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# "Default" Generation of Neonatal Regulatory T Cells

**Guohua Wang,*,†,[1] Yoshihiro Miyahara,*,[1] Zhiyong Guo,*,‡,[1] Mithun Khattar,* Stanislaw M. Stepkowski,* and Wenhao Chen***

**$CD4^+Foxp3^+$ regulatory T (Treg) cells were shown to control all aspects of immune responses. How these Treg cells develop is not fully defined, especially in neonates during development of the immune system. We studied the induction of Treg cells from neonatal T cells with various TCR stimulatory conditions, because TCR stimulation is required for Treg cell generation. Independent of the types of TCR stimulus and without the addition of exogenous TGF-β, up to 70% of neonatal $CD4^+Foxp3^-$ T cells became $CD4^+Foxp3^+$ Treg cells, whereas generally <10% of adult $CD4^+Foxp3^-$ T cells became $CD4^+Foxp3^+$ Treg cells under the same conditions. These neonatal Treg cells exert suppressive function and display relatively stable Foxp3 expression. Importantly, this ability of Treg cell generation gradually diminishes within 2 wk of birth. Consistent with in vitro findings, the in vivo i.p. injection of anti-CD3 mAb to stimulate T cells also resulted in a >3-fold increase in Treg cells in neonates but not in adults. Furthermore, neonatal or adult $Foxp3^-$ T cells were adoptively transferred into $Rag1^{-/-}$ mice. Twelve days later, the frequency of $CD4^+Foxp3^+$ T cells converted from neonatal cells was 6-fold higher than that converted from adult cells. Taken together, neonatal $CD4^+$ T cells have an intrinsic "default" mechanism to become Treg cells in response to TCR stimulations. This finding provides intriguing implications about neonatal immunity, Treg cell generation, and tolerance establishment early in life.** ***The Journal of Immunology*****, 2010, 185: 71–78.**

In 1995, Sakaguchi et al. (1) identified CD25 as a cell surface marker of $CD4^+$ regulatory T (Treg) cells. This finding led to our current understanding that thymus-derived $CD4^+CD25^+$ Treg cells maintain immunologic self-tolerance and negatively regulate various immune responses. In 2003, forkhead box P3 (Foxp3) was discovered as a master transcription factor determining the $CD4^+CD25^+$ Treg cell lineage (2–4). It was also found that stable expression of Foxp3 is predominantly restricted to Treg cells (2–4) and that sustained Foxp3 expression is required to maintain their phenotype and suppressor function (5, 6). In contrast, unstable expression of Foxp3 in Treg cells could lead to the generation of pathogenic memory T cells (7).

Similar to other T cell lineages, Treg cell commitment requires TCR engagement (reviewed by Josefowicz and Rudensky in Ref. 8). Yet, it is unclear whether TCR- and CD28-derived signals are sufficient to induce Foxp3 expression in thymocytes. Recently, a two-step model of thymic Treg cell generation revealed that TCR/ligand interactions result in elevated CD25 expression on $CD4^+Foxp3^-$ cells, followed by IL-2 signals that subsequently induce Foxp3 expression in some of these $CD4^+CD25^+Foxp3^-$ Treg precursor cells (9, 10). Therefore, IL-2 signaling through Stat5 is essential for Treg cell generation and survival. TGF-β–induced Smad signals also have a profound effect on thymic Treg cell generation, because ablation of TGF-β or its cognate receptors results in deficiency of thymic $CD4^+Foxp3^+$ cells during the first week of life (11–13). In addition, TGF-β seems to be critical for extrathymic generation of inducible Treg (iTreg) cells in vitro and in vivo (14–16). Importantly, signals negatively regulating the generation of Treg cells in the thymus are beginning to be unveiled. Recently, Liu et al. (17) showed that sphingosine 1-phosphate receptor type 1 delivers an intrinsic negative signal to block the differentiation of thymic Treg cells.

Although the signals shaping Treg cell generation are being revealed, a question remains as to why early neonatal mice lack Treg cells. It is likely that elucidation of the processes of Treg cell generation in neonates will advance our understanding of Treg cell development (10, 18). Indeed, initial insights about the existence of thymic Treg cells came from neonatal thymectomy experiments (19). Removal of the thymus gland within 3 d after birth led to the development of autoimmune diseases, which could be prevented by adoptive transfer of adult T cells. Thus, a population of thymic cells generated after 3 d of life were believed to control the immune tolerance, which led to the discovery of $CD4^+CD25^+$ Treg cells (19). Studies by Fontenot et al. (18) conclusively verified that the development of $CD4^+Foxp3^+$ Treg cells was substantially delayed relative to nonregulatory single-positive (SP) thymocytes. Consequently, Burchill et al. (10) suggested that neonates may lack factors required for Treg cell development. They recently showed that expression of a constitutively active Stat5b transgene allowed for $CD4^+Foxp3^+$ Treg cell generation in the thymus 1 d after birth, which led to the formulation of the above-mentioned two-step model. Given the importance of understanding how neonatal Treg cells develop, our current study investigated the induction of $CD4^+Foxp3^+$ Treg cells from neonatal versus adult T cells in various TCR-stimulatory conditions.

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Abbreviations used in this paper: Adult Spl, adult $CD4^+Foxp3/GFP^-$ splenocyte; Adult Thy, adult $CD4^+CD8^-Foxp3/GFP^-$ thymocyte; DC, dendritic cell; Foxp3, forkhead box P3; iTreg, inducible regulatory T; mTOR, mammalian target of rapamycin; Neo Thy, neonatal $CD4^+CD8^-Foxp3/GFP^-$ thymocyte; neoTreg, neonatal regulatory T; nTreg, naturally occurring regulatory T; SP, single positive; Tconv, conventional T; Treg, regulatory T; TSLP, thymic stromal lymphopoietin.



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## Materials and Methods

### *Mice*

C57BL/6 (B6), OT-II TCR transgenic, Foxp3/GFP (20), Rag1$^{-/-}$ (all with B6 background), and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). We crossed female Foxp3/GFP ($^{+/+}$) mice and male OT-II ($^{+/+}$) TCR transgenic mice, and their $F_1$ progeny, which are OT-II TCR ($^{+}$) Foxp3/GFP ($^{+}$), were used in some experiments. Animals were maintained at the University of Toledo specific pathogen-free animal facility, according to the institutional guidelines.

### *Reagents*

Anti–CD3-FITC, anti–CD4-FITC, anti–CD4-PE-Cy5, anti–CD8-allophycocyanin, anti–CD25-PE, anti–CD69-PE-Cy5, anti–glucocorticoid-induced TNFR-PE, anti–CTLA-4-PE, purified anti-CD3, and purified anti-CD28 mAbs were purchased from BD Biosciences (San Jose, CA). TGF-β–neutralizing Ab (clone 1D11), mouse TGF-β1, and IL-2 DuoSet ELISA kits were purchased from R&D Systems (Minneapolis, MN). IL-2–neutralizing mAb (JES6-1A12), anti–Foxp3-PE mAb, and an intracellular staining kit were purchased from eBioscience (San Diego, CA). Murine IL-1β, -2, -3, -6, -7, -13, -15, -21, and -33; thymic stromal lymphopoietin (TSLP); and human TGF-β cytokines were purchased from PeproTech (Rocky Hill, NJ). PI3K inhibitor (LY294002), mammalian target of rapamycin (mTOR) inhibitor (rapamycin), calcineurin inhibitor (cyclosporine), and an inhibitor of TGF-β superfamily type I activin receptor-like kinase receptors (SB-431542) were purchased from Sigma-Aldrich (St. Louis, MO); Jak3 inhibitor (CP690550) was purchased from Axon Biochemicals (Groningen, The Netherlands).

