Expansion of activated regulatory T cells inversely correlates with clinical severity in septic neonates

To the Editor:

Current knowledge about the function of regulatory T (Treg) cells in early life is very limited. Treg cells represent a heterogeneous $CD4^+$ T-cell population addressed to maintain immunologic self-tolerance and immune homeostasis in various immune-mediated diseases, including infectious processes. It is known that numbers of circulating $CD4^+CD25^+$ Treg cells increase during septic shock in adults,[1] whereas very few data exist about Treg cells in the setting of neonatal infection. The aim of our study was to analyze the frequency/heterogeneity of Treg cells in neonates with infectious and noninfectious systemic inflammatory response syndrome (SIRS; see Table E1 in this article's Online Repository at www.jacionline.org).

First, we found that the percentage of forkhead box protein 3 $(FOXP3)^+CD127^{low}$ Treg cells within the $CD4^+$ T-cell gate in mononuclear cells enriched from peripheral blood of neonates with sepsis or SIRS, as well as in healthy neonatal control subjects (CTRLs) and pediatric control subjects (PEDs), was similar in all cohorts (see Fig E1, *A* and *B*, in this article's Online Repository at www.jacionline.org). However, when different subsets of Treg cells were analyzed ($CD45RA^{high}FOXP3^{low}$ resting, $CD45RA^{low}FOXP3^{high}$ activated, and $CD45RA^{low}FOXP3^{low}$ nonsuppressive Treg cells; complete methodology is available in the Methods section in this article's Online Repository at www.jacionline.org),[2] a marked increase in circulating activated Treg cell counts was observed in both septic neonates and those with SIRS compared with CTRLs (see Fig 1, *A*, and Fig E1).

Using the European Medicines Agency clinical and laboratory criteria to diagnose neonatal sepsis,[3,4] we developed a composite score to evaluate the clinical severity of enrolled patients. Importantly, an inverse correlation between activated Treg cell frequency and clinical score was detected in septic neonates (Fig 1, *B*), suggesting that activated Treg cells might limit excessive sepsis-specific immunopathology. This hypothesis was supported by the evidence that CD39, a membrane ectonucleotidase hydrolyzing ATP and ADP to AMP and ultimately contributing to the peculiar suppressive functions of Treg cells,[5] was expressed on Treg cells more than on conventional T (Tconv) cells (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org) and on activated Treg cells more than on resting or nonsuppressive Treg cells (Fig 1, *C*).

To explore the mechanism of the high interindividual variability in CD39 expression on Treg cells (Fig 1, *D* and *E*), we analyzed the single nucleotide polymorphism (SNP) rs10748643 (A vs G) within the ectonucleoside triphosphate diphosphohydrolase 1 *(ENTPD1)* gene.[6] The allelic variation was closely related to CD39 levels in Treg cells but not in Tconv cells; indeed, GG homozygous and/or AG heterozygous subjects had a higher frequency of $CD39^+$ Treg cells compared with that seen in AA homozygous subjects within each group (Fig 1, *D* and *E*). In addition, a further increase in $CD39^+$ Treg cell counts but not Tconv cell counts (Fig 1, *E* and *F*) was shown in both septic neonates and those with SIRS compared with counts in CTRLs on stratification by genotype, suggesting that both genetic signature and inflammatory milieu contribute to CD39 overexpression. As a control, we analyzed the genetic contribution of the *ENTPD1* SNP in determining CD39 levels in cord blood (CB), a context mostly spared from T-cell activation events (see Fig E2, *B-F*).

To evaluate whether Treg cells can modulate their phenotype during sepsis or SIRS, we quantified the expression of OX40, a receptor belonging to the TNF receptor family that is specifically upregulated in highly suppressive tumor-infiltrating Treg cells.[7] OX40 was significantly more expressed in $CD39^+$ compared with $CD39^-$ cells, principally within the gate of Treg rather than Tconv cells (see Fig E2, *G*). Furthermore, a significant increment of $OX40^+$ cells among $CD39^+$ Treg cells was detected in septic neonates and even more in neonates with SIRS compared with CTRLs (Fig 2, *A*). Notably, CTRLs, neonates with sepsis, and neonates with SIRS had significantly higher OX40 expression in $CD39^+$ cells than PEDs. Also in Tconv cells, OX40 levels were markedly higher in $CD39^+$ cells with respect to $CD39^-$ cells (see Fig E2, *H*) but at lower levels than seen in Treg cells (Fig 2, *A*). These data suggest that Treg cells, but not Tconv cells, upregulated OX40 during both sepsis and noninfectious SIRS, especially in suppressive $CD39^+$ cells, accounting for the possible homeostatic role of Treg cells in these pathologic conditions. Consistent with this hypothesis, the percentage of $OX40^+$ Treg cells was inversely correlated with the clinical score in septic neonates (Fig 2, *B*), suggesting OX40 induction on Treg cells as a protective mechanism in neonatal sepsis. Interestingly, $OX40^+$ and $CD39^+$ Treg cells were more represented within $Helios^{high}$ than $Helios^{low}$ cells, irrespective of cohort (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org), strongly suggesting they belong to a stable Treg cell population. Indeed, the transcription factor Helios has been described to distinguish thymic-derived (committed/stable) $FOXP3^+$ Treg cells from peripherally induced $FOXP3^+$ Treg cells.[8]

To investigate whether particular cytokines might provide selective signals inducing OX40 upregulation during neonatal sepsis or SIRS, we analyzed the percentage of circulating T cells producing IFN-γ, TNF-α, or both using flow cytometry. Notably, IFN-γ single-producing or IFN-γ/TNF-α double-producing T cells were poorly detectable in both $CD4^+$ and $CD8^+$ cells of neonates compared with those of PEDs and adult healthy donors (see Fig E3, *B* and *C*), suggesting a neonatal incompetence to generate IFN-γ–mediated adaptive responses. Consistent with this hypothesis, T cells from all neonatal groups prevalently expressed a naive phenotype (data not shown). By contrast, TNF-α–producing T cells ($CD4^+$ and $CD8^+$) were similarly represented in all neonatal cohorts (see Fig E3, *B* and *C*), as well as in PEDs and adult healthy donors (see Fig E3, *B* and *C*), despite detection of an increased plasma TNF-α level in septic neonates (data not shown). Further studies are needed to investigate whether additional sources (monocytes and dendritic cells) might contribute to increased TNF-α levels in septic neonates.

