

1 Letter to the Editor

2 **Expansion of activated Treg cells inversely correlates with clinical severity in septic neonates.**

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24 **Capsule summary**

25 Our work contributes to the definition of a possibly protective role of Tregs in neonatal severe  
26 infections with particular attention to different molecules that may better define the phenotype and  
27 role of the Treg subset.

28

29 **Key words**

30 Regulatory T cells; Infants; Neonates; Sepsis; Systemic Inflammatory Response Syndrome.

31

32 **Abbreviations**

33 act, activated; HD, healthy donor; MFI, mean fluorescence intensity; pts, patients; SIRS,  
34 systemic inflammatory response syndrome; Tconvs, conventional T cells; Tregs, regulatory T  
35 cells.

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37 To the Editor:

38 Current knowledge about regulatory T cells' (Tregs) function in early life is very limited. Tregs  
39 represent a heterogeneous CD4<sup>+</sup> T cell population addressed to maintain immunological self-  
40 tolerance and immune homeostasis in various immune-mediated diseases including infectious  
41 processes. It is known that circulating CD4<sup>+</sup>CD25<sup>+</sup> Tregs increase during septic shock in adult,<sup>1</sup>  
42 while very few data exist about Tregs in neonatal infections. The aim of our study was to analyse  
43 frequency/heterogeneity of Tregs in neonates with infectious and non-infectious systemic  
44 inflammatory response syndrome (SIRS) (see Table E1 in Online Repository).

45 Firstly, we found that the percentage of FOXP3<sup>+</sup>CD127<sup>low</sup> Tregs within the gate of CD4<sup>+</sup> T cells in  
46 mononuclear cells enriched from peripheral blood of neonates with sepsis or SIRS, as well as in  
47 neonatal (CTRLs) and paediatric (PED) controls, resulted similar in all cohorts (Fig.E1A-B) (see  
48 Figure E in Online Repository). However, when different subsets of Tregs were analysed  
49 (CD45RA<sup>high</sup>FOXP3<sup>low</sup> resting [rest], CD45RA<sup>low</sup>FOXP3<sup>high</sup> activated [act], and  
50 CD45RA<sup>low</sup>FOXP3<sup>low</sup> non-suppressive [non] Tregs),<sup>2</sup> (complete methodology available as Online  
51 Repository Methods) a marked increase of circulating actTregs was observed in both septic and  
52 SIRS neonates compared to CTRLs (Fig.E1A-C; Fig.1A). Using the EMA clinical and laboratory  
53 criteria to diagnose neonatal sepsis,<sup>3, 4</sup> we developed a composite score in order to evaluate the  
54 clinical severity of enrolled patients. Importantly, an inverse correlation between actTreg frequency  
55 and clinical score was detected in septic neonates (Fig.1B), suggesting that actTregs may limit  
56 excessive sepsis-specific immunopathology. This hypothesis was supported by the evidence that  
57 CD39, a membrane ectonucleotidase hydrolysing ATP and ADP to AMP and ultimately  
58 contributing to the peculiar suppressive functions of Tregs,<sup>5</sup> was expressed on Tregs more than  
59 Tconvs (Fig.E2A), and on actTregs more than restTregs or nonTregs (Fig.1C). To explore the  
60 mechanism of the high inter-individual variability in the CD39 expression on Tregs (Fig. 1D-E), we  
61 analysed the single-nucleotide polymorphism (SNP) rs10748643 (A vs G) within the *ENTPDI*  
62 gene.<sup>6</sup> The allelic variation was closely related to CD39 levels in Tregs, but not in Tconvs; indeed,  
63 GG homozygous and/or AG heterozygous individuals showed a higher frequency of CD39<sup>+</sup> Tregs  
64 compared to AA homozygous within each group (Fig.1D-E). In addition, a further increase of  
65 CD39<sup>+</sup> Tregs, but not Tconvs (Fig.1E-F), was shown in both sepsis and SIRS neonates as compared  
66 with CTRLs, upon stratification by genotype, suggesting that both genetic signature and  
67 inflammatory milieu contribute to the CD39 overexpression. As control, we analysed the genetic  
68 contribution of the *ENTPDI* SNP in determining CD39 levels in cord blood, a context mostly  
69 spared from T cell activation events (Fig.E2B-F).