### *Cell preparation*

Single-cell suspensions were obtained from thymi and spleens of neonatal (2–4 d old), adult (8 wk old), or mice at various ages, as indicated in the figures. Foxp3/GFP$^{-}$ thymocytes were sorted out from Foxp3/GFP mice. The CD4$^{+}$CD8$^{-}$Foxp3/GFP$^{-}$ thymocytes were isolated from Foxp3/GFP or (Foxp3/GFP × OTII)$F_1$ mice with FACS sorting, using anti–CD4-PE-Cy5 and anti–CD8-allophycocyanin. CD4$^{+}$Foxp3/GFP$^{+}$ and CD4$^{+}$Foxp3/GFP$^{-}$ adult splenocytes were isolated and used in some experiments. Thymocytes obtained from B6 mice were also stained with anti–CD4-FITC, anti–CD8-allophycocyanin, anti–CD25-PE, and anti–CD69-PE-Cy5 mAbs to isolate the CD4$^{+}$CD8$^{-}$CD25$^{-}$, CD4$^{+}$CD8$^{-}$CD25$^{-}$CD69$^{+}$, or CD4$^{+}$CD8$^{-}$CD25$^{-}$CD69$^{-}$ populations, respectively. CD3$^{-}$ APCs were isolated from splenocytes of adult B6 and BALB/c mice by sorting using anti–CD3-FITC mAb, whereas CD4$^{+}$CD25$^{-}$ splenocytes were isolated by sorting using anti–CD4-PECy5 and anti–CD25-PE mAbs. All cell sorting was performed with a FACSAria cell sorter (BD Biosciences), and the purity of cells was determined to be >97%.

### *In vitro induction of Foxp3$^{+}$ cells*

For allogeneic APC stimulation, 5 × 10$^4$/well Foxp3/GFP$^{-}$ thymocytes or splenocytes from Foxp3/GFP mice were cultured in 96-well round-bottom plates in the presence of 1.5 × 10$^5$/well allogeneic APCs (CD3$^{-}$ BALB/c splenocytes). For syngeneic APC stimulation, sorted cells were cultured in the presence of 1.5 × 10$^5$/well syngeneic APCs (CD3$^{-}$ B6 splenocytes) and 0.5 μg/ml soluble anti-CD3 mAb. In some experiments, sorted thymocytes or splenocytes were cultured in 96-well flat-bottom plates coated by 4 μg/ml anti-CD3 mAb and in the presence of 2 μg/ml soluble anti-CD28 mAb. Some thymocytes were CFSE labeled prior to cultivation. In other experiments, CD4$^{+}$CD8$^{-}$Foxp3/GFP$^{-}$ thymocytes from neonate or adult (Foxp3/GFP × OTII)$F_1$ mice were stimulated by bone-marrow derived dendritic cells (DCs) pulsed with 10 ng/ml OT-II peptide (OVA323–339). Five μg/ml TGF-β–neutralizing mAb, 5 μM TGF-β signaling inhibitor (SB-431542), 10 μg/ml IL-2–neutralizing mAb, 20 IU/ml murine IL-2, 2 ng/ml human TGF-β, 10 μM LY294002, 100 nM CP690550, 10 nM rapamycin, 40 nM cyclosporine, 10 ng/ml IL-1β, -3, -6, -7, -13, -15, -21, or -33, or TSLP was added in some cultures. Foxp3 expression was determined using a FACSCalibur flow cytometer (BD Biosciences) by Foxp3/GFP expression or intracellular Foxp3 staining on day 3.

### *ELISA*

CD4$^{+}$CD8$^{-}$Foxp3/GFP$^{-}$ thymocytes, isolated from neonatal or adult Foxp3/GFP mice, were cultured in 96-well flat-bottom plates (5 × 10$^4$

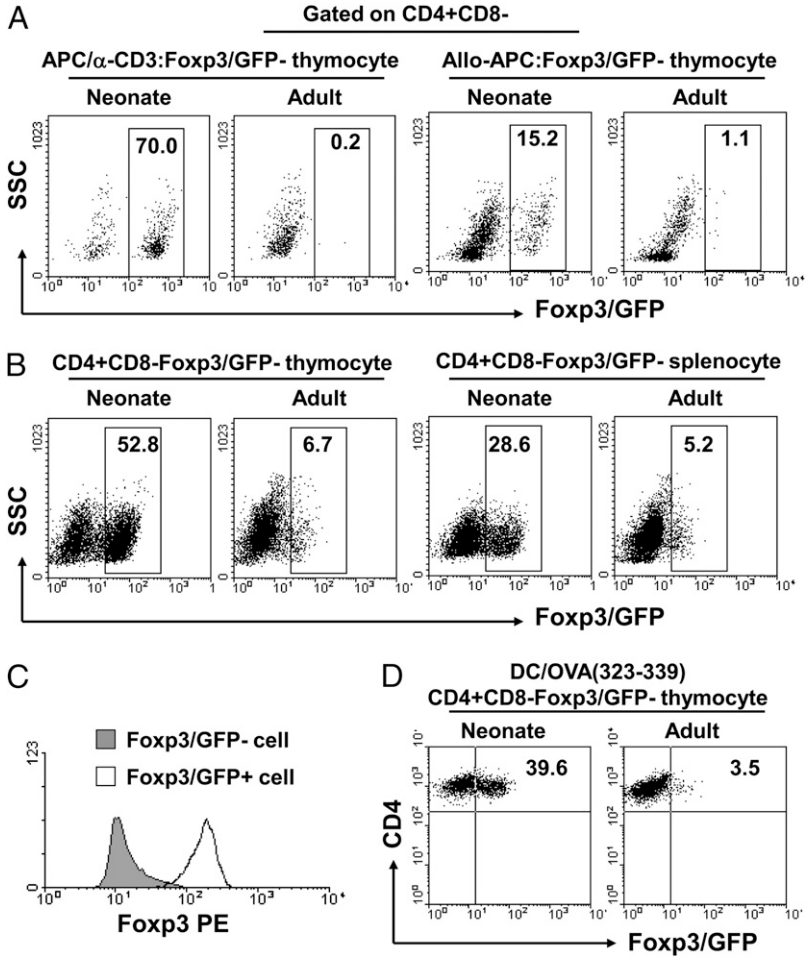


**FIGURE 1.** Conversion of neonatal, but not adult, CD4$^{+}$CD8$^{-}$Foxp3/GFP$^{-}$ cells into Foxp3/GFP$^{+}$ Treg cells. *A*, Foxp3/GFP$^{-}$ thymocytes from 2-d-old neonatal and 8-wk-old adult Foxp3/GFP mice were cultured with syngeneic APCs plus soluble anti-CD3 mAb (*left two panels*) or with allogeneic APCs (*right two panels*). Dot plots show the frequencies of Foxp3/GFP$^{+}$ cells within the CD4$^{+}$CD8$^{-}$ population in day-3 cultures. *B*, CD4$^{+}$CD8$^{-}$Foxp3/GFP$^{-}$ thymocytes (*left two panels*) and splenocytes (*right two panels*) from 3-d-old neonatal and 8-wk-old adult Foxp3/GFP mice were stimulated with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb. Frequencies of Foxp3/GFP$^{+}$ cells in the day-3 cultures are shown. *C*, Foxp3/GFP$^{+}$ and Foxp3/GFP$^{-}$ cells were sorted out from the day-3 cultures of CD4$^{+}$CD8$^{-}$Foxp3/GFP$^{-}$ neonatal thymocytes stimulated by anti-CD3/anti-CD28 mAbs. Sorted cells underwent intracellular staining with Foxp3-PE. The overlay plot represents the difference in Foxp3 expression between Foxp3/GFP$^{+}$ and Foxp3/GFP$^{-}$ cells. *D*, CD4$^{+}$CD8$^{-}$Foxp3/GFP$^{-}$ thymocytes from neonatal and adult (OT-II × Foxp3/GFP)$F_1$ mice were cultured with OVA(323–339)-pulsed syngeneic DCs. Frequencies of Foxp3/GFP$^{+}$ cells in the day-3 cultures are shown. Data are representative of at least three independent experiments.