Notably, we found that CD120b (TNFR2) was expressed at higher levels in Treg cells than in Tconv cells (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org), as well as in $CD39^+$ with respect to $CD39^-$ Treg cells (see Fig E4, *A*), and even in septic neonates or neonates with SIRS significantly more than in CTRLs (Fig 2, *C*). Interestingly, the percentage of $OX40^+$ cells within $CD39^+$ Treg cells directly correlated with the percentage of $CD120b^+$ cells (Fig 2, *D*), so as to support that the preferential OX40 expression on $CD39^+$

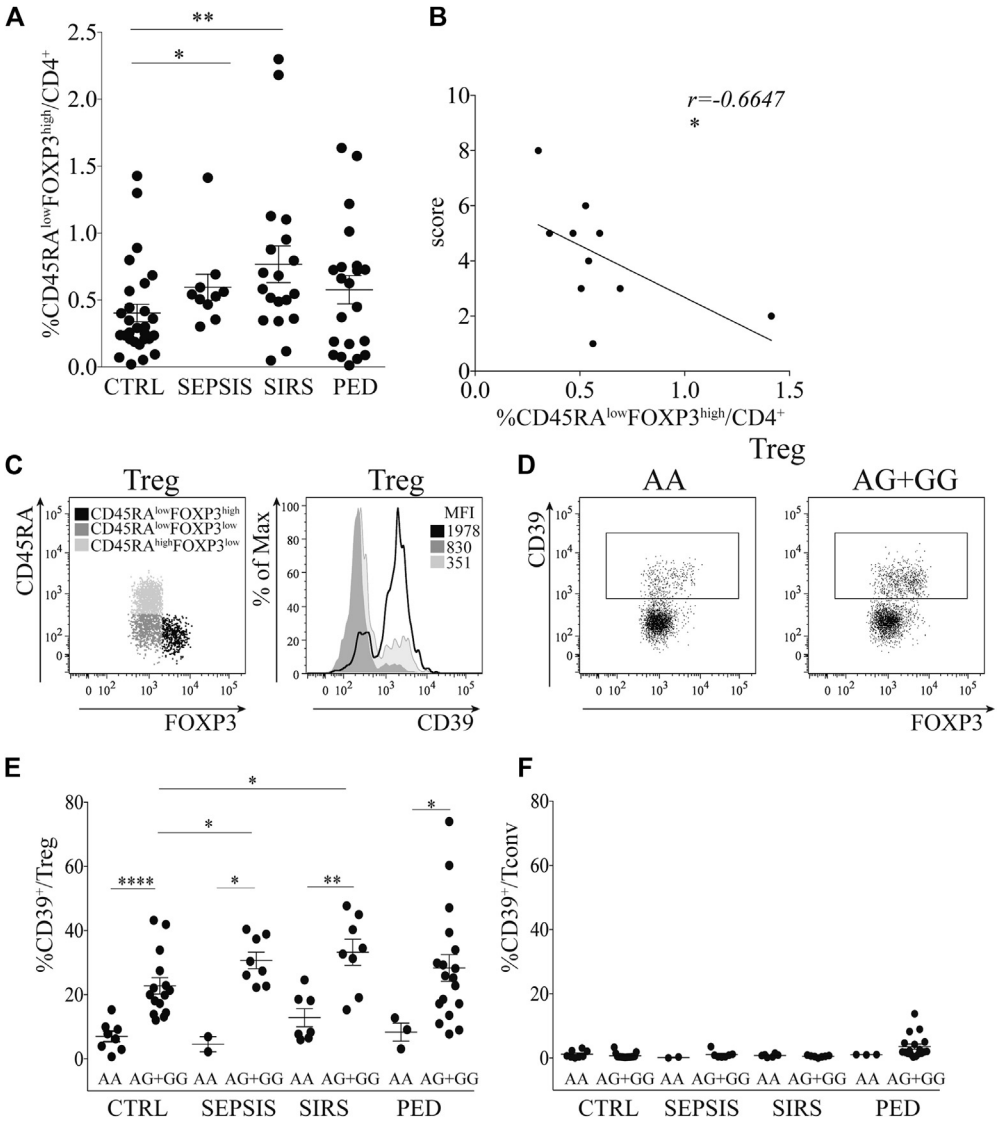

FIG 1. Frequency of activated Treg cells inversely correlates with septic score. **A,** Activated Treg cell frequency estimated by using flow cytometry as the percentage of CD45RA^{low}FOXP3^{high} Treg cells among CD4$^+$ T cells (CTRLs = 28, neonates with sepsis = 10, neonates with SIRS = 19, and PEDs = 21). **P* < .05 and ***P* < .01, Mann-Whitney test, 2-tailed. **B,** Spearman correlation *(r)* between activated Treg cell/CD4$^+$ T-cell percentage and clinical severity score in septic neonates. **P* < .05. **C,** *Left*, Representative flow cytometric data (CTRL sample) showing CD45RA versus FOXP3 profile. *Right*, Overlay of CD39 mean fluorescence intensity of CD45RA^{low}FOXP3^{high} (activated Treg cell), CD45RA^{high}FOXP3^{low} (resting Treg cell), and CD45RA^{low}FOXP3^{low} (nonsuppressive Treg cell) subsets. **D,** Representative flow cytometric plots of CD39 versus FOXP3 expression in gated Treg cells showing the frequency of CD39$^+$ cells according to AA or AG+GG genotypes in the *ENTPD1* gene. **E,** Frequency of CD39$^+$ cells in gated Treg cells in all cohorts according to AA or AG+GG genotype in the *ENTPD1* gene (CTRLs = 23, neonates with sepsis = 10, neonates with SIRS = 15, and PEDs = 21). **P* < .05, ***P* < .01, and *****P* < .001, Mann-Whitney test, 2-tailed. **F,** Frequency of CD39$^+$ cells in gated Tconv cells in all cohorts according to AA or AG+GG genotype in the *ENTPD1* gene (CTRL = 23, sepsis = 10, SIRS = 15, and PED = 21).

