

Letter to the Editor

Defective T_H17 development in human neonatal T cells involves reduced RORC2 mRNA content

To the Editor:

The neonatal immune system shows a different response on activation when compared with the adult system.¹ Several mechanisms contribute to this phenomenon, which involves both functioning of the innate system² and adaptive mechanisms of the immune system³ of which the T_H2 bias has been investigated intensively. We previously showed an important role for human regulatory T (Treg) cells⁴ in modulation of immune activation in the neonatal setting, which is induced by the interaction between antigen-presenting cells (APCs) and naive T cells. Especially programmed death-1 ligation by programmed death-L1 contributes significantly to the induction of Treg cells from human cord blood (CB)-derived precursors.⁴

T_H17 and Treg cells have a reciprocal development pathway.⁵ Both cells play an important role in the induction and perpetuation of inflammatory responses during infection, and also in the pathogenesis of autoimmune and allergy-related diseases. On T-cell activation in the presence of TGF- β , both signature transcription factors for T_H17 and Treg-cell lineage are upregulated: retinoid acid-related orphan receptor (ROR) γT for T_H17 cells and forkhead box (FOXP) 3 for Treg cells. In the absence of proinflammatory cytokines, FOXP3 dominates ROR γT function and prevents T_H17 development. Because of the propensity of Treg-cell induction in CB, we hypothesized there to be a regulatory mechanism in CB that inhibits T_H17 cell development in neonatal

T cells derived from CB cells as compared with adult peripheral blood (APB) cells. Activation of $CD4^+CD25^-CD45RO^-$ T cells in the presence of APCs (see this article's Methods section in the Online Repository at www.jacionline.org) shows increased numbers of FOXP3⁺ cells in the CB (shown to be functional Treg cells earlier⁴) than in the APB (Fig 1, A and D). IL-17 production, however, was not observed in the CB (Fig 1, A and B). Replacement of APCs by anti-CD28 antibody prevented T_H17 induction in the APB (see Fig E1, A, in the Online Repository at www.jacionline.org). The lack of IL-17, IL-21, IL-22, and GM-CSF (Fig 1, C) in the supernatants of CB cell cultures confirms that T_H17 phenotype is not induced in the CB after TCR stimulation. Other key cytokines, including IFN- γ and IL-13, were also reduced in CB cell cultures than in APB cells (Fig E1, B), confirming earlier work.⁶ IL-21 and IL-22 in the APB were only partly derived from IL-17-producing cells (Fig E1, C).

T_H17 cells may derive from any naive T cell when activated in a conducive cytokine milieu, or from a defined precursor cell. In humans, $CD161^+CD4^+$ T cells are thought to be such precursors to the T_H17 cell lineage.⁷ It has also been suggested that T_H17 cells more readily develop from $CD45RO^+$ memory T cells.⁸ Therefore, we tested whether memory cell contamination within our naive cells at the start of culture accounts for the increased ability of APB to yield T_H17 cells. Fluorescence-activated cell sorting-sorted populations of $CD25^-CD45RO^-CD161^-$ cells from the APB can still develop into T_H17 cells, whereas the same population in the CB develops into Treg cells (Fig E1, D). Thus, in our culture system with viable APCs present, CB-naive T cells cannot differentiate into T_H17 .