cells per well) and were stimulated by plate-bound anti-CD3 mAb and soluble anti-CD28 mAb for 3 d. The levels of TGF-β1 and IL-2 in culture supernatants were assessed by ELISA, using commercial kits following the manufacturer's instructions.

*In vivo induction of Foxp3$^+$ cells*

Two different experimental models were used. In the first model, a dose of 1 mg/kg purified anti-CD3 mAb was injected i.p. into 2-d-old neonatal and 8-wk-old Foxp3/GFP mice. Three days later, thymocytes were isolated followed by the flow cytometric analysis of frequencies of Foxp3/GFP$^+$ cells within the population of CD4 SP thymocytes. In the second model, Rag1$^{-/-}$ mice were transferred i.v. with $8 \times 10^6$ Foxp3/GFP$^-$ thymocytes from 3-d-old neonatal or 8-wk-old adult Foxp3/GFP mice. Twelve days later, the frequencies of CD4$^+$Foxp3/GFP$^+$ cells within the peripheral lymphoid organs of these mice were determined by flow cytometric analysis.

*Suppression assay*

A total of $5 \times 10^4$/well CD4$^+$CD25$^-$ T effector cells sorted from B6 mice were used as responder cells. Suppressors were Foxp3/GFP$^+$ cells sorted out from day-3 cultures, which were further cultured in 20 IU/ml IL-2–containing medium for an additional 2 d after Foxp3/GFP conversion. Foxp3/GFP$^+$ naturally occurring Treg (nTreg) cells were sorted ex vivo from Foxp3/GFP mice. Treg cells were seeded in different numbers, according to the suppressor/responder ratios. Cells were cultured in the presence of $1.5 \times 10^5$/well CD3$^-$ syngeneic APCs and soluble anti-CD3 mAb (0.5 μg/ml) for 3 d. The cultures were labeled with 1 μCi/well [$^3$H] thymidine during the final 18 h of culture, and incorporated radioactivity was determined by a microplate scintillation counter (Packard, Ramsey, MN).

*Statistical analysis*

Statistical analysis and *p* values were calculated using an unpaired, two-tailed Student *t* test.

## Results

*Stimulation through TCR induces Foxp3/GFP expression in neonatal, but not adult, T cells*

To investigate how neonatal Treg cells develop, we first studied the in vitro induction of Foxp3 expression in neonatal versus adult T cells. Foxp3/GFP$^-$ thymocytes from 2-d-old neonatal and 8-wk-old adult B6 Foxp3/GFP mice were isolated by FACS sorting. These two populations were then cultured for 3 d with T cell-depleted B6 splenocytes (syngeneic APCs) and soluble anti-CD3 mAb or with T cell-depleted allogeneic BALB/c splenocytes (allogeneic APCs). Surprisingly, stimulation of Foxp3/GFP$^-$ neonatal thymocytes with syngeneic APC/anti-CD3 mAb resulted in the generation of 70% Foxp3/GFP$^+$ cells within the CD4$^+$CD8$^-$ population. In contrast, only 0.2% CD4$^+$CD8$^-$Foxp3/GFP$^+$ adult thymocytes were found in a similar culture (Fig. 1*A*, *left two panels*). Likewise, stimulation of Foxp3/GFP$^-$ neonatal thymocytes with allogeneic APCs resulted in the generation of 15.2% CD4$^+$CD8$^-$ Foxp3/GFP$^+$ cells compared with only 1.1% of adult thymocytes (Fig. 1*A*, *right two panels*). Less induction of Foxp3/GFP$^+$ T cells under allogeneic stimulation might reflect the fact that allogeneic APCs activated only a fraction of T cells. Thus, neonatal CD4$^+$ CD8$^-$ thymocytes have a much greater propensity to become Foxp3$^+$ cells than do adult thymocytes. These dramatic results motivated us to isolate CD4$^+$CD8$^-$Foxp3/GFP$^-$ cells from the thymus and spleen of neonatal and adult Foxp3/GFP mice to culture them with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb. After 3 d of cultivation, >50% of neonatal thymic and almost 30% of neonatal splenic CD4$^+$CD8$^-$Foxp3/GFP$^-$ cells converted into Foxp3/GFP$^+$ T cells (Fig. 1*B*). In contrast, <7% of adult CD4$^+$CD8$^-$ thymocytes and splenocytes became Foxp3/GFP$^+$ T cells following stimulation (Fig. 1*B*). Intracellular staining confirmed that the induced Foxp3/GFP$^+$ cells expressed high levels of Foxp3 (Fig. 1*C*). Finally, CD4$^+$CD8$^-$Foxp3/GFP$^-$ thymocytes isolated from 2-d-old neonatal and 8-wk-old adult (OTII × Foxp3/GFP)F$_1$ mice were cultured for 3 d with OTII peptide-pulsed DCs. Ag-specific stimulation by peptide-pulsed DCs resulted in a 10-fold elevation of Foxp3/GFP$^+$ T cells in neonatal thymocytes compared with adult thymocytes (Fig. 1*D*), demonstrating Ag-specific induction of Foxp3$^+$ T cells. Taken together, these data reveal a surprising finding that neonatal CD4$^+$ T cells intrinsically default to CD4$^+$Foxp3$^+$ cells upon TCR stimulations.

