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## The $\beta$ 1-Adrenergic Receptor Contributes to Sepsis-Induced Immunosuppression Through Modulation of Regulatory T-Cell Inhibitory Function\*

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1 **The  $\beta_1$ -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  $\beta_1$ -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  $\beta_1$ -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

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16  
17 61 *Subjects:* C57BL/6 mice

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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  $\beta_1$ -adrenoreceptor blocker),  
23  
24 64 or in  $\beta_1$ -adrenoreceptor knockout mice ( $\beta_1^{-/-}$ ). At 18 hours after surgery  
25  
26  
27 65 echocardiography was performed with blood and spleen collected to analyze  
28  
29  
30 66 lymphocyte function.

31  
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  
38  
39  
40 70 cell proportions. Pharmacologic and genetic  $\beta_1$ -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.  $\beta_1$ -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:*  $\beta_1$ -adrenoreceptor activation enhances Treg inhibitory function and thus  
55  
56 77 contributes to sepsis-induced immunosuppression. This can be attenuated by  $\beta_1$ -  
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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 ( $\beta_1$ -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  $\beta_1$ -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  $\beta_1$ -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  $\beta_1$ -AR blockade on the early adaptive immune response (18, 19). We hypothesized  
136 that  $\beta_1$ -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  $\beta_1$ -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**.  $\beta_1$ -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  $\beta_1$ -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  $\mu$ l/h, 120  $\mu$ 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<sup>hi</sup> CD127<sup>low</sup>, Foxp3) in viable cells. Th1 (INF- $\gamma$ +), 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  $\mu$ M/10<sup>6</sup> cells; Biolegend, San Diego, CA) according to the manufacturer's instructions  
183 and stimulated with phytohemagglutinin (PHA; 4  $\mu$ g/10<sup>6</sup> 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  $\mu$ M dobutamine ( $\beta_1$ -AR agonist), norepinephrine ( $\alpha_1$ -

197 and  $\beta_1$ -AR agonist), phenylephrine ( $\alpha_1$ -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% CO<sub>2</sub>) 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 In 5–6 mice from each group, complete blood counts were measured (Micros 60

214 ABX, Radeberg, Germany).

215 *Quantification of inflammatory mediators*

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2  
3 216 In 5–6 mice from each group, the plasma levels of 46 inflammatory mediators were  
4  
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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  
10  
11 219 manufacturer’s instructions.

12  
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14 220 *Immunohistochemistry*

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17  
18 221 A full description is provided in **Supplemental Digital Content – Methods**. Hearts  
19  
20 222 were sectioned into transverse slices from apex to base and embedded in optimal  
21  
22  
23 223 cutting temperature compound (CellPath, Newtown, Powys, UK) for  
24  
25 224 immunohistochemistry. Leukocytes were stained on 7- $\mu$ 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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35  
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  
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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.

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50 233  
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53 234 *Statistical analysis*

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56  
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  **$\beta_1$ -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  $\beta_1$ -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<sup>high</sup> CD127<sup>low</sup> 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  $\beta_1$ -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,  $\beta_1$ -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- $\gamma$ ), 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**).

278

279  **$\beta_1$ -AR activation induces a decrease in CD4 T cell proliferation during sepsis.**

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2 280 After culturing splenocytes for 72 hours with PHA stimulation, the proliferative  
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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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9  
10 283 15% vs. sham,  $p=0.04$ ). However, administration of a  $\beta_1$ -AR blocker limited this effect  
11  
12  
13 284 (-8% vs. sham,  $p=0.38$ ). In  $\beta_1^{-/-}$  mice, sepsis did not decrease the proliferative  
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**  
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19  
20 287 **Content – Figure 8**). Of note,  $\beta_1$ -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  **$\beta_1$ -AR stimulation of Treg cells increases their immunosuppressive capacities.**