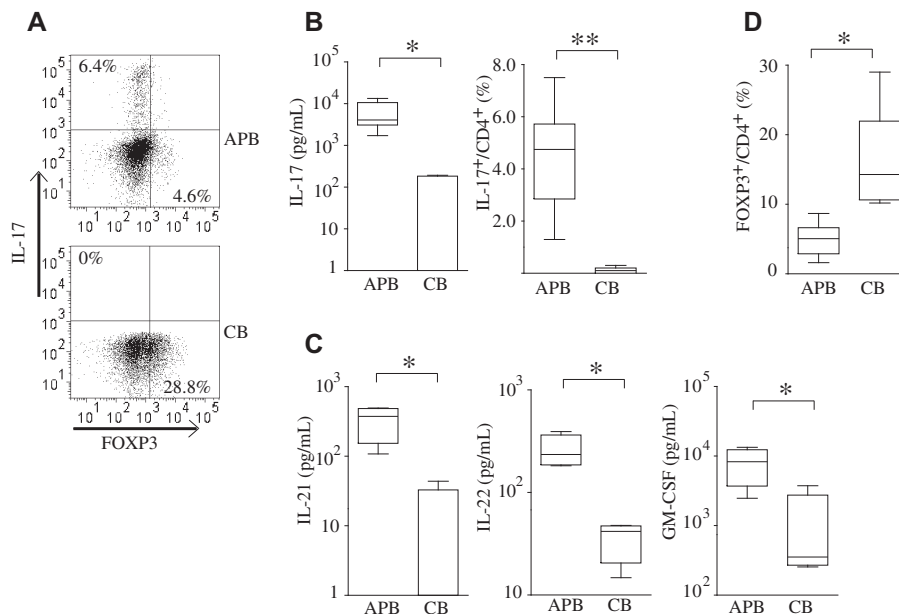


FIG 1. CB T cells are deficient in T_H17 development but do upregulate FOXP3. APB and CB-naive T cells were activated by plate-bound anti-CD3 in the presence of viable APC for 6 days. IL-17⁺ and FOXP3⁺ CD4⁺ T-cell numbers were assessed by flow cytometry. **A**, Representative cytometry plots. **B**, Supernatant IL-17 concentration (n = 8 APB, n = 5 CB). **C**, Supernatant IL-21, IL-22, and GM-CSF (n = 4 APB, n = 5 CB). **D**, Percentage of FOXP3⁺ CD4⁺ cells (n = 8). * $P < .05$, and ** $P < .01$ Mann-Whitney U test.