*In vitro characterization of neonatal Treg cells*

To characterize CD4$^+$Foxp3$^+$ T cells derived from neonatal T cells, we examined additional membrane markers, the suppressive function, and the stability of Foxp3 expression of these cells. Ex vivo-isolated nTreg cells and exogenous TGF-β–induced iTreg cells were used as controls. First, neonatal CD4$^+$CD8$^-$ Foxp3/GFP$^-$ thymocytes were isolated from 2-d-old Foxp3/

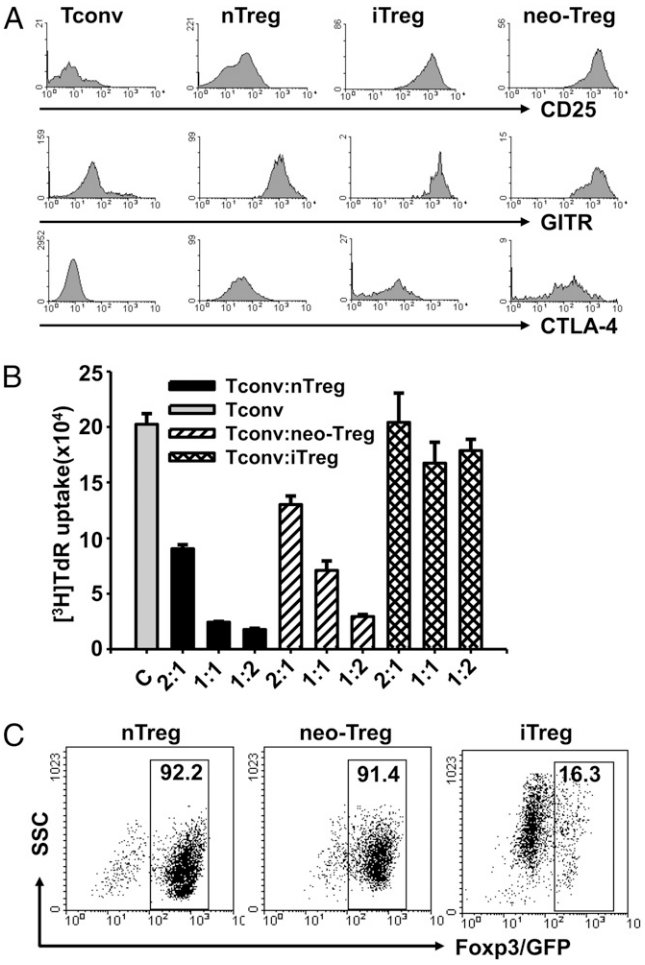


**FIGURE 2.** In vitro characterization of neonatal Treg cells. Foxp3/GFP$^+$ neo-Treg cells were sorted out from the day-3 culture of neonatal CD4$^+$ CD8$^-$Foxp3/GFP$^-$ thymocytes stimulated by anti-CD3/anti-CD28 mAbs. Foxp3/GFP$^+$ iTreg cells were sorted out from the day-3 culture of adult CD4$^+$Foxp3/GFP$^-$ splenocytes stimulated by anti-CD3/anti-CD28 mAbs in the presence of TGF-β. The sorted cells were cultured in 20 IU/ml IL-2–containing medium for an additional 2 d. CD4$^+$Foxp3/GFP$^+$ nTreg cells and CD4$^+$Foxp3/GFP$^-$ Tconv cells were ex vivo-sorted out from the splenocytes of adult Foxp3/GFP mice. *A*, Graphs show the expression of indicated surface markers. *B*, Purified B6 CD4$^+$CD25$^-$ T cells were cultured with syngeneic APCs, soluble anti-CD3 mAb, and various numbers of iTreg, nTreg, or neo-Treg cells. Cell proliferation was assessed on day 3 by [$^3$H]thymidine incorporation. *C*, iTreg, nTreg, or neo-Treg cells were restimulated by anti-CD3/anti-CD28 mAbs for 3 d. Dot plots show the percentage of Foxp3/GFP$^+$ cells in the day-3 cultures. Data are representative of three experiments. neo-Treg, neonatal Treg; Tconv, conventional T.

GFP mice and stimulated for 3 d with anti-CD3/anti-CD28 mAbs. Generated Foxp3/GFP$^+$ T cells were then sorted out from the culture and rested in 20 IU/ml IL-2–containing medium for 2 d. Flow cytometric analysis showed that these neonatal Foxp3/GFP$^+$ T cells expressed high levels of CD25, glucocorticoid-induced TNFR, and CTLA4 on their surface (Fig. 2*A*), a cell-surface phenotype similar to iTreg cells (Foxp3/GFP$^+$ cells sorted out from the anti-CD3/anti-CD28/TGF-β culture of adult T cells followed by a 2-d resting period) and nTreg cells (ex vivo-sorted Foxp3/GFP$^+$ cells; Fig. 2*A*). Next, to examine suppressive function, the same neonatal CD4$^+$Foxp3$^+$ T cells, nTreg cells, and iTreg cells were cultured together with B6 CD4$^+$CD25$^-$ T effector cells stimulated by syngeneic APCs and anti-CD3 mAb (Fig. 2*B*). Cell proliferation determined 3 d later by [$^3$H]thymidine incorporation showed that nTreg cells and neonatal CD4$^+$Foxp3$^+$ cells suppressed T effector cell proliferation in a similar fashion. Furthermore, neonatal CD4$^+$Foxp3$^+$ cells and nTreg cells stably maintained their Foxp3 expression upon repeated stimulation (Fig. 2*C*). In contrast, adult iTreg cells generated in these cultures were less effective in the suppressor assay (Fig. 2*B*) and down-regulated Foxp3 expression upon TCR restimulation (Fig. 2*C*), as reported previously (21). Thus, we concluded that neonatal T cell-derived CD4$^+$Foxp3$^+$ cells display a Treg cell phenotype, which exhibits a relatively stable Foxp3 expression and exerts a potent suppressive effect.

*The capability of default generation of neonatal Treg cells diminishes during ontogeny*

We next determined the kinetics of the "default" Treg generation from 2 d to 8 wk after birth. CD4$^+$CD8$^-$CD25$^-$ thymocytes were isolated from B6 mice at various ages and were stimulated for 3 d with anti-CD3/anti-CD28 mAb. As shown in Fig. 3*A* and 3*B*, the percentage and total number of induced Foxp3$^+$ cells were reduced dramatically in cultures of thymocytes from 2-wk-old mice. These data indicated that the intrinsic default inclination to generate Treg cells is restricted to the neonatal period of life. Cultures of CFSE-labeled CD4$^+$CD8$^-$CD25$^-$ thymocytes showed that the pattern of cell division was similar among neonatal Foxp3$^+$ cells, neonatal Foxp3$^-$ cells, and adult Foxp3$^-$ cells (Fig. 3*C*). Thus, Foxp3 induction in neonatal T cells does not interfere with cell proliferation.

Positive selection in the thymus leads to CD69$^+$ SP thymocytes, which decrease their CD69 levels upon further maturation (22). We isolated CD69$^+$ and CD69$^-$ populations from CD4$^+$CD25$^-$ SP thymocytes of neonatal and adult B6 mice followed by 3-d cultures; CD69$^+$CD4 SP thymocytes were more susceptible to Treg cell generation than CD69$^-$CD4 SP thymocytes (Fig. 3*D*, *middle* versus *right panels*). Nevertheless, neonatal CD4 SP thymocytes always exhibited superior capability in Treg cell generation compared with adult CD4 SP thymocytes (Fig. 3*D*, *upper* versus *lower panels*). We showed previously that neonatal CD4