Treg cells *in vivo* might be due to the higher susceptibility to TNF-α by CD39$^+$CD120b$^+$ Treg cells, 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 to a greater degree in Treg cells than in Tconv cells (see Fig E4, *B*). TNF-α induced significant OX40 upregulation principally on CD39$^+$ Treg cells (Fig 2, *E*), suggesting that CD39$^+$ Treg cells are particularly prone to upregulate OX40.

Then, based on the evidence of increased plasma levels of IL-33 (cytokine belonging to the IL-1 superfamily) in neonates with SIRS (data not shown) and of a previous report demonstrating IL-33–dependent upregulation of OX40 on mouse Treg cells *in vitro*,[9] we investigated the role of this cytokine in

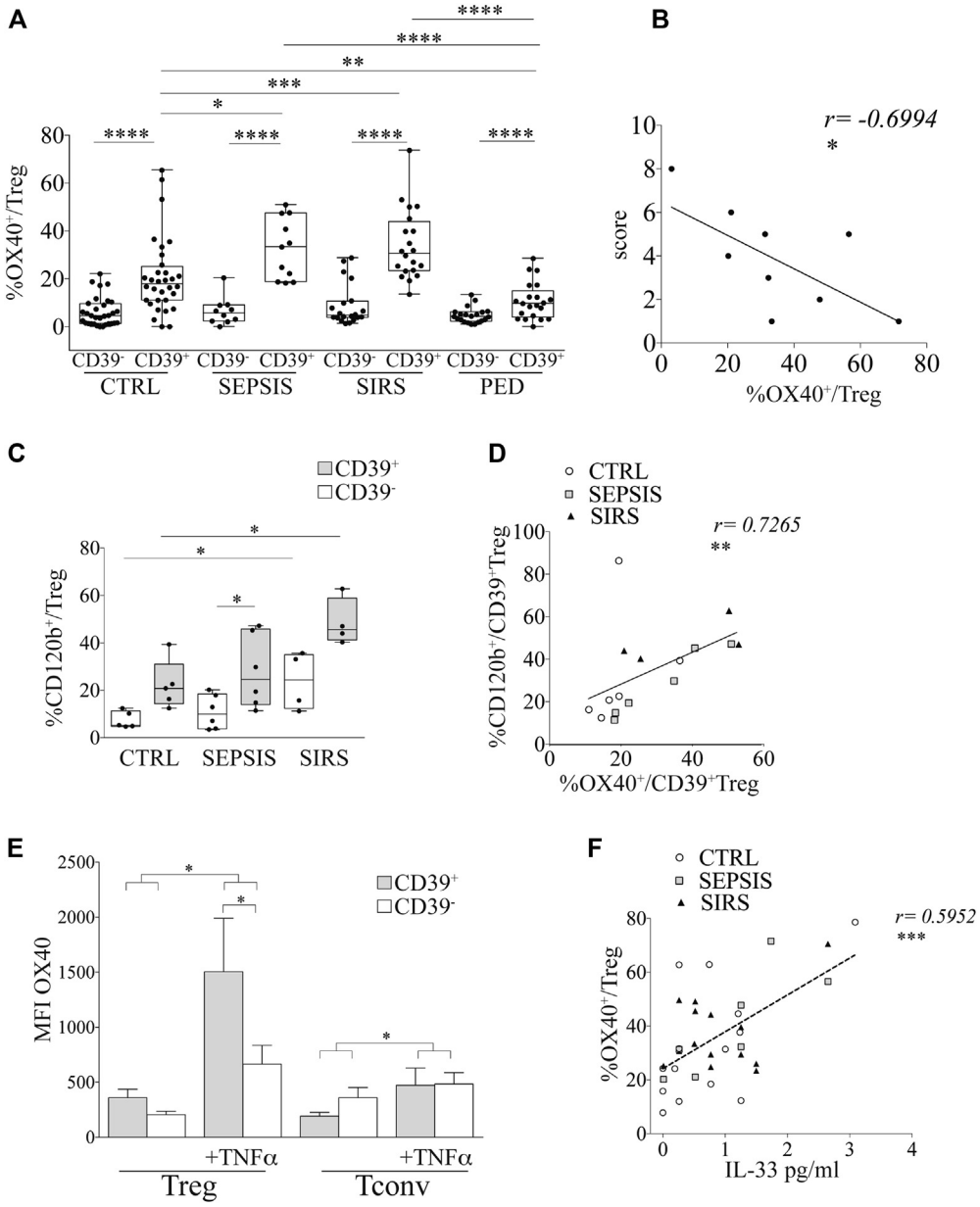

FIG 2. Frequency of OX40$^+$ Treg cells is upregulated in septic neonates and neonates with SIRS and inversely correlates with septic score. **A,** Frequency of OX40$^+$ cells in subdivided CD39$^+$ and CD39$^-$ Treg cell subsets in all groups (CTRLs = 32, neonates with sepsis = 11, neonates with SIRS = 20, and PEDs = 21). *P < .05, **P < .01, ***P < .005, and ****P < .0001, Mann-Whitney test, 2-tailed, and Wilcoxon matched pairs test, 2-tailed. **B,** Spearman correlation *(r)* between frequency of OX40$^+$ Treg cells and clinical severity score in septic neonates (Table I). *P < .05. **C,** Frequency of CD120b$^+$ cells in CD39$^+$ and CD39$^-$ Treg cells (CTRLs = 5, neonates with sepsis = 6, and neonates with SIRS = 4). *P < .05, Mann-Whitney test, 2-tailed, and Wilcoxon matched pairs test, 2-tailed. **D,** Pearson correlation *(r)* between frequency of CD120b$^+$ cells and OX40$^+$ cells within CD39$^+$ Treg cells in all cohorts. **P < .01. **E,** PBMCs from CTRLs were cultured overnight with or without TNF-α. OX40 mean fluorescence intensity was evaluated in Treg and Tconv cells. *P < .05, paired *t* test, 2-tailed. **F,** Spearman correlation *(r)* between frequency of OX40$^+$ Treg cells and IL-33 plasma concentration in all cohorts. ***P < .005.