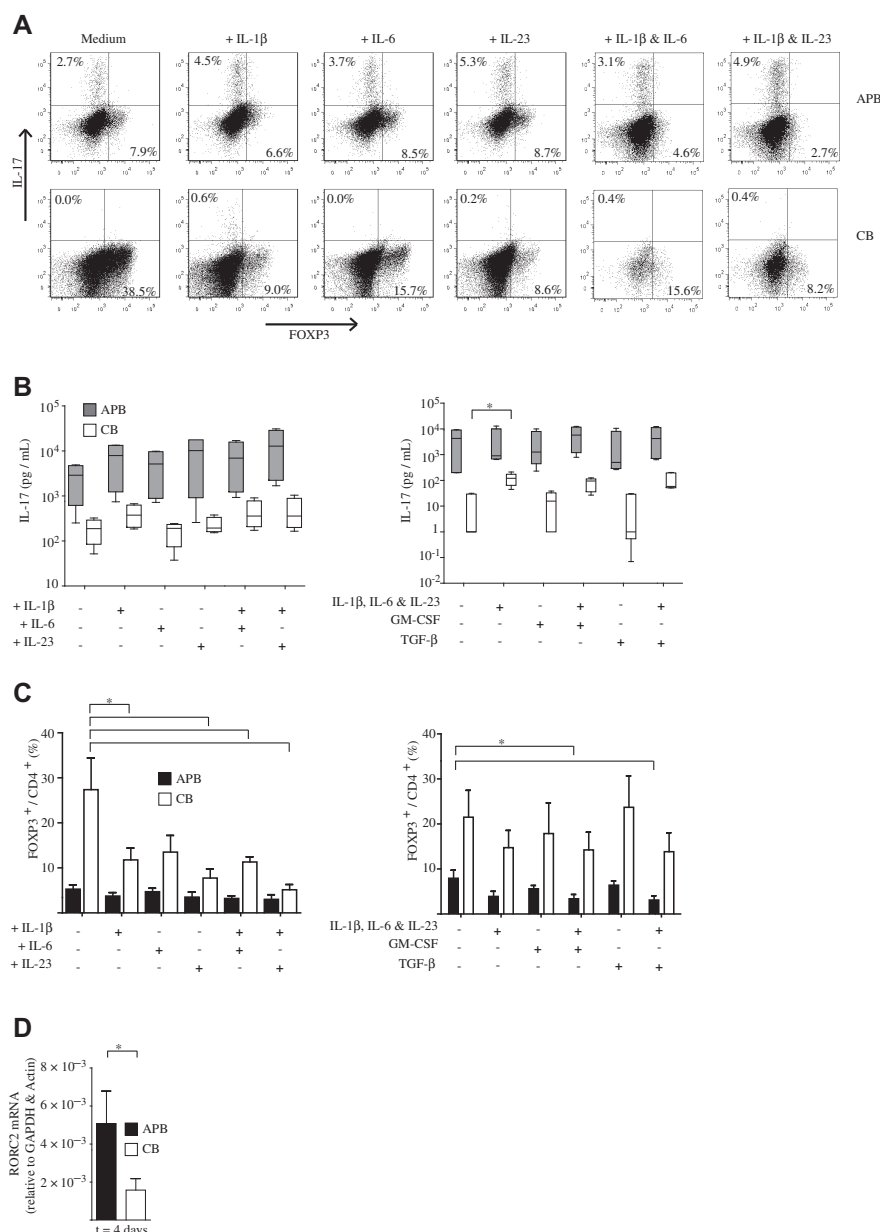


FIG 2. CB T cells cannot be forced to T_H17 development and do not upregulate RORC. APB and CB-naïve T cells were activated by plate-bound anti-CD3 in the presence of viable APC and recombinant cytokines as indicated for 6 days. **A**, Representative cytometry plots. **B**, IL-17 in the culture supernatants ($n = 6$ left plot and $n = 4$ APB, $n = 5$ CB right plot). **C**, Percentage of FOXP3⁺ CD4⁺ cells ($n = 6$ APB, $n = 5$ CB left plot, $n = 4$ APB, $n = 5$ CB right plot). **D**, Relative RORC2 mRNA expression ($n = 4$). * $P < .05$, Kruskal-Wallis with Dunn post hoc (Fig 2, B, C) and Student t test (Fig 2, D).

When IL-6 (mouse) or IL-1 β , IL-6, and IL-23 (human) are present during T-cell activation, FOXP3 is downregulated and ROR γ T enforces T_H17 development. Neonatal innate cells differ in their activation-induced cytokine production, compared with APB cells.² Does this biased cytokine production profile account for the difference in T_H17 development capacity? Indeed, culture supernatants of CB cells show reduced concentrations of both IL-1 β and IL-6 during the culture period of 6 days as compared with APB cultures where these cytokines were readily produced. APB cultures yield an increased concentration of IL-17 at the end of the culture (see Fig E2, A, in the Online Repository at www.jacionline.org).

To further investigate whether the absence of these cytokines in CB cell cultures had a role in the disability of T_H17 development, we added recombinant human cytokines to the cultures of naïve APB and CB CD4⁺ T cells and monitored again both IL-17 production and FOXP3 expression. Addition of IL-1 β , IL-6, and IL-23 resulted in an increased number of IL-17-producing APB T cells without affecting the number of cells that upregulated FOXP3 (Fig 2, A-C). Only the combination of IL-1 β , IL-6, and IL-23 induced IL-17 production in CB cells but never to the extent observed in APB cells. The number of FOXP3⁺ cells was reduced when these cytokines were included alone or in

combination in the cultures. Addition of GM-CSF or TGF- β did not clearly affect IL-17 production alone, nor in combination with the inflammatory cytokines (Fig 2, B). The combination of inflammatory cytokines and these cytokines did show a reduced FOXP3 expression in APB cells. Thus, CB T cells respond to the cytokines as shown by reduced FOXP3 expression and the increase in IL-10 and IFN- γ production (Fig E2, B), suggesting that the lack of T_H17 differentiation in these experiments is not due to the absence of the specific receptors for these cytokines.