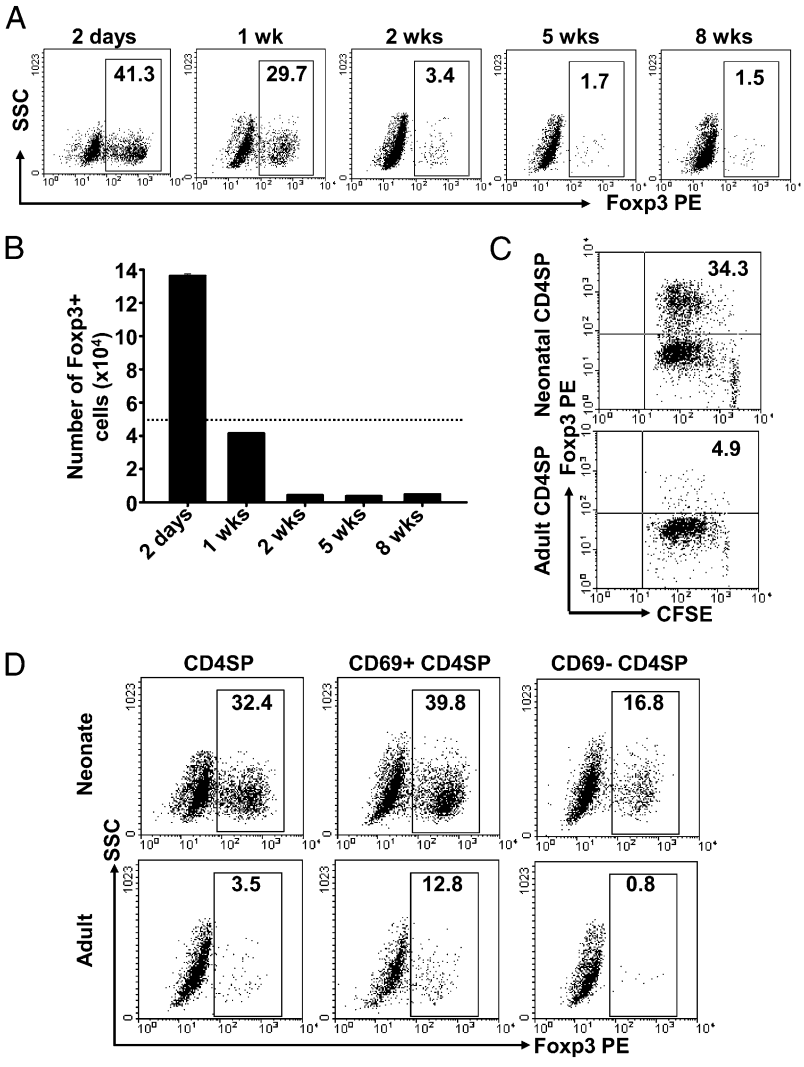


**FIGURE 3.** Default generation of Treg cells inversely correlates with age. Thymocytes purified from neonatal or adult mice were stimulated by plate-bound anti-CD3 and soluble anti-CD28 mAbs. *A* and *B*, CD4$^+$CD8$^-$CD25$^-$ thymocytes were sorted out from B6 mice at various ages. *A*, Dot plots show the frequency of Foxp3$^+$ cells in the day-3 cultures. *B*, Bar graphs show the total number of Foxp3$^+$ cells in each well after 3 d of culture. The dashed line represents the initial cell number added in each well. *C*, CD4$^+$CD8$^-$CD25$^-$ thymocytes sorted out from neonatal or adult B6 mice were CFSE labeled prior to in vitro stimulation. Dot plots show CFSE fluorescence versus intracellular Foxp3 staining in the day-3 cultured cells. Numbers represent the percentage of Foxp3$^+$ cells. *D*, CD4$^+$CD8$^-$CD25$^-$ (CD4 SP) thymocytes from neonatal (*upper panels*) and adult (*lower panels*) mice were separated into CD69$^+$ and CD69$^-$ populations before cultivation. The frequency of Foxp3$^+$ cells in day-3 cultures are shown. Data are representative of three experiments.

splenocytes also maintained the intrinsic propensity to express Foxp3 upon activation (Fig. 1*B*). Taken together, although the capability of Treg cell generation relates to different stages of T cell maturation, these findings show that neonatal $CD4^+$ T cells are prone to become Treg cells upon TCR stimulations.

### *The default generation of neonatal Treg cells is TGF-β and IL-2 dependent*

TCR stimulation in conjunction with an extrinsic source of TGF-β is the major method to generate adult iTreg cells (23, 24). All of our previously described experiments were performed in cultures that were not supplemented with exogenous TGF-β. For comparison, we performed cultures of neonatal and adult Foxp3/$GFP^-$ thymocytes that were supplemented with TGF-β and IL-2 (Fig. 4*A*) and compared the cultures to those without exogenous cytokines (Fig. 4*B*). Plate-bound anti-CD3 mAb and soluble anti-CD28 mAb were used to stimulate Foxp3/$GFP^-$ thymocytes. Confirming the role of TGF-β and IL-2 in the generation of Treg cells in vitro, all examined thymic cell populations of neonates and adults among $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ thymocytes displayed a significant percentage of Foxp3/$GFP^+$ cells (Fig. 4*A*). Moreover, neonatal and adult cells exhibited very similar patterns of Foxp3/GFP expression, indicating that the combination of TGF-β and IL-2 potently induced the expression of Foxp3 (23, 24). In contrast, different patterns of Foxp3 expression were observed among thymocytes stimulated without exogenous TGF-β and IL-2, because a substantial percentage of Foxp3/$GFP^+$ Treg cells were again seen only in the population of neonatal $CD4^+CD8^-$ cells (Fig. 4*B*). These results verify the finding in Fig. 1 that neonatal $CD4^+CD8^-$ cells are highly prone to become $CD4^+Foxp3^+$ Treg cells.

To further examine the role of TGF-β and IL-2 in the generation of neonatal Treg cells, we added neutralizing anti-TGF-β mAb (clone 1D11), TGF-β signaling inhibitor (SB 431542), neutralizing anti–IL-2 mAb (JES6-1A12), or IL-2 to the culture of $CD4^+CD8^-$ thymocytes that were isolated from wild-type neonates. Following 3 d of stimulation with anti-CD3/anti-CD28 mAbs, intracellular staining of Foxp3-PE identified that the percentage of $Foxp3^+$ cells was dramatically reduced in the presence of neutralizing anti–TGF-β mAb or TGF-β signaling inhibitor (Fig. 4*C*). Although the TGF-β levels were low in all cultures (Fig. 4*E*, *left panel*), the results from these experiments suggest that the generation of neonatal $CD4^+$ $Foxp3^+$ Treg cells is TGF-β dependent. Neutralizing IL-2 also abrogated Foxp3 induction in neonatal cells (Fig. 4*D*, *middle panel*). Thus, the generation of neonatal $CD4^+Foxp3^+$ Treg cells is also IL-2 dependent, despite the fact that the IL-2 level in the culture of neonatal cells was lower than in the culture of adult cells (Fig. 4*E*, *right panel*). Addition of exogenous IL-2 alone did not dramatically upregulate Foxp3 expression (Fig. 4*D*, *right panel*), indicating that the low amount of endogenous IL-2 was sufficient for neonatal Treg cell generation. Thus, neonatal $CD4^+$ T cells default to Foxp3-expressing cells in response to TCR stimulations, but this process is TGF-β and IL-2 dependent.