70 To evaluate whether Tregs may modulate their phenotype during sepsis or SIRS, we quantified the  
71 expression of OX40, a receptor belonging to the tumour necrosis factor receptor family specifically  
72 up-regulated in highly suppressive tumour-infiltrating Tregs.<sup>7</sup> OX40 was significantly more  
73 expressed in CD39<sup>+</sup> compared to CD39<sup>-</sup>, principally within the gate of Tregs rather than Tconvs  
74 (Fig.E2G). Furthermore, a significant increment of OX40<sup>+</sup> cells among CD39<sup>+</sup> Tregs was detected  
75 in sepsis and even more in SIRS neonates as compared with CTRLs (Fig.2A). Notably, CTRLs,  
76 sepsis and SIRS neonates showed significantly higher OX40 expression in CD39<sup>+</sup> than PED. Also  
77 in Tconvs, OX40 was markedly higher in CD39<sup>+</sup> respect to CD39<sup>-</sup> cells (Fig.E2H), but at lower  
78 levels than Tregs (Fig.2A). These data suggest that Tregs, but not Tconvs, up-regulated OX40  
79 during both sepsis and non-infectious SIRS, especially in suppressive CD39<sup>+</sup> cells accounting for  
80 the possible homeostatic role of Tregs in these pathologic conditions. Consistent with this  
81 hypothesis, the percentage of OX40<sup>+</sup> Tregs was inversely correlated with the clinical score in septic  
82 neonates (Fig.2B), suggesting OX40 induction on Tregs as a protective mechanism in neonatal  
83 sepsis. Interestingly, OX40<sup>+</sup> and CD39<sup>+</sup> Tregs were more represented within Helios<sup>high</sup> than  
84 Helios<sup>low</sup> cells irrespective of cohorts (Fig.E3A), strongly suggesting their belonging to a stable  
85 Treg population. Indeed, the transcription factor Helios has been described to distinguish thymic-  
86 derived (committed/stable) FOXP3<sup>+</sup> Treg from peripherally induced FOXP3<sup>+</sup> Tregs.<sup>8</sup>

87 To investigate whether particular cytokines may provide selective signals inducing OX40 up-  
88 regulation during neonatal sepsis or SIRS, first we analysed the percentage of circulating T cells  
89 producing IFN- $\gamma$  and/or TNF- $\alpha$  by flow cytometry. Notably, IFN- $\gamma$ -single or IFN- $\gamma$ /TNF- $\alpha$ -double  
90 producing T cells were poorly detectable in both CD4<sup>+</sup> and CD8<sup>+</sup> of neonates compared to PED and  
91 adult healthy donors (AD) (Fig.E3B-C), suggesting a neonatal incompetence to generate IFN- $\gamma$ -  
92 mediated adaptive responses. Consistently with this hypothesis, T cells from all neonatal groups  
93 prevalently expressed a naïve phenotype (data not shown). By contrast, TNF- $\alpha$ - producing T cells  
94 (CD4<sup>+</sup> and CD8<sup>+</sup>) were similarly represented in all neonatal cohorts (Fig.E3B-C), as well as in PED  
95 and AD (Fig.E3B-C), despite an increased plasma TNF- $\alpha$  level was detected in septic neonates  
96 (data not shown). Further studies are needed to investigate whether additional sources (monocytes,  
97 dendritic cells) may contribute to increase TNF- $\alpha$  levels in septic neonates. Notably, we found that  
98 CD120b (TNFR2) was expressed at higher levels in Tregs than in Tconvs (Fig.E4A), as well as in  
99 CD39<sup>+</sup> respect to CD39<sup>-</sup> Tregs (Fig.E4A), and even in septic or SIRS neonates significantly more  
100 than in CTRLs (Fig.2C). Interestingly, the percentage of OX40<sup>+</sup> cells within CD39<sup>+</sup> Tregs directly  
101 correlated with the percentage of CD120b<sup>+</sup> cells (Fig.2D), as to support that the preferential OX40  
102 expression on CD39<sup>+</sup> Tregs *in vivo* might be particularly due to the higher susceptibility to TNF- $\alpha$   
103 by CD39<sup>+</sup>CD120b<sup>+</sup> Tregs, principally during neonatal sepsis and SIRS. In line with this finding, *in*

104 *in vitro* experiments revealed that the overnight TNF- $\alpha$  treatment of fresh PBMCs obtained from  
105 healthy CTRLs enhanced the level of OX40 expression at a greater degree in Tregs than in Tconvs  
106 (Fig.E4B). TNF- $\alpha$  induced a significant OX40 up-regulation principally on CD39<sup>+</sup> Tregs (Fig.2E),  
107 suggesting that CD39<sup>+</sup> Tregs are particularly prone to up-regulate OX40.