As the development of T_H17 cells inversely correlates to the development of Treg cells,⁵ we considered that the induction of T_H17 cells in the APB could be responsible for the diminished induction of Treg cells in APB cells as compared with CB cells. Pharmacologic inhibition of proinflammatory cytokine signaling, through the addition of IL-1RA- or IL-6R- blocking antibody (anakinra and tocilizumab, respectively), decreased the number of IL-17-producing T cells and lowered concentrations of supernatant IL-17 (Fig E2, C-E). Nevertheless, the number of APB cells expressing FOXP3 after 6 days of culture remained stable and was not affected by these treatments. Because CB cell cultures did not produce IL-1 β and IL-6, blockade of the signaling pathways did not affect the percentages of IL-17⁺ or FOXP3⁺ T cells.

Finally, as CB precursor cells did not form T_H17 cells, while we did see a signaling effect of inflammatory cytokines, we hypothesized that the blockade in T_H17 differentiation should be upstream of the transcription of IL-17 but downstream of cytokine signaling. We therefore analyzed the T_H17 defining transcription factor ROR γ T, in humans encoded by transcript variant 2 of the *RORC* gene.^{9,10} Indeed, quantitative PCR of *RORC2* mRNA after 3 and 4 days of activation shows significantly lower *RORC2* mRNA content in CB as compared with APB T cells (Fig 2, D), resulting in a reduced IL-17 mRNA (Fig E2, F) and prevention of IL-17 production.

We propose that neonatal T cells have an intrinsic mechanism that prevents T_H17 differentiation through the regulation of ROR γ T expression. This may be of functional relevance to prevent adverse immune reactions against self or nonpathogenic microbes during the establishment of T-cell repertoire and bacterial colonization. Exact mechanisms preventing *RORC* transcription in CB cells are not known yet but might be found in upstream signaling or DNA methylation and histone acetylation. Appropriate activation, for example, via innate signaling, can overcome this deficiency.¹

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REFERENCES

1. Schaub B, Liu J, Schleich I, Höppler S, Sattler C, Von Mutius E. Impairment of T helper and T regulatory cell responses at birth. *Allergy* 2008;63:1438.
2. Belderbos ME, van Bleek GM, Levy O, Blanken MO, Houben ML, Schuijff L, et al. Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life. *Clin Immunol* 2009;133:228-37.
3. Wang G, Miyahara Y, Guo Z, Khattar M, Stepkowski SM, Chen W. "Default" generation of neonatal regulatory T cells. *J Immunol* 2010;185:71-8.
4. de Roock S, Hoeks SBEA, Meurs L, Steur A, Hoekstra MO, Prakken BJ, et al. Critical role for programmed death 1 signaling and protein kinase B in augmented regulatory T-cell induction in cord blood. *J Allergy Clin Immunol* 2011;128:1369.
5. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006;441:235.
6. Nitsche A, Zhang M, Clauss T, Siegert W, Brune K, Pahl A. Cytokine profiles of cord and adult blood leukocytes: differences in expression are due to differences in expression and activation of transcription factors. *BMC Immunol* 2007;8:18.
7. Cosmi L, De Palma R, Santarlasci V, Maggi L, Capone M, Frosali F, et al. Human interleukin 17-producing cells originate from a CD161⁺CD4⁺ T cell precursor. *J Exp Med* 2008;205:1903-16.
8. van Beelen AJ, Zelinkova Z, Taanman-Kueter EW, Muller FJ, Hommes DW, Zaat SAJ, et al. Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. *Immunity* 2007;27:660.
9. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelletier A, Lafaille JJ, et al. The orphan nuclear receptor ROR γ T directs the differentiation program of proinflammatory IL-17⁺ T helper cells. *Cell* 2006;126:1121.
10. Unutmaz D. RORC2: the master of human Th17 cell programming. *Eur J Immunol* 2009;39:1452.