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30 291 The direct effect of  $\beta_1$ -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  $\beta_1$ -AR agonist dobutamine, in comparison  
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37 294 to untreated Treg cells ( $p=0.08$ , **Figure 2, panel A**). This lower cell count, noted when  
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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  
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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  **$\beta_1$ -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  
59 303 **Digital Content – Table 2**). In  $\beta_1^{+/+}$  septic mice, administration of a  $\beta_1$ -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  $\beta_1$ -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  $\beta_1$ -AR had no influence on myocardial leukocyte infiltration (Supplemental**  
320 **Digital Content – Figure 10).**

### 322 **$\beta_1$ -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  $\beta_1$ -AR blocker  
1  
2 330 reduced plasma TNF- $\alpha$  by 66% vs. CLP ( $p=0.02$ ), and IL-10 by 82% vs. CLP ( $p=0.01$ ).  
3  
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**).  
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12  **$\beta_1$ -AR modulates survival during sepsis.**  
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15 335 The survival rate was documented in  $\beta_1^{+/+}$  CLP,  $\beta_1^{+/+}$  CLP + esmolol and  $\beta_1^{-/-}$  CLP mice  
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17 336 (**Figure 4**). Survival at 110 hours decreased from 60% in  $\beta_1^{-/-}$  CLP mice, to 37% in  $\beta_1^{+/+}$   
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20 337 CLP + esmolol mice and 35% in  $\beta_1^{+/+}$  CLP mice (log rank  $p=0.13$ ).  
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25 339 **Sex does not affect the  $\beta_1$ -AR effect.**  
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28 340 Sensitivity analyses were performed on results obtained for major hemodynamic  
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30 341 parameters (**Supplemental Digital Content – Figure 11**) and immune  
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32 342 (**Supplemental Digital Content – Figure 12**) effects according to sex. No differences  
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35 343 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  $\beta_1$ -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  $\beta_1$ -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  $\beta_1$ -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 β<sub>2</sub>-AR but not β<sub>1</sub>-AR subtypes were found on T cells (38,  
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21  
22 378 39). As a consequence, β<sub>1</sub>-AR stimulation was not considered to have a major role in  
23  
24 379 inflammation (40). β<sub>1</sub>-AR were found to be widely expressed on splenic CD4 T cells  
25  
26 380 only recently. In cirrhotic mice, the β<sub>1</sub>-AR agonist dobutamine could re-activate  
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29 381 inhibited Treg cells, suggesting the presence of β<sub>1</sub>-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 β<sub>1</sub>-AR activity, dobutamine also  
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36 384 has activity on the β<sub>2</sub>-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 β<sub>1</sub> 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  
45  
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 β<sub>1</sub>-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  
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2 395 decrease in pro- and anti-inflammatory mediators in animals with mid-grade sepsis  
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5 396 treated by  $\beta_1$ -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  
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10 398 sympathetic adrenergic stimulation) inhibited the host T cell response and increased  
11  
12 399 cytokine production (46). In this model, pharmacological blockade of the  $\beta_1$ -AR but not  
13  
14 400 the  $\beta_2$ -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  $\beta_1$ -AR blocked animals with mid-grade sepsis, we found the decrease in Treg cells  
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24 404 in the spleen was associated with a concomitant increase in CD4 T cells, as reported  
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26 405 previously (48). In addition, we found that the proliferative capacity of splenic CD4 T  
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29 406 cells in septic animals was maintained when the  $\beta_1$ -AR was blocked either  
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31 407 pharmacologically or genetically. This suggests that  $\beta_1$ -AR activation contributes to  
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34 408 sepsis-induced immunosuppression, which could be reversed by  $\beta_1$ -blockade. By  
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36 409 comparison, volunteers challenged with lipopolysaccharide, and then being infused  
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39 410 with norepinephrine for 5 hours showed a shift to an anti-inflammatory state  
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41 411 (decreased TNF- $\alpha$ /IL-10 ratio) which could be counterbalanced by  $\beta$ -AR blockade  
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43  
44 412 (increased TNF- $\alpha$ /IL-10 ratio) (49).  $\beta_1$ -AR blockade in our septic animals reduced the  
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46 413 inflammatory burden and, taken as a whole, helped to shift from an  
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49 414 immunosuppressive state to a state of immune homeostasis.

