 manuscript. All authors were involved in critical revision of the manuscript. All authors
26
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28 494 read and approved the final manuscript.
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30

31
32 495 **Availability of supporting data.**

33
34
35 496 Raw data and statistical analyses of all presented results are available at the following:
36
37
38 497 <https://osf.io/nz5u9/> or DOI 10.17605/OSF.IO/NZ5U9.
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40

41 498 MD takes responsibility for the integrity of the data.
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653 **Figures Legends**

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2 654 **Figure 1: β_1 -AR contributes to the post-septic immunosuppressive phenotype,**
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5 655 **which is reversed by β_1 -AR blockade.** The counts of white blood cells (A) and
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7 656 lymphocytes (B) in whole blood were determined in each group. Splenocytes were
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10 657 immunophenotyped 18 hours after sepsis induction. The proportion of CD4 T cells
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12 658 among live cells (C), the proportion of CD8 T cells among live cells (D), and the
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14 659 proportion of Treg cells (CD25^{high} CD127^{low}) among live CD4 T cells (E) were
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17 660 assessed. (F) Proliferation of CD4 T cells according to cell tracking dye staining after
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19 661 72 hours of culture with a mitogen (PHA). Data are expressed as mean \pm SD; each
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22 662 point represent a mouse. * p<0.05; ** p<0.01; *** p<0.001.

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27 664 **Figure 2: In vitro β_1 -AR stimulation of Treg cells increases their**
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29 665 **immunosuppressive capacities.** Treg cells isolated from $\beta_1^{+/+}$ mice were treated with
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32 666 dobutamine or PBS for 30 minutes. Pre-treated Treg cells were then co-cultured with
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34 667 CD4 T cells at a 1:4 ratio in the presence of anti-CD3 and anti-CD28 beads for 5 days.
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36 668 A fluorescent green reagent was added for counting dead cells. (A) shows the real-
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39 669 time cell count normalized to the first count at H0, and (B) shows the dead cell count
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41 670 as a percentage of total cells. Data shown are the means of 4 images per well in
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44 671 duplicate with 4 mice per time point \pm SEM (total 32 images/time/group). Images were
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46 672 taken at 20 \times magnification on the IncuCyte[®] imager.

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50 673
51 674 **Figure 3: β_1 -AR modulates the level of both pro- and anti-inflammatory**
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54 675 **circulatory mediators.** Plasma levels of 46 inflammatory mediators were measured
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56 676 in 5–6 animals per group. Data are expressed as Log₁₀(mean).

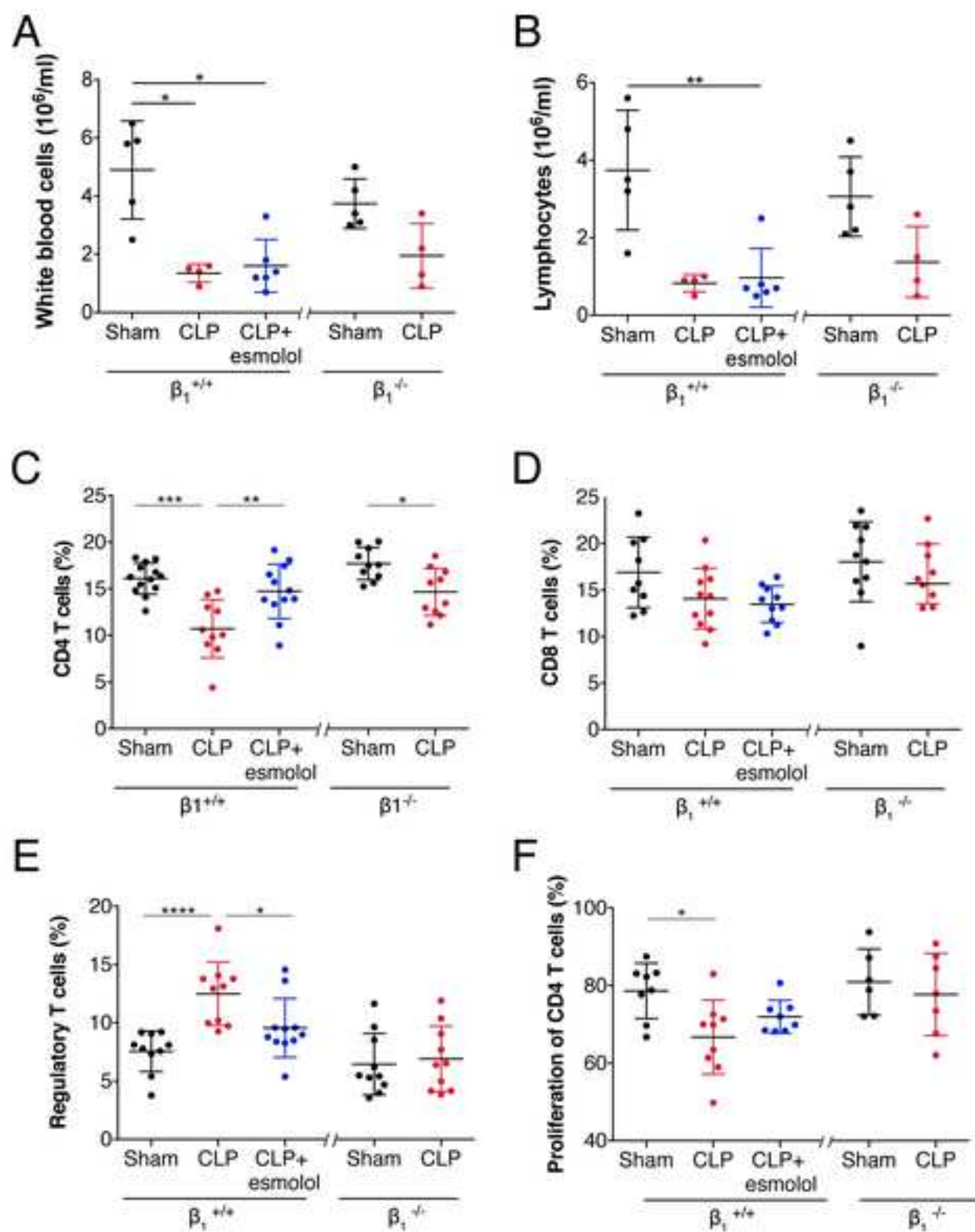
677 **Figure 4: β_1 -AR blockade modulates survival during sepsis.** Survival curve of $\beta_1^{+/+}$
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2 678 CLP mice, $\beta_1^{+/+}$ CLP mice receiving esmolol for 24 hours, and $\beta_1^{-/-}$ CLP mice. The one
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5 679 animal that died before the first count was considered a technical failure and excluded
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7 680 from the study.