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METHODS

CB samples were obtained from normal deliveries of full-term neonates (approved by the Medical Ethical Committee of the University Medical Center Utrecht). Following ethical guidelines of our hospital, no further information (eg, exact gestational age or sex) on the CB samples was provided, which limited the selection criteria for these samples. Adult samples were collected from healthy volunteers, aged between 21 and 35 years. Mononuclear cells from CB and APB were isolated by using Ficoll-Isopaque (Pharmacia, Uppsala, Sweden). Naive T cells and APCs were isolated by using magnetic cell sorting (CD4-T-cell enrichment and CD25⁺CD45RO⁺ T-cell depletion and CD3⁺ cell-depletion, respectively; BD Bioscience, San Jose, Calif, cell purity after magnetic cell sorting >95%) or by fluorescence-activated cell sorting by using ARIA II. A total of 50,000 T cells were cultured for 6 days with 100,000 autologous, nonirradiated (viable) APCs or 1 μ g/mL anti-CD28 (eBioscience, San Diego, Calif), activated with 1 μ g/mL plate-bound anti-CD3 (OKT3, eBioscience) and 30 units recombinant human IL-2/mL (Novartis, Arnhem, The Netherlands) in RPMI 1640 (Gibco, Breda, The Netherlands) supplemented with 10% human AB serum (Sanquin, Amsterdam, The Netherlands), 1% L-glutamine (Gibco), and 0.5% penicillin-streptomycin (Gibco), restimulated with phorbol 12-myristate 13-acetate (20 ng/mL) and ionomycin

(1 μ g/mL) in the presence of monensin (GolgiStop, BD Bioscience) for 4 hours and subsequently analyzed for IL-17 (eBio64DEC17, eBioscience, phycoerythrin-conjugated) and FOXP3 (PHC101, eBioscience, eFluor450-conjugated) expression by using flow cytometric analysis. In other experiments, IL-21 (clone 3A3-N2) and IL-22 (clone 22URTI) production was also analyzed by flow cytometry.

Recombinant IL-1 β (Miltenyi Biotec, Bergisch Gladbach, Germany; 10 ng/mL), IL-6 (50 ng/mL), IL-23 (BD Bioscience, 10 ng/mL), GM-CSF (Immunotools, Friesoythe, Germany; 500 units/mL), and TGF- β (Peprotech, NJ, 5 ng/mL) were added to the cultures. Signaling of IL-1 β and IL-6 was blocked with IL-1RA anakinra (Kineret) or IL-6 receptor blocker tocilizumab (RoActemra), both added as 1 μ g/mL.

Culture supernatants were analyzed for cytokine production by multiplex analysis, and cultured cells were analyzed for *RORC2* and *IL-17* mRNA expression. Detection limits for luminex were (lower limit-upper limit, pg/mL) as follows: IL-1 β (0.6-4141), IL-6 (2.4-22643), IL-13 (0.43-10173), IL-17 (0.57-9940), IL-21 (12.2-87363), IL-22 (0.8-2294), IFN- γ (6.24-20733), and GM-CSF (2.62-40413).

Significance of the differences was tested by using Prism 5.0 for Windows. Specific tests are mentioned in the figure legends.