activating Treg cells in terms of OX40 upregulation *in vitro* by using PBMCs from adult healthy donors. Interestingly, PBMCs treated with IL-33 were able to upregulate OX40 expression at a significantly higher level in total or CD39$^+$ Treg cells than in their Tconv cell counterparts (see Fig E4, *C*). Notably, a positive correlation between IL-33 plasma concentration and the percentage of OX40$^+$ cells in total Treg cells was observed in all neonatal cohorts (Fig 2, *F*).

Taken together, our data support the hypothesis that activated Treg cells, especially OX40$^+$ Treg cells showing CD39$^+$, Helios$^+$, and CD120b$^+$ phenotypes, counteract excessive immunopathology in neonatal sepsis and that their functions are

modulated through prominent inflammatory cytokines, such as TNF-α and IL-33, during infective and not infective SIRS.

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REFERENCES

1. Monneret G, Debard AL, Venet F, Bohe J, Hequet O, Bienvenu J, et al. Marked elevation of human circulating CD4+CD25+ regulatory T cells in sepsis-induced immunoparalysis. Crit Care Med 2003;31:2068-71.
2. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity 2009;30:899-911.
3. Report on the Expert Meeting on Neonatal and Paediatric Sepsis. Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Report/2010/12/WC500100199.pdf. Accessed February 10, 2015.
4. Goldstein B, Giroir B, Randolph A. International Consensus Conference on Pediatric S. International pediatric sepsis consensus conference: definitions for sepsis and organ dysfunction in pediatrics. Pediatr Crit Care Med 2005;6:2-8.
5. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood 2007;110:1225-32.
6. Friedman DJ, Kunzli BM, A-Rahim YI, Berberat J, Berberat PO, Enjyoji K, et al. From the cover: CD39 deletion exacerbates experimental murine colitis and human polymorphisms increase susceptibility to inflammatory bowel disease. Proc Natl Acad Sci U S A 2009;106:16788-93.
7. Piconese S, Timperi E, Pacella I, Schinzari V, Tripodo C, Rossi M, et al. Human OX40 tunes the function of regulatory T cells in tumor and nontumor areas of hepatitis C virus-infected liver tissue. Hepatology 2014;60:1494-507.
8. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J Immunol 2010;184:3433-41.
9. Schiering C, Krausgruber T, Chomka A, Frohlich A, Adelmann K, Wohlfert EA, et al. The alarmin IL-33 promotes regulatory T-cell function in the intestine. Nature 2014;513:564-8.

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METHODS

Study design

The study was conducted at the Bambino Gesù Children's Hospital in Rome between July 2013 and July 2014. It was a prospective observational study involving full-term neonates (≥37 weeks of gestational age) admitted to selected departments. Neonates affected by sepsis or SIRS were enrolled and compared with CTRLs admitted for elective minor surgeries and healthy PEDs aged 2 to 14 years. Septic patients were defined according to the criteria established by an Expert Meeting on Neonatal and Pediatric Sepsis on behalf of the European Medical Agency.[E1]

Neonates who underwent major surgery were enrolled if fulfilling criteria for SIRS, as defined by Goldstein et al.[E2]

CB specimens were obtained from healthy full-term newborns at Policlinico Umberto I Hospital. The study was approved by the local ethics committee, and informed consent was obtained from parents or guardians of all participants.

Demographic and clinical characteristics of enrolled patients

A total of 73 full-term neonates fulfilling the inclusion criteria were enrolled in the study. The median age was 12 days (interquartile range, 6-19 days; Table E1). Thirteen neonates with sepsis and 20 neonates with noninfectious SIRS were compared with 40 CTRLs. Thirteen CB specimens and 21 specimens from PEDs and adult healthy donors were also collected.

Sample collection and processing

PBMCs were obtained from complete blood count residuals (100-500 μL). CB samples were collected from the umbilical vein immediately after delivery. PBMCs were isolated by means of Ficoll density gradient centrifugation from peripheral blood or CB and collected in complete RPMI 1640 medium containing 10% FBS, L-glutamine (2 mmol/L), penicillin (60 μg/mL), streptomycin (100 μg/mL), nonessential amino acids (1×), sodium pyruvate (1 mmol/L), and 2-mercaptoethanol (50 μmol/L). Plasma was obtained from whole blood. PBMCs were analyzed by means of flow cytometry.

Flow cytometry and *in vitro* stimulation

For surface staining, cells were pretreated with Fc-blocking reagent (eBioscience, San Diego, Calif), and then antibodies were added and incubated for 20 minutes at 4°C for surface markers. Intracellular staining was performed according to the manufacturer's instructions for FOXP3 analysis (eBioscience). Data were acquired on an LSR Fortessa (Becton Dickinson, San Jose, Calif) and analyzed with FlowJo software (version 8.8.7; TreeStar, Ashland, Ore) and Spice software (version 5). Antibodies to the following molecules were used. IFN-γ (Alexa Fluor 488, 4S.B3), OX40 (phycoerythrin, Ber-ACT35), CD8 (phycoerythrin-Cy7, SK1), CD39 (BV421, A1), TNF-α (BV421, MAb11), CD4 (BV510, OKT4), and CD14 (allophycocyanin-Cy7 HDC14) were from BioLegend (San Diego, Calif). CD4 (Alexa Fluor 488, OKT4), Helios (allophycocyanin, 22F6), FOXP3 (PerC-Cy5.5 PHC101), CD127 (PE-Cy7, ebioRDR5), CD45RA (eF605, HI100), and CCR7 (PE, 3D12) were from eBioscience. Before IFN-γ and TNF-α staining, cells were stimulated for 4 hours with Cell Stimulation Cocktail (plus protein transport inhibitors, eBioscience).