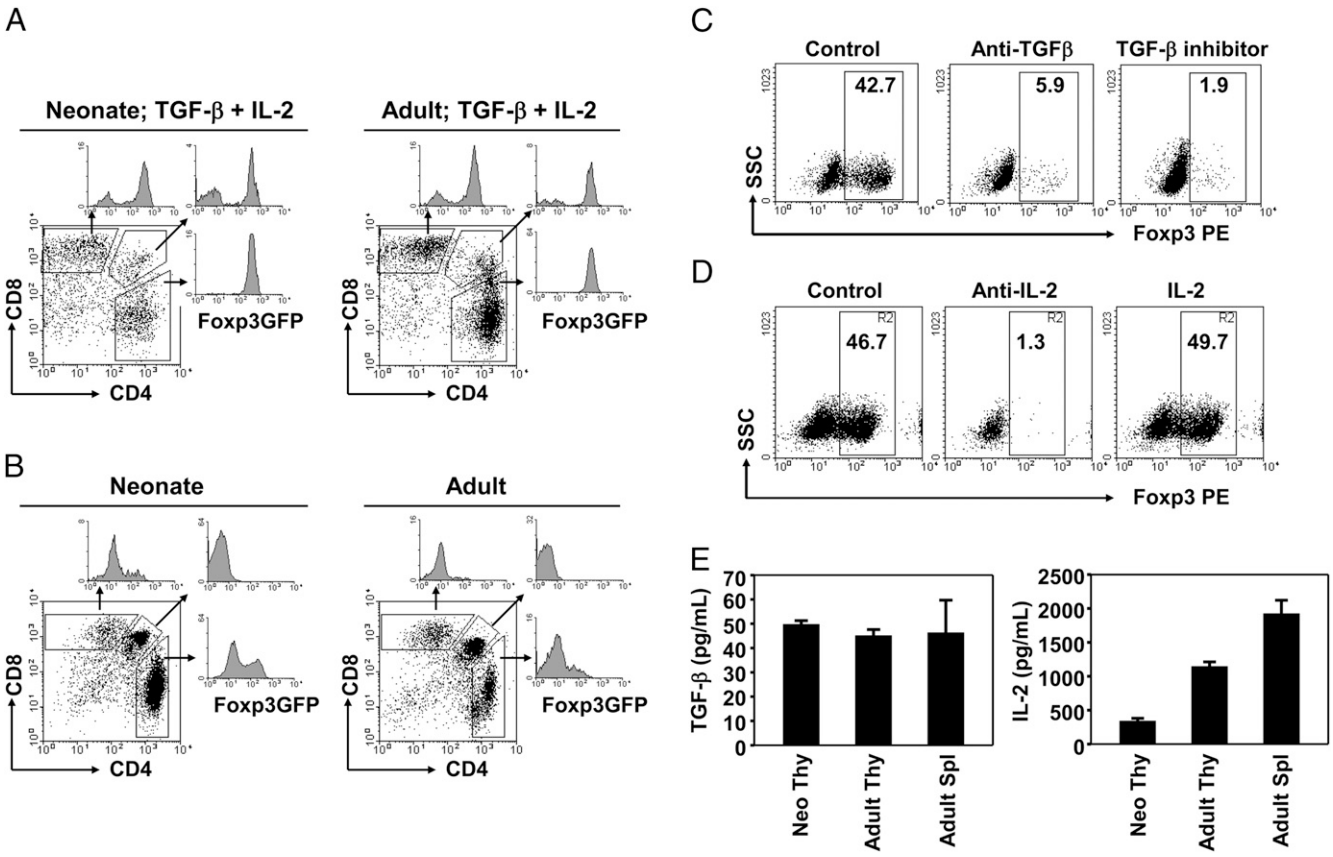


**FIGURE 4.** Generation of neonatal Treg cells is TGF-β and IL-2 dependent but does not require exogenous TGF-β and IL-2. Foxp3/$GFP^-$ thymocytes from neonatal and adult Foxp3/GFP mice were cultured for 3 d with anti-CD3/anti-CD28 mAbs in the presence (*A*) or absence (*B*) of TGF-β/IL-2. Graphs show the expression of Foxp3/GFP in each population of thymocytes. *C* and *D*, $CD4^+CD8^-CD25^-$ thymocytes from neonatal wild type B6 mice were cultured for 3 d with anti-CD3/anti-CD28 mAbs. Some cultures were supplemented with anti–TGF-β mAb (1D11), TGF-β signaling inhibitor (SB-431542), anti–IL-2 mAb (JES6-1A12), and IL-2. The expression of Foxp3 was assessed by intracellular staining. The percentage of $Foxp3^+$ cells in cultures is shown. *E*, Neonatal $CD4^+CD8^-$Foxp3/$GFP^-$ thymocytes (Neo Thy), adult $CD4^+CD8^-$Foxp3/$GFP^-$ thymocytes (Adult Thy), and adult $CD4^+$Foxp3/$GFP^-$ splenocytes (Adult Spl) were stimulated by anti-CD3/anti-CD28 mAbs for 3 d. The bar graphs show the levels of TGF-β and IL-2 in culture supernatants assessed by ELISA. Data are representative of three experiments.

*In vivo generation of neonatal Treg cells*

To determine whether Treg cells can be generated in vivo in neonates upon TCR stimulation, we injected anti-CD3 mAb or PBS once i.p. into 2-d-old or 8-wk-old Foxp3/GFP mice. Three days later, thymocytes were isolated and analyzed by flow cytometry. Neonates injected with PBS had ~3% Foxp3/GFP$^+$ Treg cells among CD4$^+$CD8$^-$ thymocytes. In contrast, neonates injected with anti-CD3 mAb displayed ~9% Foxp3/GFP$^+$ Treg cells within CD4 SP thymocytes ($n = 4$; $p < 0.001$ versus PBS control). The frequencies of Foxp3/GFP$^+$ CD4 SP thymocytes in adult mice injected with anti-CD3 mAb compared with mice injected with PBS were not significantly increased (Fig. 5*A*, *5B*). These results showed that CD4$^+$Foxp3$^+$ Treg cells can be generated in high numbers in neonates upon in vivo stimulation.

Next, we adopted the T cell transfer model (25) to compare the in vivo conversion of neonatal versus adult Foxp3$^-$ T cells into Foxp3$^+$ T cells. Rag1$^{-/-}$ mice were transferred i.v. with $8 \times 10^6$ Foxp3/GFP$^-$ thymocytes from 3-d-old or 8 wk-old Foxp3/GFP mice. Twelve days after adoptive transfer, lymph nodes and spleens were obtained for flow cytometric analysis. As shown by representative dot plots in Fig. 5*C*, a high frequency of CD4$^+$ T cells expressed the activation marker CD25 in peripheral lymphoid organs of both reconstituted groups. Within the CD4$^+$CD25$^+$ population generated from neonatal cells, about half of the cells expressed Foxp3 (Fig. 5*C*, *upper panels*). By contrast, significantly less CD4$^+$CD25$^+$Foxp3$^+$ Treg cells were converted from adult cells (Fig. 5*C*, *lower panels*; Fig. 5*D*). Thus, neonatal T cells are prone to differentiate into Foxp3-expressing cells upon activation in lymphopenic mice.

*IL-7 inhibits the generation of neonatal Treg cells*

We studied several factors in modulating the in vitro generation of neonatal CD4$^+$Foxp3$^+$ Treg cells. In particular, CD4$^+$CD8$^-$ thymocytes isolated from 2-d-old B6 neonates were stimulated by anti-CD3/anti-CD28 mAbs in the presence of LY294002 (PI3K inhibitor), rapamycin (mTOR inhibitor), cyclosporine (calcineurin inhibitor), CP690550 (Jak3 inhibitor), IL-7, and other cytokines. When the expression of Foxp3 was determined 3 d later by intracellular staining, we found that the induction of neonatal CD4$^+$ Foxp3$^+$ Treg cells was reduced by cyclosporine and the Jak3 inhibitor, but it was not affected by the PI3K inhibitor and rapamycin (Fig. 6*A*). Interestingly, although inhibition of Jak3 reduced the Foxp3 induction in neonatal T cells, a Jak3-dependent cytokine IL-7 displayed a robust inhibition of neonatal CD4$^+$Foxp3$^+$ Treg cell generation (Fig. 6*A*). TSLP, an IL-7–like cytokine, also dramatically reduced the development of neonatal Treg cells (Fig. 6*B*). The results from these experiments suggest the existence of unknown intrinsic or extrinsic signals affecting Foxp3 induction in T cells.