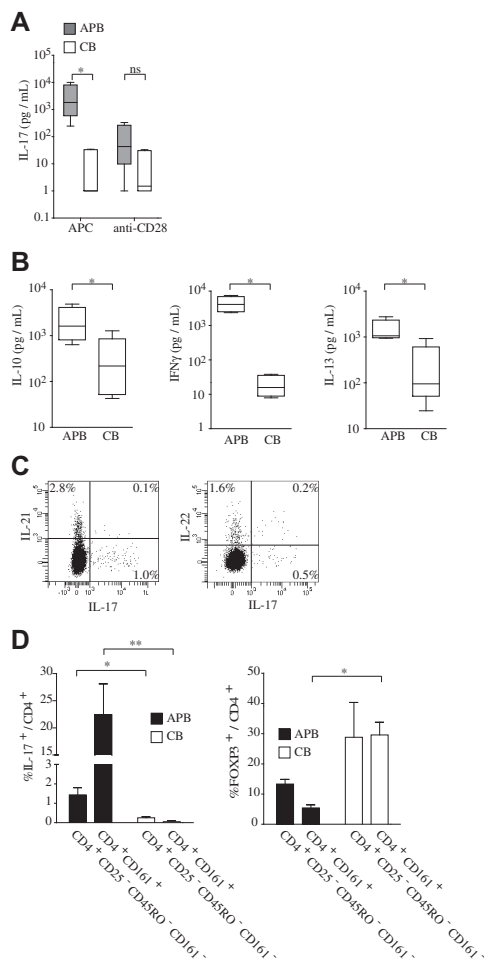


FIG E1. A, Culture supernatant IL-17 concentration after culture for 6 days with anti-CD3 and APCs or with APCs replaced by anti-CD28. $n = 4$ for APB and 5 for CB. **B,** IL-10, IFN- γ , and IL-13 concentration in APB and CB cell culture supernatants. APB and CB-naïve T cells were cultured with APCs and plate-bound anti-CD3 for 6 days. Supernatants were subsequently analyzed for cytokine. $n = 4$ for APB and 5 for CB. **C,** Flow cytometric analysis of IL-17 and IL-21 or IL-22 production in APB cells. Naïve T cells were cultured with APCs and plate-bound anti-CD3 for 6 days, restimulated with phorbol 12-myristate 13-acetate, ionomycin, and monensin and subsequently analyzed for production of IL-17, IL-21, and IL-22. Representative for $n = 5$. **D,** CD161 expression does influence T_H17 development in CB. APB and CB T cells were sorted as $CD25^-CD45RO^-CD161^-$ or $CD4^+CD161^+$ and subsequently activated by anti-CD3 in the presence of viable APCs without addition of recombinant cytokines. *Left plot,* APB $CD161^+$ cells become IL-17 producers rather than APB $CD161^-$ cells. CB cells do not produce IL-17 regardless of their CD161 expression. *Right plot,* CB T cells upregulate FOXP3 independently of CD161 expression. $n = 3$. * $P < .05$ and ** $P < .01$, Mann-Whitney U Test.