PBMCs from CTRLs were seeded with anti-CD3 (1 μg/mL, eBioscience) plus TNF-α (50 ng/mL; R&D Systems, Minneapolis, Minn) overnight to assess OX40 induction *in vitro*. PBMCs from adult healthy donors were treated overnight with IL-33 (10 ng/mL, R&D Systems). Flow cytometric analysis was performed in gated Treg and Tconv cells.

DNA extraction, real-time PCR, and genotyping assay

DNA was extracted from frozen PBMCs of 23 CTRLs, 10 neonates with sepsis, 15 neonates with SIRS, 21 PEDs, and 13 CB specimens, according to the manufacturer's protocol (DNeasy Blood and Tissue Kit; Qiagen, Hilden, Germany). Analysis of the SNP (rs10748643) in the *ENTPD1* gene (chromosome 10: 97516764) was performed by using specific TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, Calif). Real-time PCR was performed on a StepOne system (Applied Biosystems).

Clinical score

Using the EMA clinical and laboratory criteria,[E2] we developed a composite score to evaluate the clinical severity of enrolled patients.

Statistical analysis

The 2-tailed paired Student *t* test was used to analyze *in vitro* data. The 2-tailed Mann-Whitney test was applied to compare groups of *ex vivo* samples. Correlations were calculated by using Pearson and Spearman analysis. All data are shown as means ± SEMs. Differences were considered statistically significant in all tests at a *P* value of less than .05. Statistical analysis was performed with Prism software (version 6; GraphPad Software, La Jolla, Calif).

REFERENCES

E1. Report on the Expert Meeting on Neonatal and Paediatric Sepsis. Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Report/2010/12/WC500100199.pdf. Accessed February 10, 2015.

E2. Goldstein B, Giroir B, Randolph A. International Consensus Conference on Pediatric Sepsis. International pediatric sepsis consensus conference: definitions for sepsis and organ dysfunction in pediatrics. Pediatr Crit Care Med 2005;6:2-8.

