

Letter to the Editor

Critical role for programmed death 1 signaling and protein kinase B in augmented regulatory T-cell induction in cord blood

To the Editor:

In newborns the induction of productive immune responses is generally blunted in comparison with adults, resulting in tolerogenic immune reactivity.¹ This immune status results in an increased potency to engraft neonatal animals, ineffective vaccination responses in newborns, and reduced occurrence of graft-versus-host disease when cord blood (CB)-derived allografts are used.^{2,3} We hypothesized that forkhead box protein 3 (FOXP3)-positive regulatory T (Treg) cells are pivotal in this phenomenon because these cells are key players in immune homeostasis.⁴ When comparing the number of FOXP3⁺ cells in human CB and adult peripheral blood (APB), we found less FOXP3⁺ cells in CB than APB (Fig 1, A, *ex vivo*, uncultured cells). However, when naive (CD25⁻CD45RO⁻) T cells were activated by plate-bound anti-CD3, significantly more FOXP3⁺ T cells were induced from CB precursors. Remarkably, we only observed this difference between CB and APB when viable antigen-presenting cells (APCs; T cell-depleted CB or APB mononuclear cell fraction) were included in the culture; replacement with irradiated APCs abrogated the effect, whereas costimulation by soluble anti-CD28 showed a less pronounced difference (Fig 1, A). Induced CB FOXP3⁺ T cells (sorted as CD4⁺CD25⁺CD127^{low} cells) were able to suppress dose dependently the proliferation of both CD4 and CD8 effector T cells when cultured together in different ratios (not shown), confirming the Treg nature of these cells. Thus on the first activation, CB T cells have a tendency to become functional FOXP3⁺ Treg cells.

To substantiate the role of CB APCs in the induction of high percentages of Treg cells, we activated naive T cells in an alloreaction with APCs. Again we observed a higher number of Treg cells when CB T cells were cultured. CB APCs were able to induce higher numbers of Treg cells than APB APCs from both CB and APB precursor T cells (Fig 1, B). Nevertheless, CB T cells always contained higher percentages of Treg cells, indicating a T-cell intrinsic mechanism as well.

We investigated the percentages of different APC populations because the APCs used in our previous experiments consisted of a mixture of different cell types. In both APB and CB, we found comparable numbers of conventional dendritic cells (HLA-DR⁺CD11c⁺) and plasmacytoid dendritic cells (HLA-DR⁺CD123⁺), with slightly more CD14⁺ monocytes in APB (not shown). When we sorted different subsets as APCs and cultured them with anti-CD3-activated T cells, we observed that all APC subsets induced more Treg cells from CB than from APB precursor cells (Fig 1, C).

The augmented Treg cell induction was not correlated with a reduced proliferation of CB T cells, which we showed to be identical in CB and APB by using carboxyfluorescein succinimidyl ester dilution assays (data not shown). Moreover, we found no difference in the kinetics of FOXP3 upregulation when we measured the percentage of FOXP3⁺ cells daily; both APB and CB showed a peak of FOXP3⁺ cells around 4 days of culture, after which the percentage of FOXP3⁺ cells decreased to stable

expression at day 6 (Fig 1, D). Both APB and CB cells had a similar stable FOXP3-expressing population compared with the initial peak around day 4; 65% of the FOXP3⁺ T cells at day 4 remain stable FOXP3-expressing cells during the 8-day culture.

The induction of Treg cells in the periphery on activation can be mediated by several factors. Cytokines like TGF- β and IL-2 are known to induce FOXP3,⁵ whereas inflammatory cytokines prevent this. To test whether possible differences in secreted mediators, such as cytokines and chemokines, can explain the difference in Treg cell induction, we exchanged culture supernatants between CB and APB every day during the 6-day culture period of naive T cells with APCs and anti-CD3. This approach did not result in altered differences between CB and APB Treg cell induction, making a role for secreted mediators unlikely (not shown).