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12 682 **Figure 5: Summary of the findings.**

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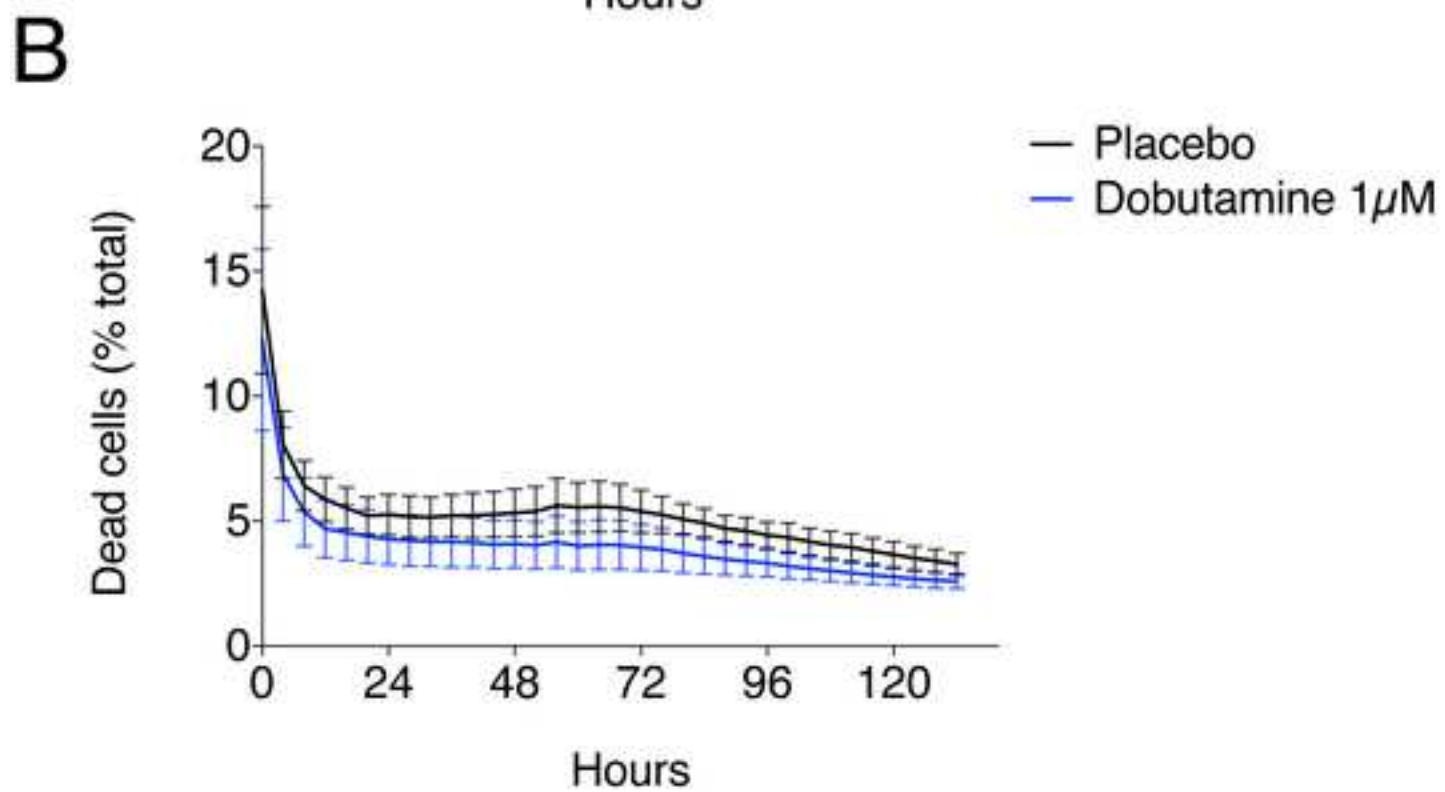
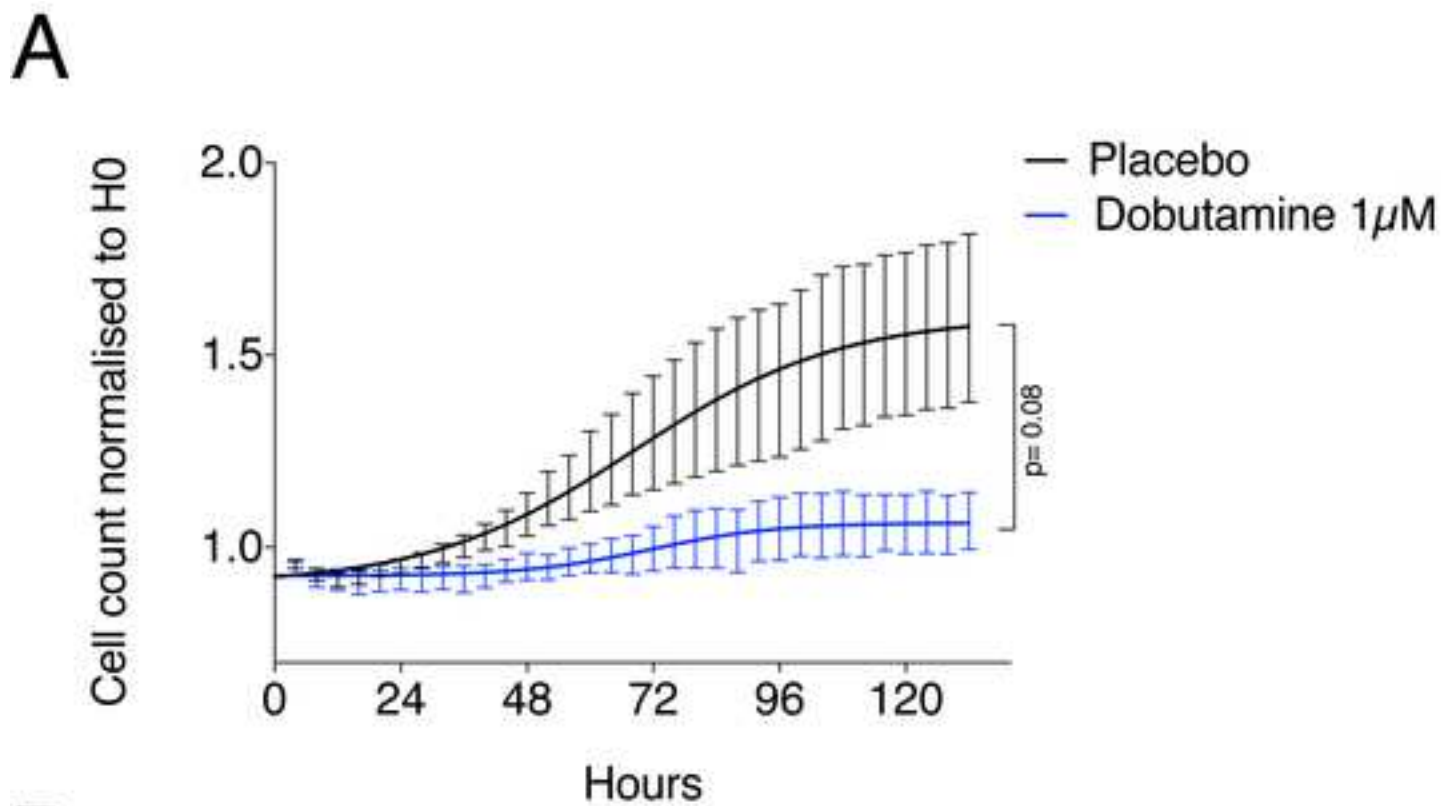


Figure 3