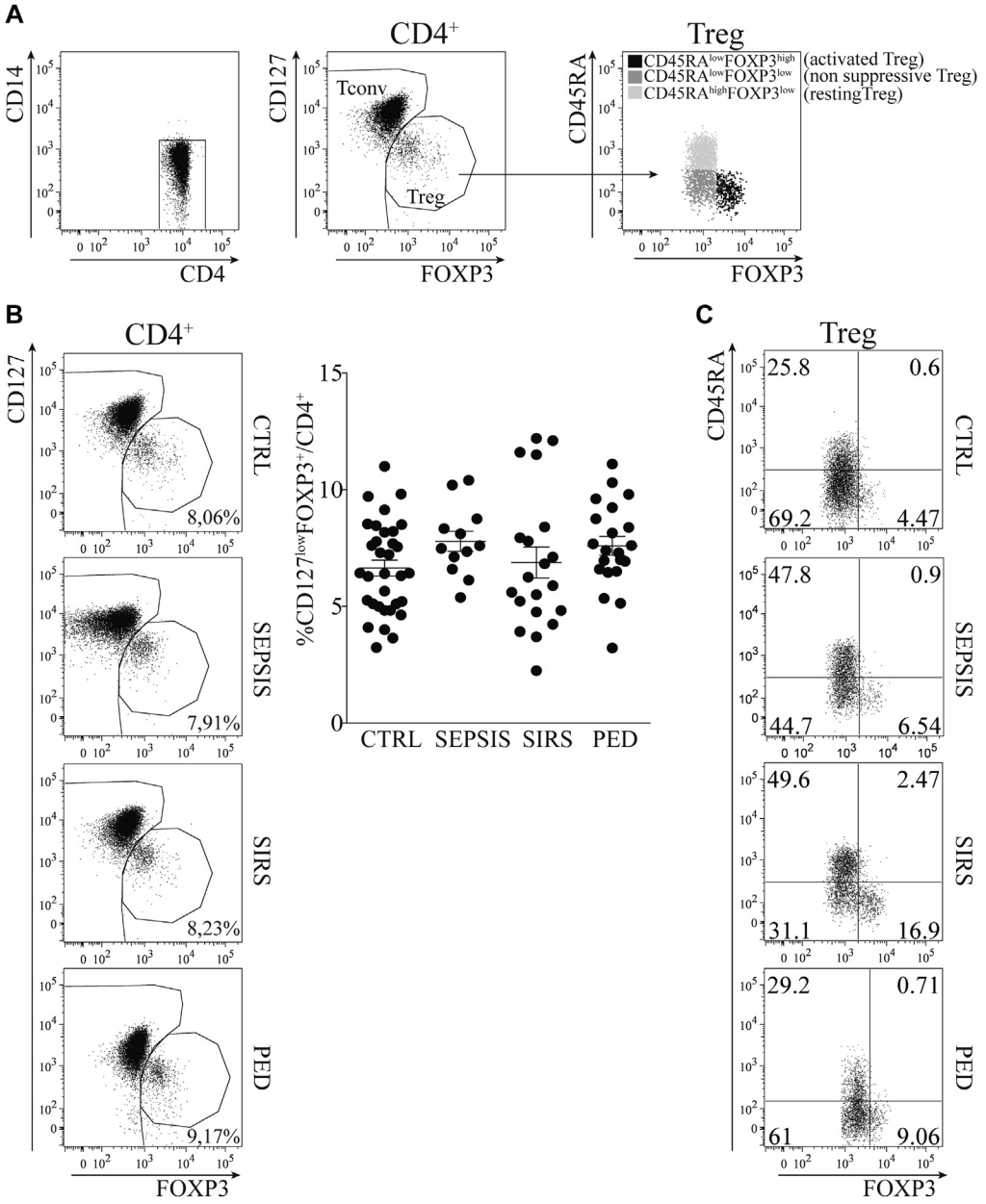

FIG E1. Percentage of Treg and activated Treg cells in neonates with sepsis and those with SIRS. **A,** Representative flow cytometric data of CTRLs indicating the gating strategy. In $CD4^+$ T cells we evaluated $CD127^{low}FOXP3^+$ Treg and $FOXP3^-$ Tconv cells, and within the Treg cell gate, we evaluated $CD45RA^{low}FOXP3^{high}$ activated, $CD45RA^{low}FOXP3^{low}$ nonsuppressive, and $CD45RA^{high}FOXP3^{low}$ resting Treg cell subpopulations. **B,** Representative flow cytometric data showing CD127 versus FOXP3 expression (Treg and Tconv cells) in the CTRL, sepsis, SIRS, and PED cohorts. Treg cell frequency (as a percentage) was estimated by means of flow cytometry as the percentage of $CD127^{low}FOXP3^+$ Treg cells among $CD4^+$ T cells (CTRL = 33, neonates with sepsis = 12, neonates with SIRS = 20, and PEDs = 21). **C,** Representative flow cytometric data showing CD45RA versus FOXP3 within the Treg cell gate in the CTRL, sepsis, SIRS, and PED cohorts.