Another factor influencing FOXP3 induction is the strength of the T-cell receptor (TCR) signal and costimulation.^{6,7} In our settings the TCR signal was standardized by plate-bound anti-CD3, but costimulation is provided by the APCs. High costimulation through CD28 signaling prevents the upregulation of FOXP3. We therefore investigated the role of the CD28 ligands CD80 and CD86 on APCs. We observed no difference in the expression of these molecules. When blocking antibodies toward CD80, CD86, or both were added to the cultures, this did not result in a difference in FOXP3 expression (Fig 2, A). This finding was confirmed by adding increasing concentrations of cytotoxic T lymphocyte-associated antigen 4-immunoglobulin (CTLA4-Ig) to the cultures, blocking both CD80 and CD86, which had no obvious effect (data not shown).

Inhibition of downstream signaling pathways of TCR and CD28 on T-cell activation is able to promote FOXP3 induction as well. The phosphoinositide 3-kinase/protein kinase B (PKB) pathway is central in T-cell differentiation.⁸ Prevention of activation of this pathway by limited costimulation or signaling through inhibitory molecules, such as programmed death 1 (PD-1), promotes FOXP3 upregulation.^{7,9} We hypothesized that differential downstream signaling on activation might be pivotal to the high levels of FOXP3 found on day 6 of activation and onward (Fig 1, D). We therefore investigated the role of PD-1 and PKB in the induction of FOXP3 in CB-naive T cells. On activation, CB CD4⁺ T cells have an increased expression of the PD-1 molecule compared with APB (Fig 2, B). When we blocked the interaction between PD-1 and its ligand PD-L1 by including a blocking mAb to PD-L1 in the culture, a significant reduction in the percentage of FOXP3⁺ T cells was observed (Fig 2, C), isotype mAb had no effect (not shown). A PD-1-blocking mAb showed comparable results. As a consequence, the phosphorylation status of PKB on activation by different concentrations of anti-CD3 was lower for CB than APB cells (Fig 2, D). Additionally, CB T cells required approximately 10-fold more TCR triggering than APB T cells before an increase in PKB phosphorylation was observed.

Shortly after birth, the immune system of the newborn encounters all kinds of neoantigens. The low percentages of Treg cells found *ex vivo* in CB are not likely to play a major role in maintaining tolerance to these neoantigens. However, we here show that PD-1 signaling in CB T cells facilitates their differentiation into induced functional FOXP3⁺ Treg cells through a