## Discussion

Our study showed that neonatal (but not adult) CD4$^+$CD8$^-$Foxp3$^-$ thymocytes and splenocytes intrinsically differentiate into CD4$^+$ Foxp3$^+$ Treg cells in response to TCR stimulation. These neonatal Treg cells exert suppressive function and display stable Foxp3 expression at levels similar to nTreg cells. In vivo activation of T cells by i.p. injection of anti-CD3 mAb also significantly increased the frequency of Foxp3$^+$ Treg cells in neonates but not in adults. Moreover, an adoptive transfer of neonatal Foxp3$^-$

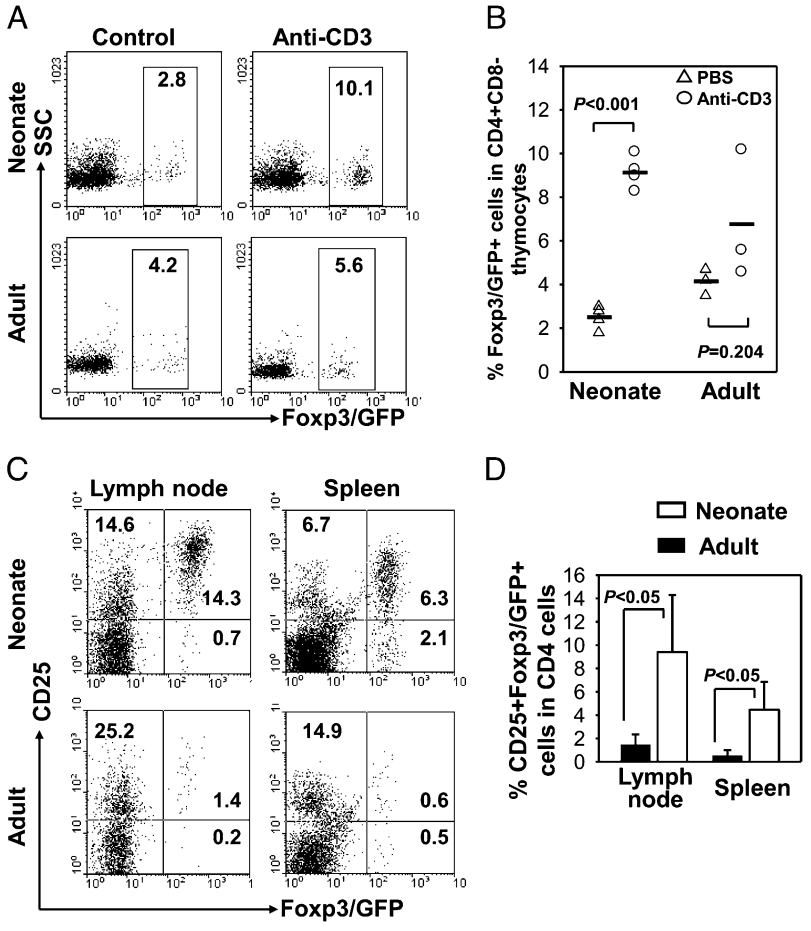


**FIGURE 5.** In vivo generation of neonatal Treg cells. *A* and *B*, Neonatal and adult Foxp3/GFP mice were injected with anti-CD3 mAb or PBS. *A*, Representative dot plots indicate the percentage of Foxp3/GFP$^+$ T cells within CD4$^+$CD8$^-$ thymocytes at day 3 after injection. *B*, Percentage of Foxp3/GFP$^+$ cells within CD4$^+$CD8$^-$ thymocytes of anti-CD3 mAb- or PBS-injected mice. The symbols represent individual mice, and the horizontal bars represent means. *C* and *D*, Rag1$^{-/-}$ mice were transferred i.v. with $8 \times 10^6$ Foxp3/GFP$^-$ thymocytes from 3-d-old neonatal or 8-wk-old adult Foxp3/GFP mice. Lymph nodes and spleens were obtained 12 d after cell transfer for analysis. *C*, Representative dot plots show the frequencies of CD25$^+$Foxp3/GFP$^-$, CD25$^+$Foxp3/GFP$^+$, and CD25$^-$Foxp3/GFP$^+$ cells within CD4$^+$ population (gated on CD4$^+$ cells). *D*, Bar graphs show the frequencies of CD25$^+$Foxp3/GFP$^+$ cells within CD4$^+$ cells ($n = 4$).

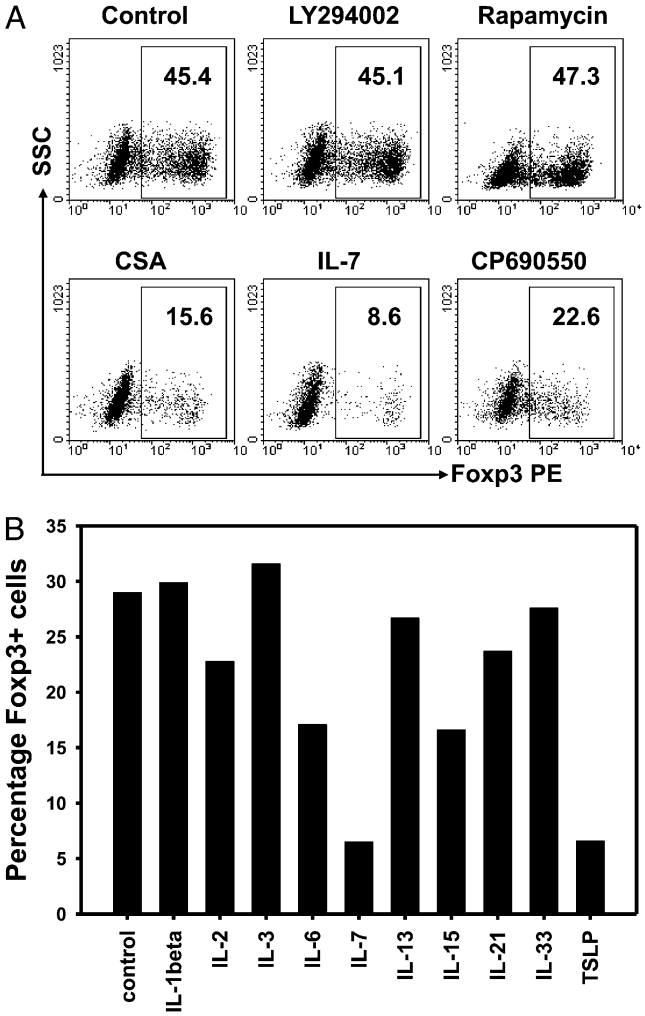


**FIGURE 6.** Factors influencing the generation of neonatal Treg cells. $CD4^+CD8^-CD25^-$ thymocytes from neonatal wild type B6 mice were cultured for 3 d with anti-CD3/anti-CD28 mAbs in the presence of PI3K inhibitor (LY294002), mTOR inhibitor (rapamycin), calcineurin inhibitor (cyclosporine), Jak3 inhibitor (CP690550), or various cytokines. Representative dot plots (*A*) and bar graphs (*B*) indicate the frequencies of $Foxp3^+$ cells in the day-3 cultures. Data are representative of three experiments.

thymocytes into $Rag1^{-/-}$ mice resulted in a 6-fold higher frequency of $CD4^+CD25^+Foxp3^+$ T cells compared with transfer of adult $Foxp3^-$ thymocytes.