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51 415 Beside this immune rebalancing in experimental mid-grade sepsis, it is important to  
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53 416 reiterate that  $\beta_1$ -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  $\beta$ -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  $\beta_1$ -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  $\beta_1$ -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  $\beta_1$ -AR and  $\beta_2$ -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  $\beta_2$ -AR (35, 53).  $\beta_2$ -AR stimulation promotes a shift from  
438 Th1 to a Th2 anti-inflammatory pattern and could activate Treg suppressive functions  
439 (36, 54).  $\beta_2$ -AR stimulation may enhance the macrophage M2 anti-inflammatory  
440 phenotype, favoring IL-10 production by CD4 T cells (37). Beneficial effects of  $\beta_2$ -AR  
441 modulation remain unclear during sepsis. In a murine endotoxemia model,  $\beta_2$ -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  $\beta_1$ -AR blockade on

1  
2 445 alteration of CD4 T cell patterns. The impact of selective  $\beta_2$ -AR modulation as well as  
3 non-specific  $\beta$ -AR blockade on CD4 T cells during sepsis merits investigation.

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5 446  
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7 447 Our initial intention was to create a CLP model with an expected 48 hour-mortality rate  
8 of 70% (26). However, despite using a larger 18G needle to puncture the cecum  
9 448 (instead of the usually recommended 22G needle for mice) and without administering  
10 antibiotics (avoided so as not to interfere with the infectious/inflammatory processes),  
11 449 the 48 hour-mortality was 30% in the non-treated CLP mice control group.  
12 Nonetheless, this remains a severe model. It would be worthwhile repeating the study  
13 with full-code resuscitative measures including ongoing fluid administration,  
14 450 catecholamines (if required), and antibiotics (24). Limitations of murine CLP models  
15 should also be acknowledged, for example youth, underlying good health, a  
16 451 homogenous infectious insult and severity, and a similar time-interval from CLP  
17 induction to evaluation. The immune system in mice is also not identical to humans  
18 452 and human sepsis is far more heterogeneous in nature. Nonetheless, such models  
19 remain useful for mechanistic understanding and an initial assessment of the impact  
20 453 of interventions. Measurements were only performed at 18 hours post-CLP. Serial  
21 assessments in a less severe model could clarify the evolving inflammatory profile of  
22 460 this disease.  
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### *Perspectives and potential clinical implications*

66 The deleterious effects of a disproportionate and prolonged activation of the adrenergic  
67 system are now well established (5, 7). In both clinical trials and cohort studies, short-  
68 and long-term administration of  $\beta$ -AR blockers are associated with a mortality benefit.  
69 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  
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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  $\beta_1$ -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  $\beta_1$ -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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21 478 In summary, in the murine model tested, the  $\beta_1$ -AR contributes to sepsis-induced  
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24 479 immunosuppression through modulation of regulatory T cell inhibitory functions.  $\beta_1$ -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  $\beta_1$ -AR blockade only for its hemodynamic effects, we suggest an  
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34 483 immunoregulatory mechanism by which  $\beta_1$ -AR blockers may also be beneficial.

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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  
26 493 manuscript. All authors were involved in critical revision of the manuscript. All authors  
27  
28 494 read and approved the final manuscript.  
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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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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:  $\beta_1$ -AR contributes to the post-septic immunosuppressive phenotype,**  
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5 655 **which is reversed by  $\beta_1$ -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<sup>high</sup> CD127<sup>low</sup>) 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  $\beta_1$ -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<sup>®</sup> imager.

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50 673  
51 674 **Figure 3:  $\beta_1$ -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 Log<sub>10</sub>(mean).