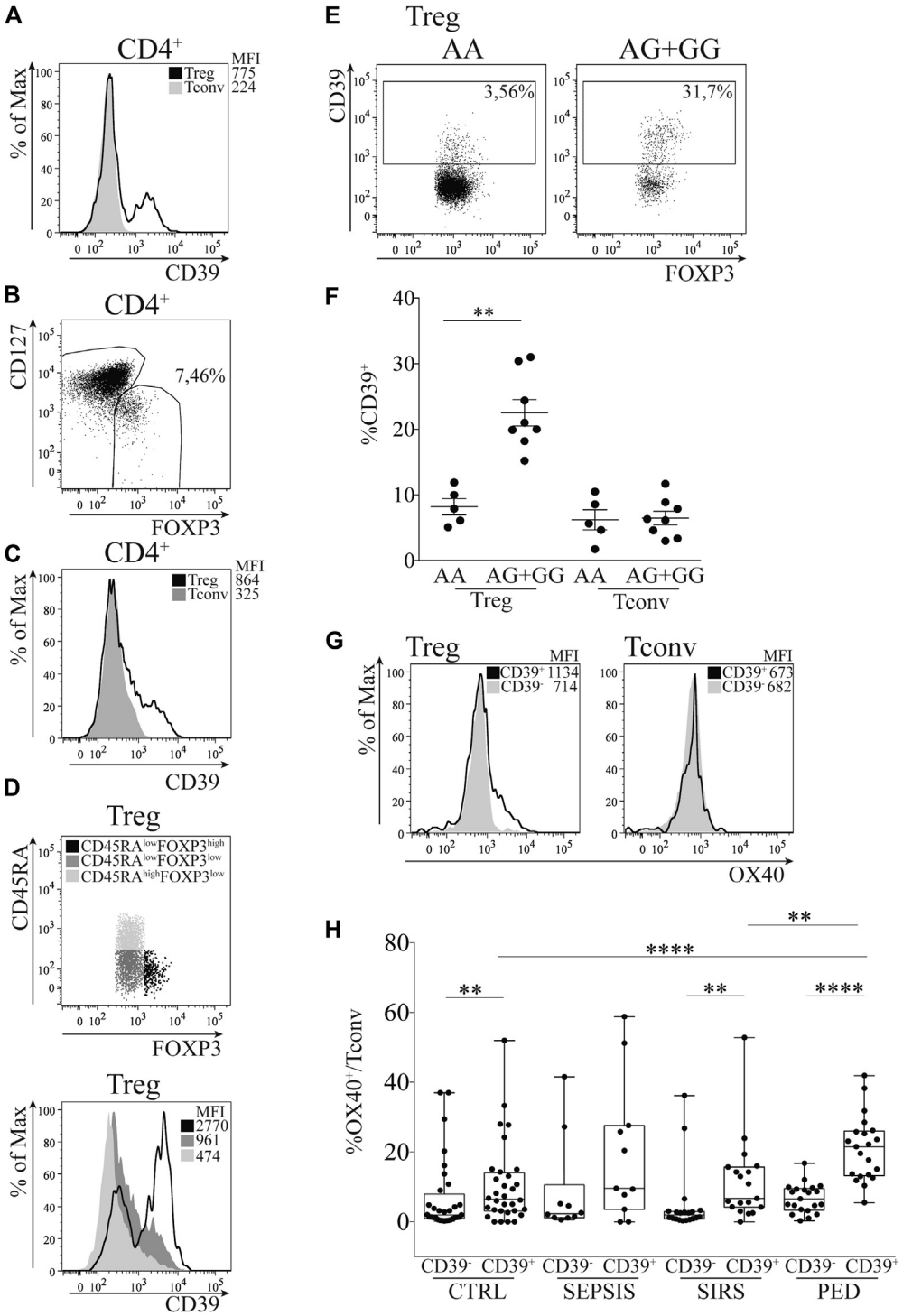

FIG E2. CD39 expression in CB samples and OX40 expression on Treg and Tconv cells. **A,** Representative overlay of CD39 mean fluorescence intensity in gated $CD4^+$ cells on Treg and Tconv cells from the CTRL cohort. **B,** Representative flow cytometric plots of CD127 versus FOXP3 expression in $CD4^+$ T cells from CB samples. **C,** Overlay of CD39 mean fluorescence intensity in Treg and Tconv cells from CB samples. **D,** Representative flow cytometric data showing CD45RA versus FOXP3 *(upper)* and CD39 median fluorescence intensity *(lower)* in activated, resting, and nonsuppressive Treg cell subsets from CB samples. **E,** Representative flow cytometric plots of CD39 versus FOXP3 in gated Treg cells, showing the frequency of $CD39^+$ cells according to AA or AG+GG genotype in the *ENTPD1* gene in CB samples. **F,** Frequency of $CD39^+$ cells within the gate of Treg and Tconv cells according to AA or AG+GG genotype in the *ENTPD1* gene (CB = 13). $^{**}P < .01$, Mann-Whitney test, 2-tailed. **G,** Representative mean fluorescence intensity of OX40 in gated Treg and Tconv cells in $CD39^+$ and $CD39^-$ cells from the CTRL cohort. **H,** Frequency of $OX40^+$ cells in subdivided $CD39^+$ and $CD39^-$ Tconv cell subsets (CTRL = 32, neonates with sepsis = 11, neonates with SIRS = 20, and PEDs = 21). $^{**}P < .01$ and $^{****}P < .0001$, Mann-Whitney test, 2-tailed, and Wilcoxon matched pairs test, 2-tailed.

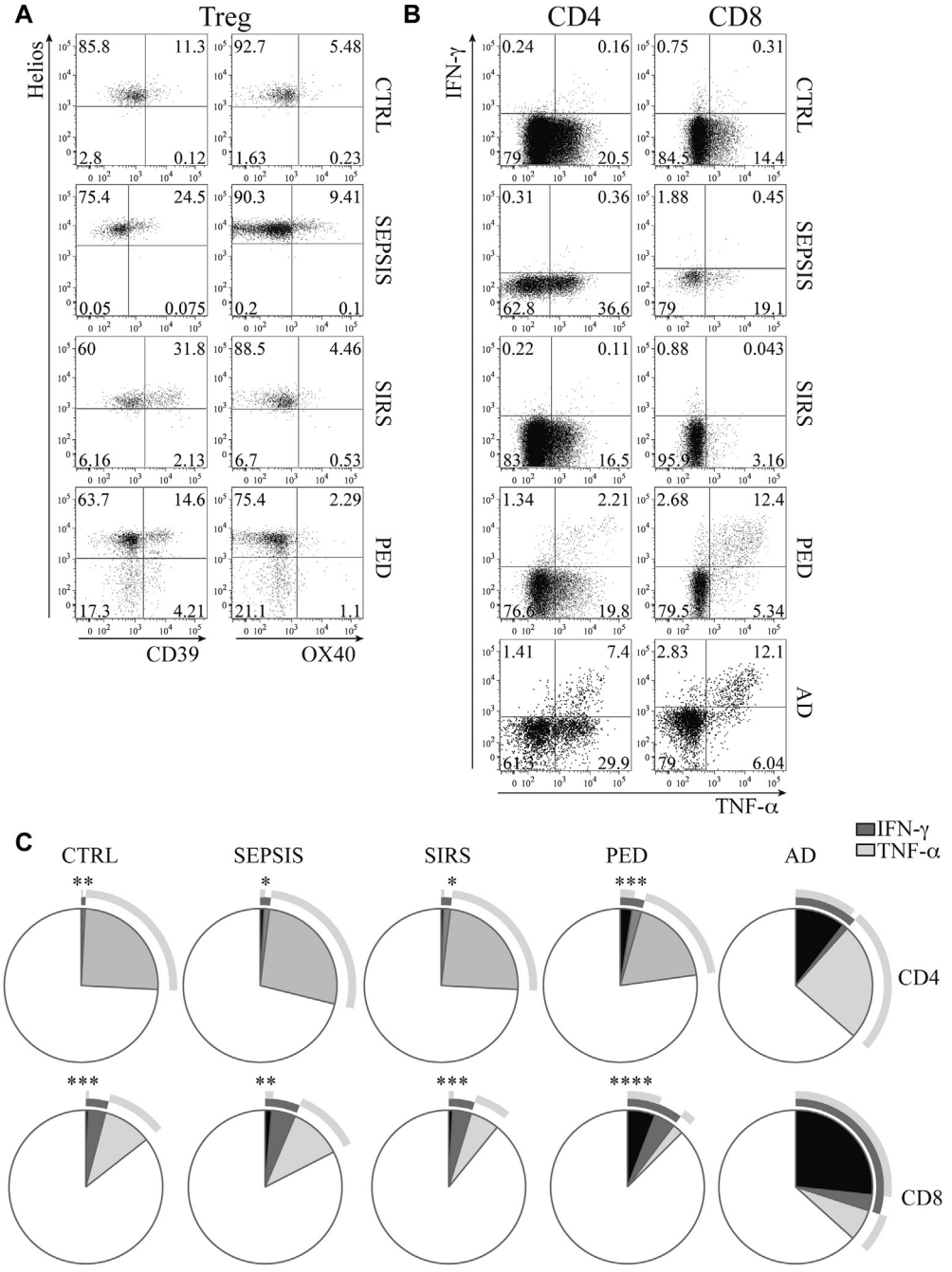

FIG E3. IFN-γ and TNF-α production by T cells. **A,** Representative plots of Helios versus CD39 and versus OX40 in gated Treg cells in the CTRL, sepsis, SIRS, and PED cohorts. **B,** Representative plots of IFN-γ versus TNF-α in gated $CD4^+$ and $CD8^+$ T cells in the CTRL, sepsis, SIRS, PED, and adult healthy donor *(AD)* cohorts. **C,** Representative pie charts (obtained through SPICE analysis) showing proportions of IFN-γ^+ or TNF-α^+ single- or double-producing cells in gated $CD4^+$ and $CD8^+$ T cells in the CTRL, sepsis, SIRS, PED, and AD cohorts. Legend refers to pie chart arcs. $^*P < .05$, $^{**}P < .01$, $^{***}P < .005$, and $^{****}P < .0001$, Student *t* test referred to IFN-γ^+TNF-α^+ double-producing cells, calculated for CTRL, sepsis and SIRS compared with PED group and PED group compared with ADs (CTRL = 12, neonates with sepsis = 4, neonates with SIRS = 14, PEDs = 15, and ADs = 6).

