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Letters to the Editor

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Comment on "A20 and CYLD Do Not Share Significant Overlapping Functions during B Cell Development and Activation"

Interleukin-6 is a cytokine that plays an important role in both innate and adaptive immunity. Its primary function in adaptive immunity is stimulation of the growth of B cells (1). In a recent article by Chu et al. (2), the authors measured IL-6 production in B cells derived from four different genotypes under five different conditions as a part of their research to examine the functional overlapping of two proteins, A20 and CYLD. In this letter, we propose a hypothesis on the mechanism of the production of IL-6 in B cells.

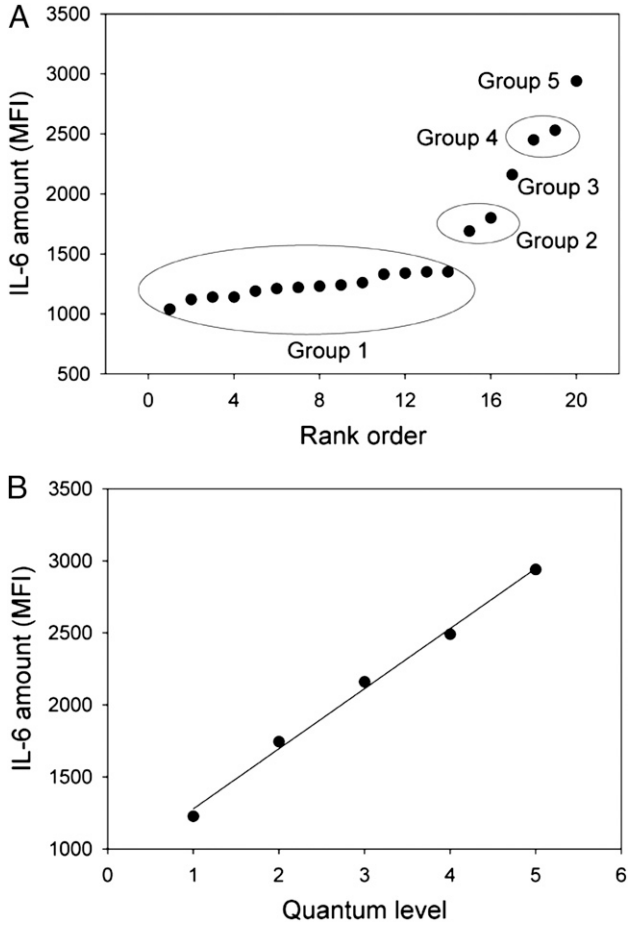

FIGURE 1. (**A**) The amount of IL-6 produced in B cells in ascending order. They are combined into five groups. When a group contains multiple elements, then the average value is taken as the representative value of the group. Each group is treated as a quantum level. (**B**) Quantized production of IL-6 as a function of quantum level. The relationship is fitted to a linear equation, $y = ax + b$, where $a = 417 \pm 17$ and $b = 860 \pm 58$, respectively (R^2: 0.9948). The IL-6 amount is expressed by mean fluorescence intensity (MFI). Fitting was conducted using SigmaPlot (version 11, Systat Software, San Jose, CA).

We grouped the 20 different measurements of IL-6 shown in the *bottom panel* of Fig. 4B of the article into five groups based on proximity of values (Fig. 1A). We found that the relationship between the quantum level (x) and the IL-6 amount (y) is described by a linear equation, $y = ax + b$ (Fig. 1B). The slope a corresponds to the quantized amount of IL-6. This means that IL-6 production in B cells may be quantized with constant spacing between each level. This type of quantized behavior has been previously observed in the release of neurotransmitters from presynaptic cells (3).

To test our hypothesis, we would like to suggest that further experiments be conducted to verify if A20/CYLD-deficient and A20-deficient B cells produce IL-6 in a quantized pattern responding to combined stimuli (such as LPS and CpG).