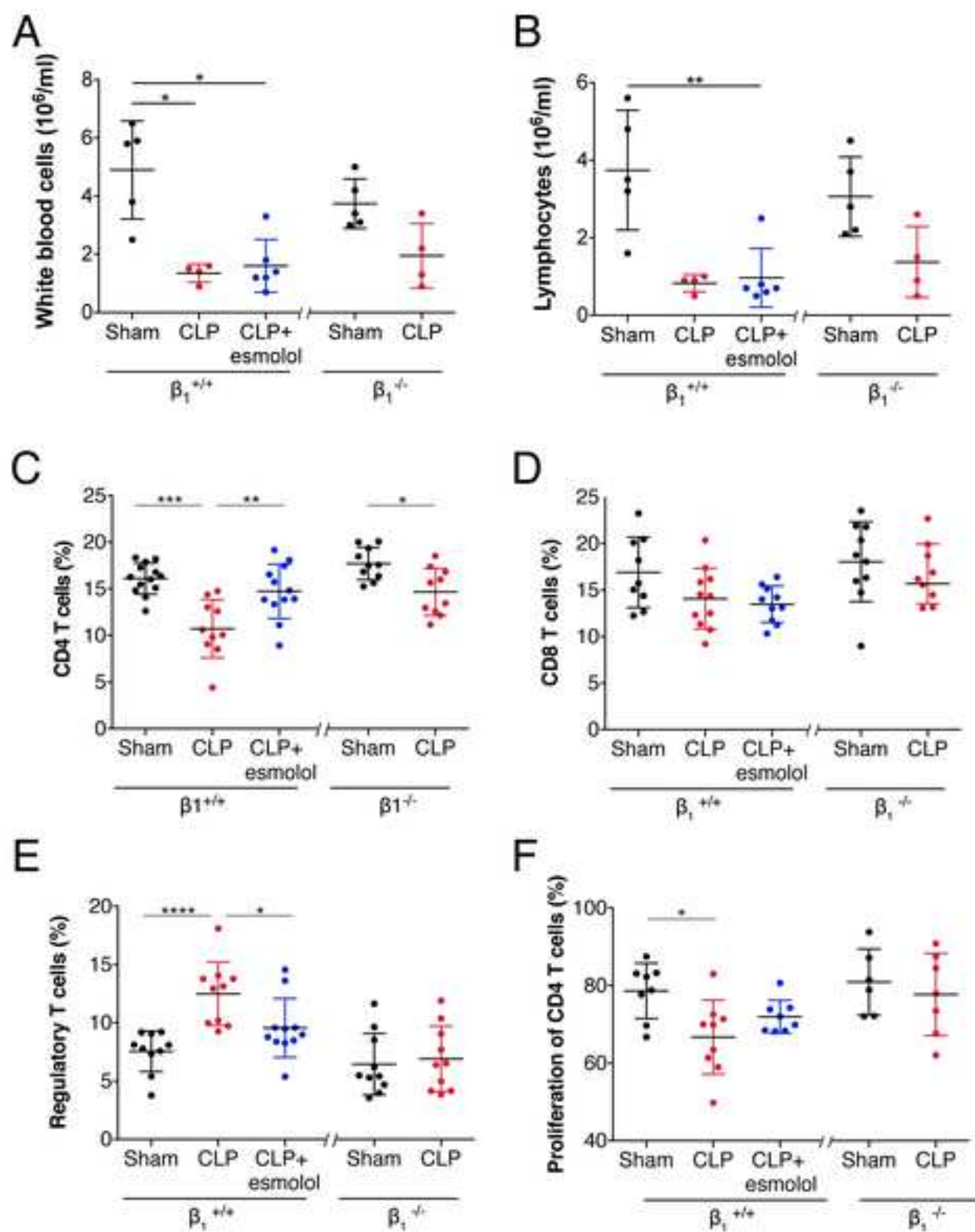
677 **Figure 4:  $\beta_1$ -AR blockade modulates survival during sepsis.** Survival curve of  $\beta_1^{+/+}$   
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2 