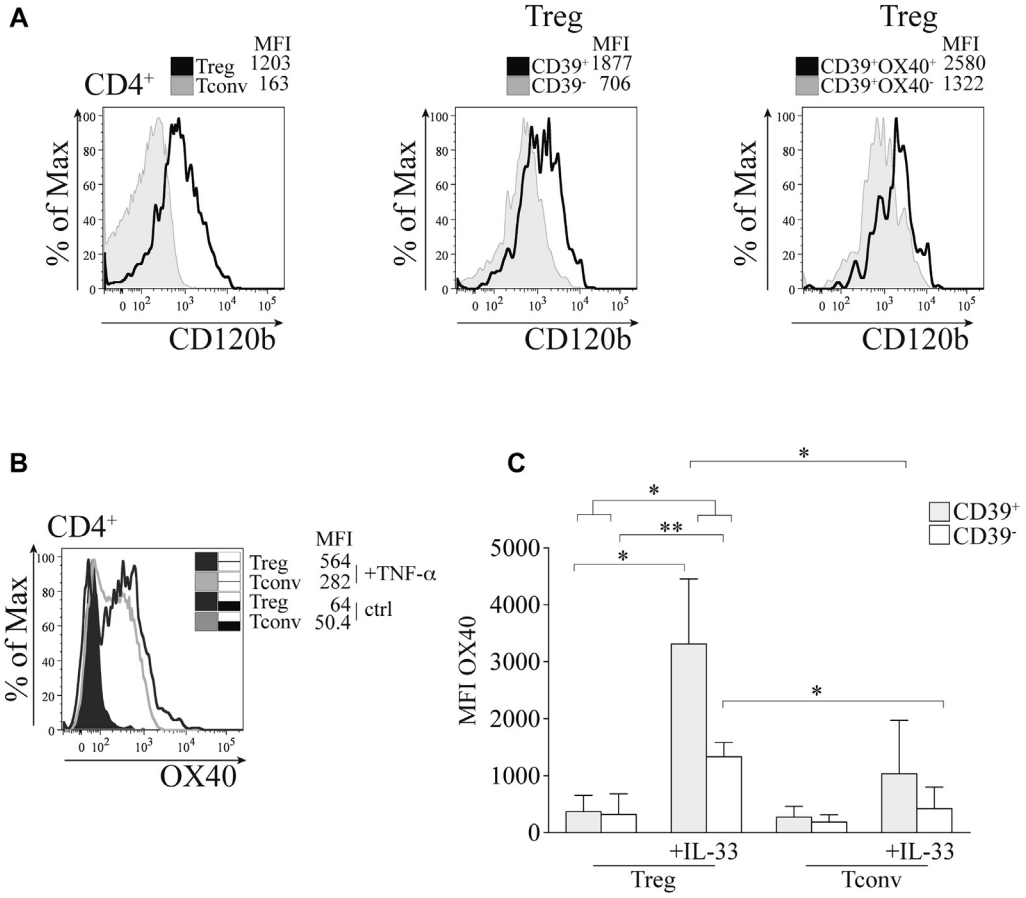

FIG E4. CD120b expression in Treg cells and IL-33 effects. **A,** *Left*, Mean fluorescence intensity of CD120b in Treg and Tconv cells. *Middle*, Overlay of CD120b mean fluorescence intensity in $CD39^+$ and $CD39^-$ Treg cells. *Right*, Overlay of CD120b mean fluorescence intensity in $CD39^+OX40^+$ and $CD39^+OX40^-$ cells within the Treg cell gate. **B,** Representative plot of OX40 mean fluorescence intensity in gated Treg and Tconv cells on overnight TNF-α treatment of PBMCs. **C,** PBMCs from adult healthy donors were cultured overnight with or without IL-33. Mean fluorescence intensity of OX40 was evaluated in $CD39^+$ versus $CD39^-$ Treg and Tconv cells. $*P < .05$ and $**P < .01$, paired *t* test, 2-tailed.

TABLE E1. Demographic and clinical characteristics of the included patients

	Total	Neonates with sepsis	Neonates with SIRS	Healthy control subjects
No.	73	13	20	40
Median age (d [IQR])	12 (6-19)	21 (13-23)	13 (7.5-19)	8.5 (4-14)
Sex				
Male	49	9	13	27
Female	24	4	7	13
Admission ward				
NICU	3	1	—	2
Neonatal other than NICU	33	7	3	23
CICU	23	3	15	5
Cardiology	4	—	2	2
PICU	1	1	—	—
Other pediatric medical	9	1	—	8
Risk factors				
Underlying conditions	51	7	20	24
CVC	31	5	17	9
Previous major surgery	26	4	20	2
Average score ± SD	—	4.8 ± 1.8	1.75 ± 1.0	—
WBC ± SD	12,778 ± 4,030	14,233 ± 4,890	11,452 ± 3,821	12,969 ± 3,724
Lymphocytes ± SD	3,943 ± 2,100	4,114 ± 2,074	2,565 ± 1,495	4,576 ± 2,083
CRP ± SD	2.2 ± 3.4	4.4 ± 3.2	4.9 ± 4.1	0

CICU, Cardiac intensive care unit; *CRP*, C-reactive protein; *CVC*, central venous catheter; *IQR*, interquartile range; *NICU*, neonatal intensive care unit; *PICU*, pediatric intensive care unit; *WBC*, white blood cell count.