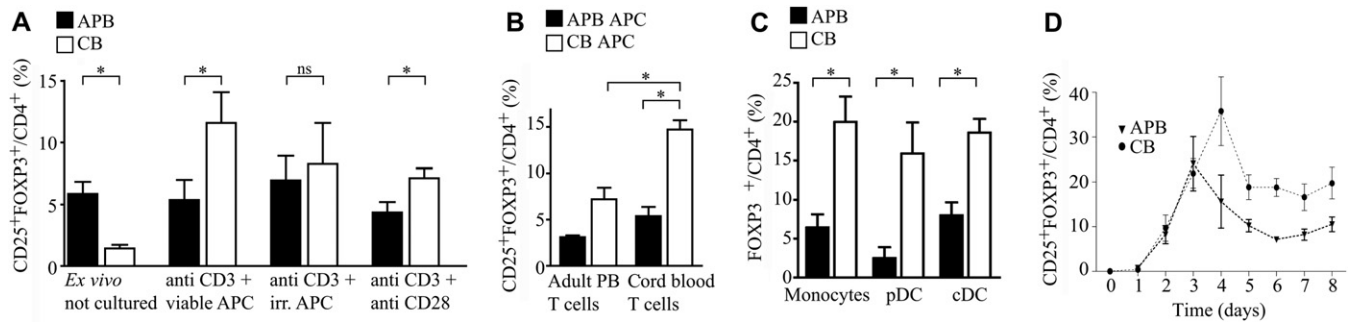


FIG 1. CB APCs stimulate Treg induction. **A**, FOXP3⁺ Treg cell percentages *ex vivo* or after 6-day naive (CD4⁺CD25⁺CD45RO⁺) T-cell stimulation, as indicated in the figure (left to right, $n = 3, 6, 4$, and 10). *ns*, Not significant. **B**, Treg cell percentage after 6-day alloreaction between naive T cells and APCs ($n = 4$). **C**, Treg cell percentage of anti-CD3-activated naive T cells after 6-day culture in the presence of sorted indicated APCs (left to right, $n = 5, 3$, and 3). *cDC*, Conventional dendritic cells; *pDC*, plasmacytoid dendritic cells. **D**, Time course of Treg cell expansion of anti-CD3-activated naive T cells cultured with viable APCs ($n = 4$). * $P \leq .05$.

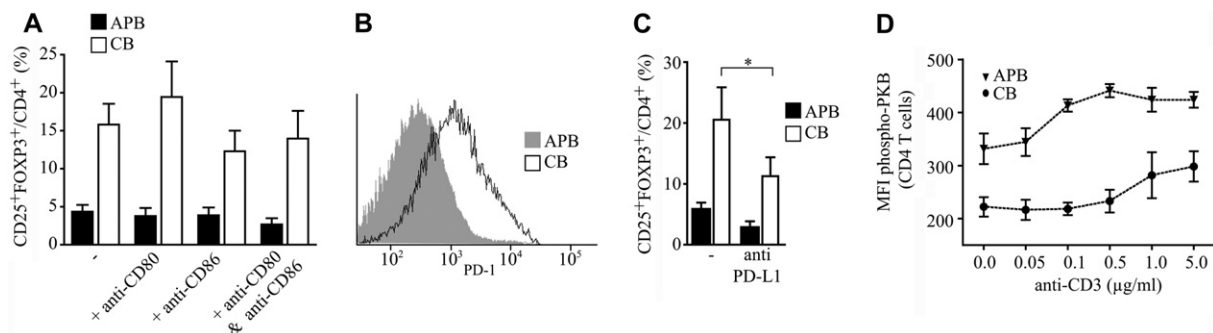


FIG 2. PD-1/PD-L1 interaction stimulates Treg cell induction. **A**, Treg cell percentage after 6-day stimulation of naive T cells in the presence of anti-CD3/viable APCs with blocking mAb as indicated ($n = 6$). **B**, PD-1 expression on 6-day anti-CD3-activated T cells in the presence of viable monocytes. **C**, Treg cell percentages of anti-CD3-activated naive T cells with viable monocytes in the presence of blocking mAb (CB, $n = 11$; APB, $n = 3$). **D**, Relative phosphorylated PKB levels in CD4⁺ T cells cultured with a graded amount of plate-bound anti-CD3 ($n = 3$). * $P \leq .05$.

mechanism involving reduced PKB signaling. This phenomenon might well represent a mechanism that is developed to ensure active tolerance in the neonatal immune system.

Sytze de Roock, MSc^{a*}

Sanne B. E. A. Hoeks, MD^{a*}

Linda Meurs^a

Anouk Steur^a

Maarten O. Hoekstra, MD, PhD^b

Berent J. Prakken, MD, PhD^a

Marianne Boes, PhD^a

Ismé M. de Kleer, MD, PhD^{a,c}

From ^athe Center for Molecular and Cellular Intervention CMCI, Wilhelmina Children's Hospital/UMC Utrecht, Utrecht, The Netherlands; ^bUniversity Medical Center Nijmegen St Radboud, Nijmegen, The Netherlands; and ^cthe Laboratory of Immunoregulation and Mucosal Immunology, Ghent University, Ghent, Belgium. E-mail: ikleer@umcutrecht.nl.

*These authors contributed equally to this work.

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