Commitment of $CD4^+$ T cells to distinct T cell lineages requires TCR engagement and cytokine polarization (26). Indeed, differentiation of adult $CD4^+Foxp3^-$ cells into $Foxp3^+$ iTreg cells generally requires TCR engagement and an extrinsic source of polarizing cytokines TGF-β (14) and IL-2 (23, 24). Strikingly, we showed in this study that TCR- and CD28-derived signals are sufficient to induce stable Foxp3 expression in neonatal T cells without an extrinsic source of TGF-β and IL-2. A common in vitro stimulation (4 μg/ml plate-bound anti-CD3 mAb plus 2 μg/ml soluble anti-CD28 mAb) induced cell proliferation (Fig. 3*C*) and resulted in substantial frequencies of $Foxp3^+$ cells in 3-d cultures of neonatal $CD4^+CD8^-Foxp3^-$ thymocytes or splenocytes (Fig. 1*B*). Because TGF-β signaling inhibitor, anti–TGF-β mAb (1D11), and anti–IL-2 mAb abrogated the induction of Foxp3 (Fig. 4), TGF-β and IL-2 signals are required for the generation of neonatal Treg cells. These results may explain why ablation of TGF-β or its cognate receptors resulted in deficiency of $Foxp3^+$ cells within 1 wk after birth (11–13), as well as why expression of a constitutively active Stat5b (IL-2 downstream signal) allowed for Treg cell generation in the thymus 1 d after birth (10). Nevertheless, an exogenous source of TGF-β and IL-2 is not required for the generation of neonatal Treg cells, indicating that this is a self-sustained default mechanism in response to TCR and CD28 stimulations.

The same TCR stimulation (4 μg/ml plate-bound anti-CD3 mAb plus 2 μg/ml soluble anti-CD28 mAb) was used to generate neonatal Treg cells and TGF-β–induced adult iTreg cells. Although Treg cells converted from neonatal cells expressed relatively stable Foxp3, the adult iTreg cells generated under the same condition expressed unstable Foxp3, as shown by a previous report (21). Selvaraj and Geiger (27) also demonstrated that strong TCR stimulation (anti-CD3/anti-CD28–coated beads) in the presence of exogenous TGF-β generated iTreg cells expressing unstable Foxp3. Later, they used a relatively weaker stimulation (soluble anti-CD3/anti-CD28 mAbs) to generate TGF-β–induced iTreg cells, which efficiently prevented the development of experimental allergic encephalomyelitis (28). Thus, suboptimal stimulation (8) could be critical for generation of functional iTreg cells.

Temporal differences in the development of an adaptive immune system vary substantially between humans and mice. Although few peripheral T cells are present in newborn mice, peripheral lymphoid tissues in the human fetus are populated by T cells. Interestingly, a high frequency of Treg cells in cord blood (15–20%) (29) and lymph nodes (~20%) (30) was observed in human fetuses at ~20 wk of gestation. As suggested by Mold et al. (31), this high frequency of human fetal Treg cells reflects a greater propensity of naive fetal T cells to differentiate into Treg cells in response to substantial numbers of maternal cells that cross the placenta to reside in the fetus. The same investigators showed that >50% of T cells expressed stable and high levels of Foxp3 in the in vitro 6-d cultures of fetal (but not adult) human T cells stimulated by alloantigens. This process was also dependent on TGF-β signaling, because inhibition of TGF-β signaling resulted in a marked reduction of Foxp3-expressing fetal T cells. Indeed, high gene expression levels of TGF-β and other TGF-β family members were identified in fetal lymph nodes (31), which is consistent with the importance of these molecules in embryogenesis (32). Taken together, the generation of human fetal Treg cells is believed to play an important role in suppressing fetal T cell responses during development (30), as well as in establishing tolerance to foreign and self-Ags (31). These findings and our current data show that human and mouse $CD4^+$ T cells are prone to become Treg cells in response to stimulations during early development of the immune system.

Because TCR-derived signals are required and sufficient for the induction of neonatal Treg cells, deficiencies in these signals [e.g., caused by DC "deficiency" (33) or incomplete structure of thymic medulla (18)] may account for the low frequency of Treg cells in newborn mice within the first 3 d after birth. Interestingly, constitutive activation of Stat5 results in the acceleration of Treg cell generation in neonates (10). This finding might be due to bypassing IL-2 signal (10) and enhancing TCR signals, because Stat5 sustains TCR-initiated gene expression under conditions of low-avidity TCR engagement (34). However, although IL-7 and TSLP are capable of activating Stat5, they exhibit robust effects in abrogating the in vitro Foxp3 induction in neonatal T cells (Fig. 6). These findings suggest a need to further delineate the role of Stat5 in Foxp3 induction. Collectively, a low frequency of Treg cells in early neonatal mice may be attributed to insufficient TCR stimulations or the presence of some cytokines restraining Foxp3 induction.

Exactly 57 y ago, Medawar and colleagues (35) showed that mice injected at birth with allogeneic splenocytes were subsequently able to accept skin allografts from the same donor strain. This finding led to the belief that the neonatal period represents an

ontogenic window for tolerance induction to self- and foreign Ags. In the ensuing decades, studies of tolerance induction in neonates revealed the key central tolerance mechanism (negative selection), which occurs in the thymus of juvenile and adult mice (36–38). The susceptibility of neonates to tolerance induction may be due to the absence of peripheral mature T cells against foreign Ags or cells, which may enter the thymus and eliminate the cognate developing thymocytes in the same way as self-reactive T cell clones. Nevertheless, human studies suggested that the generation of Treg cells is an additional mechanism by which fetuses acquire tolerance to the maternal allogeneic Ags (31). Thus, the questions remain as to whether the default generation of Treg cells is also involved in establishing tolerance to allogeneic Ags in neonatal mice (35) and whether the initial period of the immune system's development is essential for the establishment of immune tolerance.

Neonates exhibit an immature immune system in a state of development, as revealed by incomplete lymphoid structures, low numbers of T and B cells and DCs, and the absence of memory cells (33, 39). However, the low immune cell numbers may not fully account for the decreased or aberrant immune responses in neonates (39). We found that neonatal $CD4^+Foxp3^-$ splenocytes (few in number) default to $Foxp3^+$ Treg cells in response to TCR stimulation. It is critical to determine whether this generation of Treg cells impairs the neonatal T cell responses against numerous pathogenic Ags. Infectious diseases cause millions of deaths in human neonates and infants annually, indicating the necessity of examining the involvement of Treg cells in neonatal immune responses.

In summary, our data revealed a surprising finding that TCR signals are sufficient to induce stable Foxp3 expression in neonatal, but not adult, T cells. Further investigation into the mechanisms underlying this default generation of Treg cells may provide new insights about the development of Treg cells, the induction of immune tolerance, and potential treatments of infectious diseases in neonates and infants.

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## Disclosures

The authors have no financial conflicts of interest.

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