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Comment on "Helios$^+$ and Helios$^-$ Cells Coexist within the Natural FOXP3$^+$ T Regulatory Cell Subset in Humans"

We read with interest the recent study by Himmel and colleagues (1) reporting that human circulating FOXP3$^+$ naive T regulatory cells (nTreg), a population that we defined in 2005 (2), includes Helios$^+$ and Helios$^-$ cells, the latter representing, on average, 30% of total nTreg (1). Whereas Himmel et al. found that T cell clones derived from the two populations were similarly suppressive in vitro (although Helios$^-$ Treg clones produced more CCL3 and IFN-γ than Helios$^+$ clones) and carried demethylated *FOXP3* T regulatory cell–specific demethylated region, indicating stable expression of FOXP3, the origin of the circulating Helios$^-$ Treg population is yet unclear. In recent experiments performed in our laboratory, we similarly identified the Helios$^-$

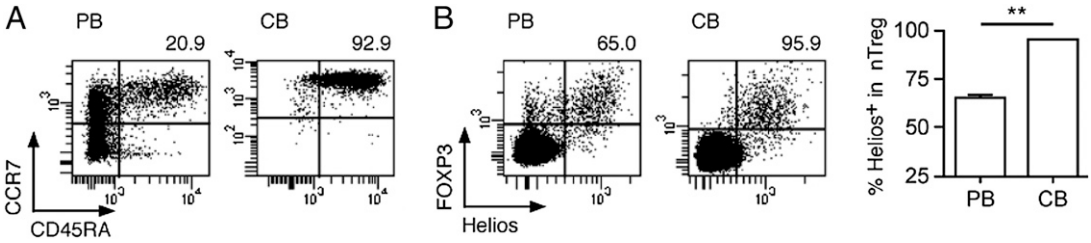

FIGURE 1. Helios$^-$ FOXP3$^+$ naive Treg are absent in cord blood. CD4$^+$ T cells, isolated from adult peripheral blood (PB) or cord blood (CB) by density gradient sedimentation (PAA Laboratories) followed by magnetic cell sorting (Miltenyi Biotec), were stained with anti-CD45RA (BD Biosciences), -CCR7 (BD Biosciences), -CD25 (Beckman Coulter), -CD127 (BD Biosciences), -FOXP3 (eBioscience), and -Helios (Biolegend) mAb and analyzed by flow cytometry (FACS Aria II; BD Biosciences). (**A**) Dot plots show CD45RA and CCR7 expression in total PB and CB CD4$^+$ T cells. Numbers correspond to the proportion of naive (CD45RA$^+$CCR7$^+$) CD4$^+$ T cells. (**B**) FOXP3 and Helios expression gated on naive CD4$^+$ T cells and the proportion of Helios$^+$ cells in FOXP3$^+$CD25$^+$CD127$^-$ nTreg are shown for one sample of PB and one sample of CB. The proportion of Helios$^+$ cells in FOXP3$^+$CD25$^+$CD127$^-$ nTreg is summarized for all samples (mean ± SEM, $n = 6$). Statistical analyses were performed using the Mann–Whitney U test. $^{**}p < 0.01$.

subpopulation, in the proportions reported by Himmel et al., in circulating nTreg from healthy donors (Fig. 1). To gain insight into the origin of this population, we assessed CD4$^+$ T cells in human cord blood, where nTreg are abundant (2). We found, however, that Helios$^-$ Treg were absent in cord blood, indicating that they develop in the periphery after birth (Fig. 1). Thus, whereas human thymically derived nTreg in cord blood are all Helios$^+$, whether Helios$^-$ circulating naive Treg in healthy adults derive from Helios$^+$ Treg that have lost Helios expression, or from another, yet undefined, population, remains to be addressed. Nonetheless, the absence of Helios$^-$ Treg, that, as suggested by Himmel et al. may be prone to secrete cytokines/chemokines, among cord blood-derived Treg, suggests that the latter should be a preferred source for use in Treg-based adoptive transfer therapy approaches.