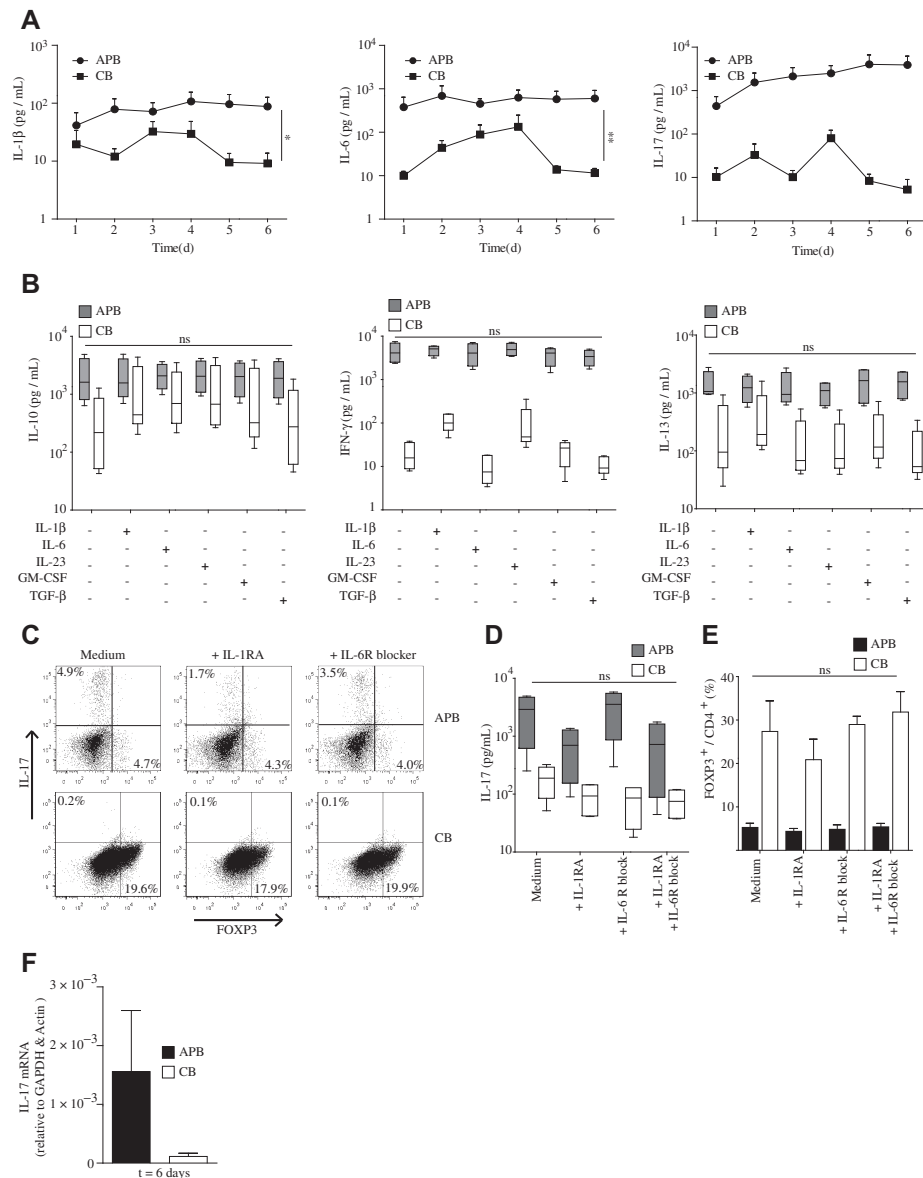


FIG E2. A, CB cell cultures produce low amounts of proinflammatory cytokines. APB and CB-naïve T cells were activated by anti-CD3 in the presence of viable APCs. Culture supernatants were harvested from separate wells daily and analyzed for IL-1 β , IL-6, and IL-17 concentration. $n = 4$. B, Addition of recombinant human cytokines does not influence APB cell culture supernatant concentration of IL-10, IFN- γ , and IL-13, but does so in CB cell cultures. $n = 4$ for APB and 5 for CB. C, Blockade of proinflammatory cytokine signaling in CB cells does not affect FOXP3 upregulation. APB and CB-naïve T cells were activated by anti-CD3 in the presence of viable APC and blocking antibodies toward IL-1 β (1 μ g/mL anakinra) and IL-6 (1 μ g/mL tocilizumab) signaling. After 6 days, IL-17-producing and FOXP3-expressing cells were assessed by flow cytometry. Representative cytometry plots for $n = 4$. D, IL-17 concentration in the culture supernatants after 6 days of culture, $n = 4$. E, Percentage of FOXP3-expressing CD4 T cells. $n = 4$. F, IL-17 mRNA content is lower in CB cells than in APB cells after 6 days of culture. $n = 4$. * $P < .05$, ** $P < .01$, *** $P < .001$, Student t test of the area under the curve (Fig E2, A), Kruskal-Wallis with Dunn *post hoc* (Fig E2, B and D, comparing the effect of blocking antibodies to medium control) and Student t test (Fig E2, F).