678 CLP mice,  $\beta_1^{+/+}$  CLP mice receiving esmolol for 24 hours, and  $\beta_1^{-/-}$  CLP mice. The one  
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5 679 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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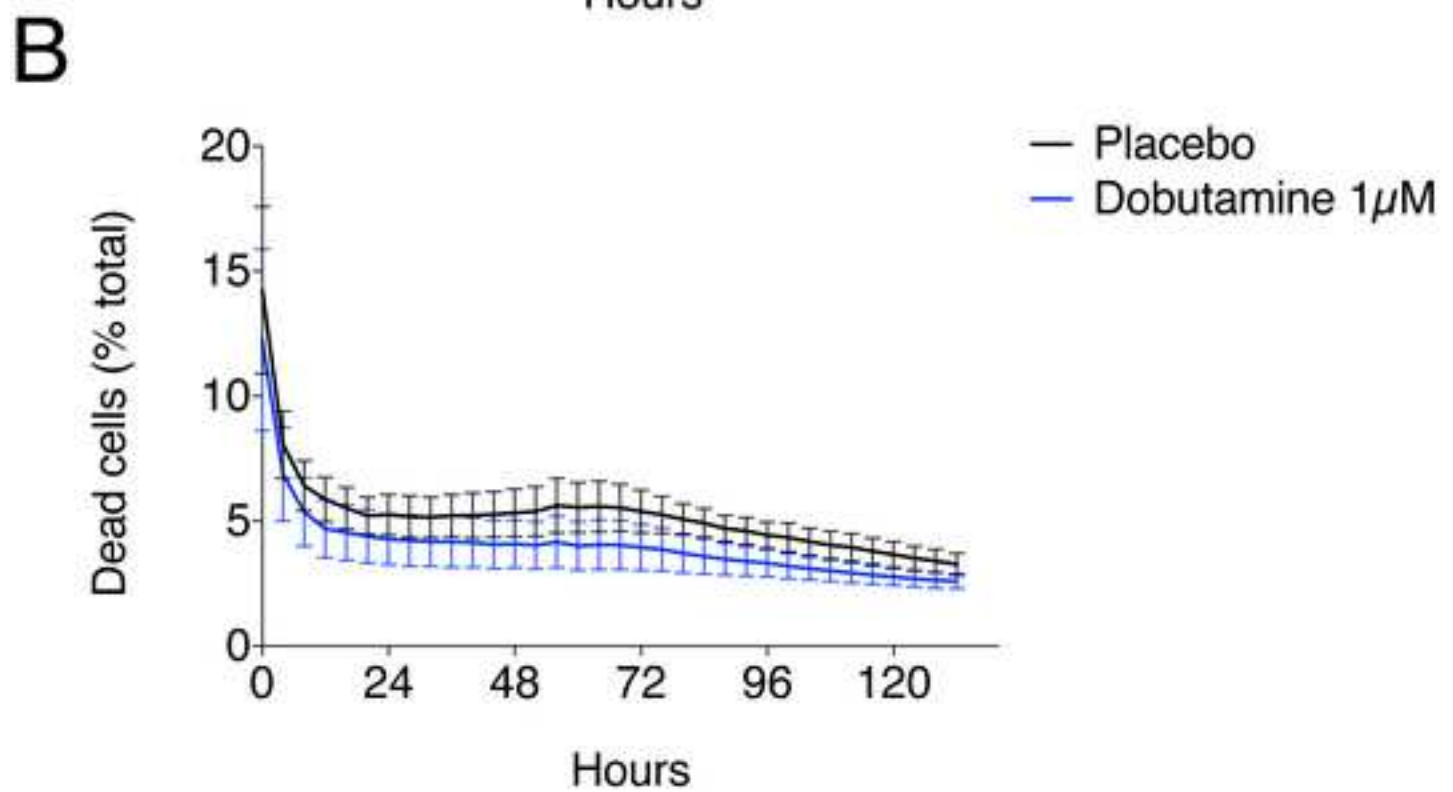
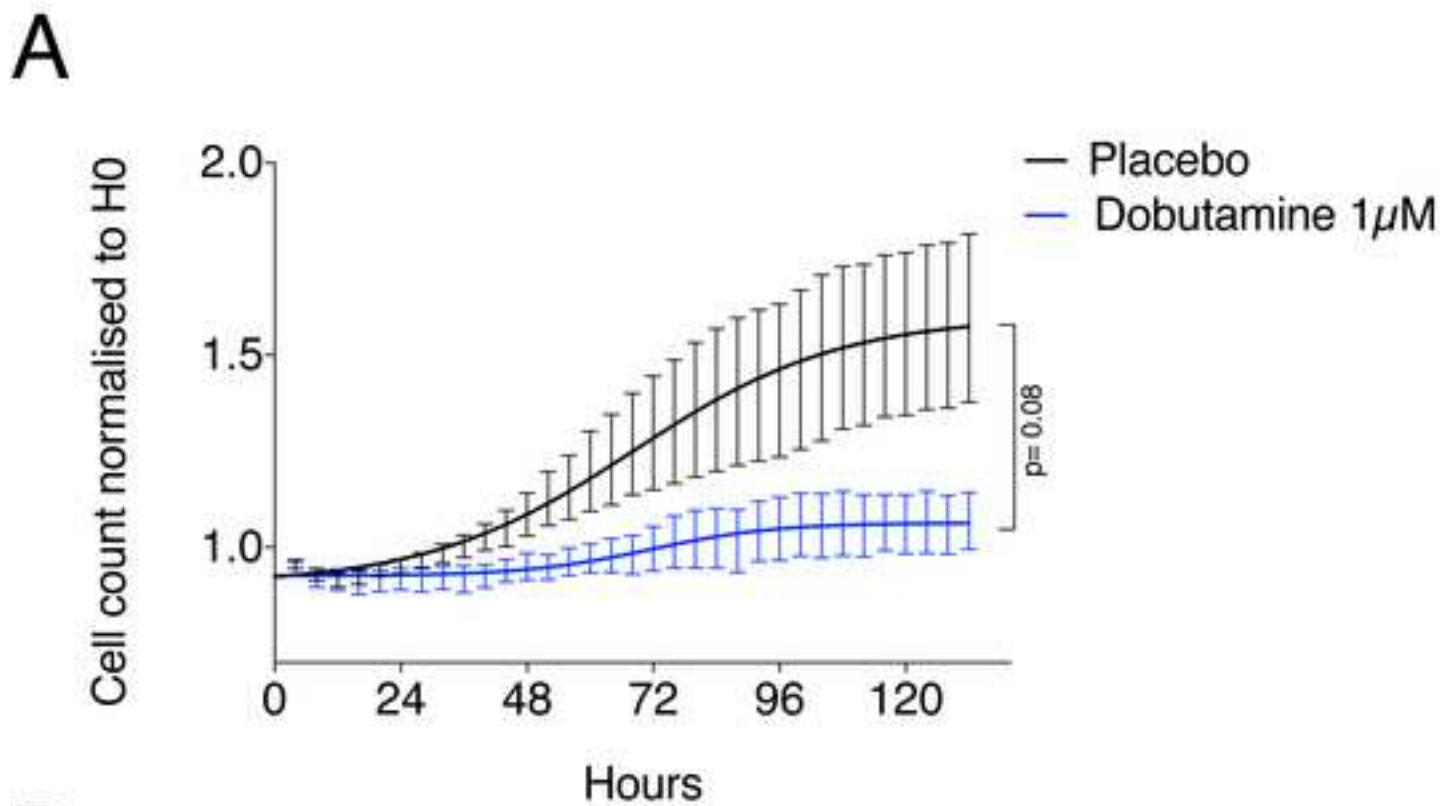


Figure 3