108 Then, based on the evidence of increased plasma levels of IL-33 (cytokine belonging to the IL-1  
109 superfamily) in SIRS neonates (data not shown), and of a previous report demonstrating an IL-33-  
110 dependent up-regulation of OX40 on mouse Tregs *in vitro*,<sup>9</sup> we investigated the role of this  
111 cytokine in activating Tregs in terms of OX40 up-regulation *in vitro* by using PBMCs from adult  
112 HDs. Interestingly, PBMCs treated with IL-33 were able to up-regulate OX40 expression at a  
113 significant higher level in total Tregs or CD39<sup>+</sup> Tregs than in Tconv counterpart (Fig.E4C).  
114 Notably, a positive correlation between IL-33 plasma concentration and the percentage of OX40<sup>+</sup> in  
115 total Tregs was observed in all neonatal cohorts (Fig.2F).

116 Taken together, our data support the hypothesis that actTregs, especially OX40<sup>+</sup> Tregs showing  
117 CD39<sup>+</sup>, Helios<sup>+</sup>, and CD120b<sup>+</sup> phenotype, counteract excessive immunopathology in neonatal  
118 sepsis, and that their functions are modulated via prominent inflammatory cytokines, such as TNF-  
119  $\alpha$  and IL-33, during infective and non-infective SIRS.

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184 **Figure legends**

185 *Figure 1. Frequency of actTregs inversely correlates with septic score.*

186 (A) actTreg frequency estimated by FCM as the percentage of CD45RA<sup>low</sup>FOXP3<sup>high</sup> Tregs among  
187 CD4<sup>+</sup> T cells (CTRL=28; sepsis=10; SIRS=19; PED=21). \**P*<0.05, \*\**P*<0.01, by Mann-Whitney's  
188 test, two-tailed.

189 (B) Spearman's correlation (*r*) between actTregs/CD4<sup>+</sup> T cell percentage and clinical severity score  
190 in septic neonates. \**P*<0.05.

191 (C) Representative FCM data (CTRL sample) showing CD45RA *versus* FOXP3 profile (left);  
192 overlay of CD39 MFI (right) of CD45RA<sup>low</sup>FOXP3<sup>+</sup> (actTreg), CD45RA<sup>high</sup>FOXP3<sup>low</sup> (restTreg),  
193 CD45RA<sup>low</sup>FOXP3<sup>-</sup> (nonTreg) subsets.

194 (D) Representative FCM plots of CD39 *vs* FOXP3 expression in gated Tregs, showing the  
195 frequency of CD39<sup>+</sup>, according to AA or AG+GG genotypes in the *ENTPDI* gene.

196 (E) Frequency of CD39<sup>+</sup> cells in gated Tregs in all cohorts according to AA or AG+GG genotypes  
197 in the *ENTPDI* gene (CTRL=23; sepsis=10; SIRS=15; PED=21). \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.005  
198 by Mann-Whitney's test, two-tailed.

199 (F) Frequency of CD39<sup>+</sup> cells in gated Tconvs in all cohorts according to AA or AG+GG genotypes  
200 in the *ENTPDI* gene (CTRL=23; sepsis=10; SIRS=15; PED=21). \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.005  
201 by Mann-Whitney's test, two-tailed.

202

203 *Figure 2. Frequency of OX40<sup>+</sup> Tregs is up-regulated in sepsis and SIRS neonates and inversely*  
204 *correlates with septic score.*

205 (A) Frequency of OX40<sup>+</sup> in subdivided CD39<sup>+</sup> and CD39<sup>-</sup> Treg subsets in all groups (CTRL=32;  
206 sepsis=11; SIRS=20; PED=21). \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.005, \*\*\*\**P*<0.0001 by Mann-  
207 Whitney's test two-tailed and Wilcoxon's matched-pairs test, two-tailed.

208 (B) Spearman's correlation (*r*) between frequency of OX40<sup>+</sup> Tregs and clinical severity score in  
209 septic neonates (Table I). \**P*<0.05.

210 (C) PBMCs from healthy neonatal controls were cultured ON with or without TNF- $\alpha$ . OX40 MFI  
211 was evaluated in Tregs and Tconvs. \**P*<0.05 by Paired T-test, two-tailed.

212 (D) Frequency of CD120b<sup>+</sup> cells in CD39<sup>+</sup> and CD39<sup>-</sup> Tregs (CTRL=5; sepsis=6; SIRS=4).

213 (E) Pearson's correlation (*r*) between frequency of CD120b<sup>+</sup> cells and OX40<sup>+</sup> cells within CD39<sup>+</sup>  
214 Tregs in all cohorts. \*\**P*<0.01.

215 (F) Spearman's correlation (*r*) between frequency of OX40<sup>+</sup> Tregs and IL-33 plasma concentration  
216 in all cohorts. \*\*\**P*<0.005.