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Response to Comment on "Helios$^+$ and Helios$^-$ Cells Coexist within the Natural FOXP3$^+$ T Regulatory Cell Subset in Humans"

Ayyoub et al. raise two long-standing questions in their comment addressing the publication by Himmel et al. (1): what is the best way to define T regulatory cells (Tregs) from different origins in humans; and which population of Tregs is optimal for cell therapy applications? A consistent observation in adult peripheral blood is that, even when only considering CD4$^+$ T cells expressing canonical naive markers, ~30% of FOXP3$^+$ cells do not express Helios (1–3). Because Helios$^-$FOXP3$^+$ Tregs have previously been characterized as cells originating in the periphery upon Ag encounter (4), these data raise the question: are Helios$^-$FOXP3$^+$ cells expressing CD45RA, CCR7, CD62L, and/or CD31 truly naive cells? This is a difficult question to definitively answer in humans, but if so, one would expect to find evidence for their existence in cord blood and thymus. Ayyoub et al. analyzed cord blood and concluded that Helios$^-$FOXP3$^+$ cells are "absent." We note, however, that although these cells are indeed present at lower proportions than in adult blood, they are not completely absent. In our own investigation of cord blood, 16.4 ± 4.9% of FOXP3$^+$ cells in the CD45RA$^+$CD4$^+$ gate were Helios$^-$ (Fig. 1A). When further gated as CCR7$^+$CD25$^+$CD127$^-$ cells, and consistent with Ayyoub et al., this proportion was reduced to 2.9 ± 0.2%. Thus some of the CD45RA$^+$Helios$^-$FOXP3$^+$ cells in cord blood do not express canonical Treg cell surface markers. We also examined the thymus and found that

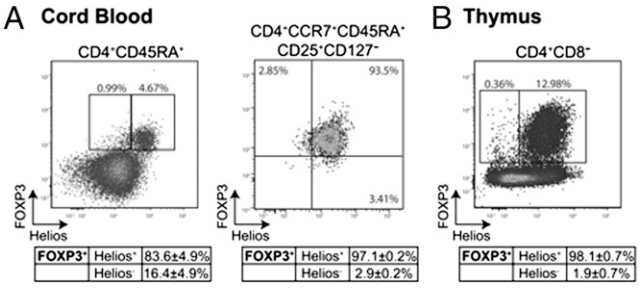

FIGURE 1. Helios$^-$FOXP3$^+$ cells are present in cord blood and thymus. (**A**) Cord blood mononuclear cells obtained from elective Cesarean section deliveries at term were stained with CD4 (eBioscience, SK3), CD8 (BioLegend, RPA-T8), CCR7 (BD Biosciences, 3D12), CD45RA (eBioscience, HI100), CD127 (eBioscience, eBioRDR5), and CD25 (BioLegend, M-A251), then fixed, permeabilized, and stained for FOXP3 (eBioscience, 236A/E7) and Helios (BioLegend, 22F6). (**B**) Thymocytes were isolated from thymi (0, 6, or 17 mo old) by mincing and filtration. Tables report the average ± SD proportion of Helios positive or negative cells within the FOXP3$^+$ gate from independent experiments: (A, *left panel*) $n = 6$, (A, *right panel*) $n = 3$, (B) $n = 3$. Gates were set based on fluorescence minus one controls.

a very small, but detectable, proportion of Helios$^-$FOXP3$^+$ cells was present (Fig. 1B). Overall, these data suggest it is premature to conclude that all Helios$^-$FOXP3$^+$ cells develop in the periphery after birth, and we agree with Ayyoub et al. that further study is required to define the origin of Helios$^-$FOXP3$^+$ cells and to identify the most accurate surface versus intracellular markers with which to track and study them.

Although cord blood is clearly enriched for Helios$^+$FOXP3$^+$ Tregs, it would be difficult to use this source of cells for applications in which delivery of autologous Tregs is desired, unless the patient has banked cord blood. In addition, despite expressing more IFN-γ and CCL3 than Helios$^+$FOXP3$^+$ Treg clones, Helios$^-$FOXP3$^+$ Treg clones are suppressive (1). Indeed, in the context of transplantation, Treg-derived production of IFN-γ is necessary for their protective effect (5, 6). Further research into the mechanisms of Treg action that are critical for their therapeutic effects in different diseases is required to determine the best way to isolate Tregs that express the immunomodulatory effector molecules required for each clinical application.

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