Letter to the Editor

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

Corresponding authors:

Vincenzo Barnaba, MD

Dipartimento di Medicina Interna e Specialità Mediche, Sapienza Università di Roma

Viale del Policlinico 155, I-00161 Rome, Italy

phone: +39 6 491268; fax: +39 6 4938 3333;

email: vincenzo.barnaba@uniroma1.it

and

Paolo Rossi, MD, PhD

Dipartimento Pediatrico Universitario Ospedaliero (DPUO), Ospedale Pediatrico Bambin Gesù

Piazza Sant'Onofrio 4, 00165 Roma, Italy

Phone: +39 06 6859 2508

email: paolo.rossi@opbg.net

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Capsule summary

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

Key words

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

Abbreviations

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

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To the Editor:

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

Firstly, we found that the percentage of $FOXP3^+CD127^{low}$ Tregs within the gate of $CD4^+$ T cells in mononuclear cells enriched from peripheral blood of neonates with sepsis or SIRS, as well as in neonatal (CTRLs) and paediatric (PED) controls, resulted similar in all cohorts (Fig.E1A-B) (see Figure E in Online Repository). However, when different subsets of Tregs were analysed ($CD45RA^{high}FOXP3^{low}$ resting [rest], $CD45RA^{low}FOXP3^{high}$ activated [act], and $CD45RA^{low}FOXP3^{low}$ non-suppressive [non] Tregs),[2] (complete methodology available as Online Repository Methods) a marked increase of circulating actTregs was observed in both septic and SIRS neonates compared to CTRLs (Fig.E1A-C; Fig.1A). Using the EMA clinical and laboratory criteria to diagnose neonatal sepsis,[3, 4] we developed a composite score in order to evaluate the clinical severity of enrolled patients. Importantly, an inverse correlation between actTreg frequency and clinical score was detected in septic neonates (Fig.1B), suggesting that actTregs may limit excessive sepsis-specific immunopathology. This hypothesis was supported by the evidence that CD39, a membrane ectonucleotidase hydrolysing ATP and ADP to AMP and ultimately contributing to the peculiar suppressive functions of Tregs,[5] was expressed on Tregs more than Tconvs (Fig.E2A), and on actTregs more than restTregs or nonTregs (Fig.1C). To explore the mechanism of the high inter-individual variability in the CD39 expression on Tregs (Fig. 1D-E), we analysed the single-nucleotide polymorphism (SNP) rs10748643 (A *vs* G) within the *ENTPD1* gene.[6] The allelic variation was closely related to CD39 levels in Tregs, but not in Tconvs; indeed, GG homozygous and/or AG heterozygous individuals showed a higher frequency of $CD39^+$ Tregs compared to AA homozygous within each group (Fig.1D-E). In addition, a further increase of $CD39^+$ Tregs, but not Tconvs (Fig.1E-F), was shown in both sepsis and SIRS neonates as compared with CTRLs, upon stratification by genotype, suggesting that both genetic signature and inflammatory milieu contribute to the CD39 overexpression. As control, we analysed the genetic contribution of the *ENTPD1* SNP in determining CD39 levels in cord blood, a context mostly spared from T cell activation events (Fig.E2B-F).

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

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

vitro experiments revealed that the overnight TNF-α treatment of fresh PBMCs obtained from healthy CTRLs enhanced the level of OX40 expression at a greater degree in Tregs than in Tconvs (Fig.E4B). TNF-α induced a significant OX40 up-regulation principally on $CD39^+$ Tregs (Fig.2E), suggesting that $CD39^+$ Tregs are particularly prone to up-regulate OX40.

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

Taken together, our data support the hypothesis that actTregs, especially $OX40^+$ Tregs showing $CD39^+$, $Helios^+$, and $CD120b^+$ phenotype, counteract excessive immunopathology in neonatal sepsis, and that their functions are modulated via prominent inflammatory cytokines, such as TNF-α and IL-33, during infective and non-infective SIRS.

Eleonora Timperi[1]*, PhD;

Laura Folgori[2]*, MD;

Donato Amodio[2], MD;

Maia De Luca[2], MD;

Sara Chiurchiù[2], MD;

Silvia Piconese[1,3], PhD;

Silvia Di Cesare[2], MS;

Ilenia Pacella[1], MS;

Carmela Martire[1], MS;

Giulia Bonatti[2], MD;

Seila Perrone[4], MD;

Terenzio Boni[4], MD;

Genni Enza Marcovecchio[2], MS;

Antonino Reale[5], MD;

Francesco Parisi[6], MD;

Andrea Dotta[7], MD;

Vincenzo Barnaba[1,3], MD

Paolo Rossi[2], MD, PhD.

*Equally contributing authors

[1]*Department of Internal Medicine and Medical Specialities,"Sapienza" Università di Roma, Policlinico Umberto I, Rome, Italy*

[2]*University Department of Pediatrics, Bambino Gesù Children's Hospital, University of Rome "Tor Vergata", Rome, Italy*

[3]*Pasteur Institute – Cenci Bolognetti Foundation, Rome, Italy*

[4]*Department of Ginecological Obstetrics Sciences and Urological Sciences, "Sapienza" Università di Roma, Policlinico Umberto I, Rome, Italy*

[5]*Emergency Department, Bambino Gesù Children's Hospital, Rome, Italy*

[6]*Department of Pediatric Cardiology and Cardiac Surgery, Bambino Gesù Children's Hospital, Rome, Italy*

[7]*Neonatal Intensive Care Unit, Department of Medical and Surgical Neonatology, Bambino Gesù Children's Hospital, Rome, Italy*

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Figure legends

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

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

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

(C) Representative FCM data (CTRL sample) showing CD45RA *versus* FOXP3 profile (left); overlay of CD39 MFI (right) of $CD45RA^{low}FOXP3^+$ (actTreg), $CD45RA^{high}FOXP3^{low}$ (restTreg), $CD45RA^{low}FOXP3^-$ (nonTreg) subsets.

(D) Representative FCM plots of CD39 *vs* FOXP3 expression in gated Tregs, showing the frequency of $CD39^+$, according to AA or AG+GG genotypes in the *ENTPD1* gene.

(E) Frequency of $CD39^+$ cells in gated Tregs in all cohorts according to AA or AG+GG genotypes in the *ENTPD1* gene (CTRL=23; sepsis=10; SIRS=15; PED=21). **P<0.05, **P<0.01, ***P<0.005* by Mann-Whitney's test, two-tailed.

(F) Frequency of $CD39^+$ cells in gated Tconvs in all cohorts according to AA or AG+GG genotypes in the *ENTPD1* gene (CTRL=23; sepsis=10; SIRS=15; PED=21). **P<0.05, **P<0.01, ***P<0.005* by Mann-Whitney's test, two-tailed.

Figure 2. Frequency of $OX40^+$ Tregs is up-regulated in sepsis and SIRS neonates and inversely correlates with septic score.

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

(B) Spearman's correlation (r) between frequency of $OX40^+$ Tregs and clinical severity score in septic neonates (Table I). **P<0.05.*

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

(D) Frequency of $CD120b^+$ cells in $CD39^+$ and $CD39^-$ Tregs (CTRL=5; sepsis=6; SIRS=4).

(E) Pearson's correlation (r) between frequency of $CD120b^+$ cells and $OX40^+$ cells within $CD39^+$ Tregs in all cohorts. ***P<0.01.*

(F) Spearman's correlation (r) between frequency of $OX40^+$ Tregs and IL-33 plasma concentration in all cohorts. ****P<0.005.*