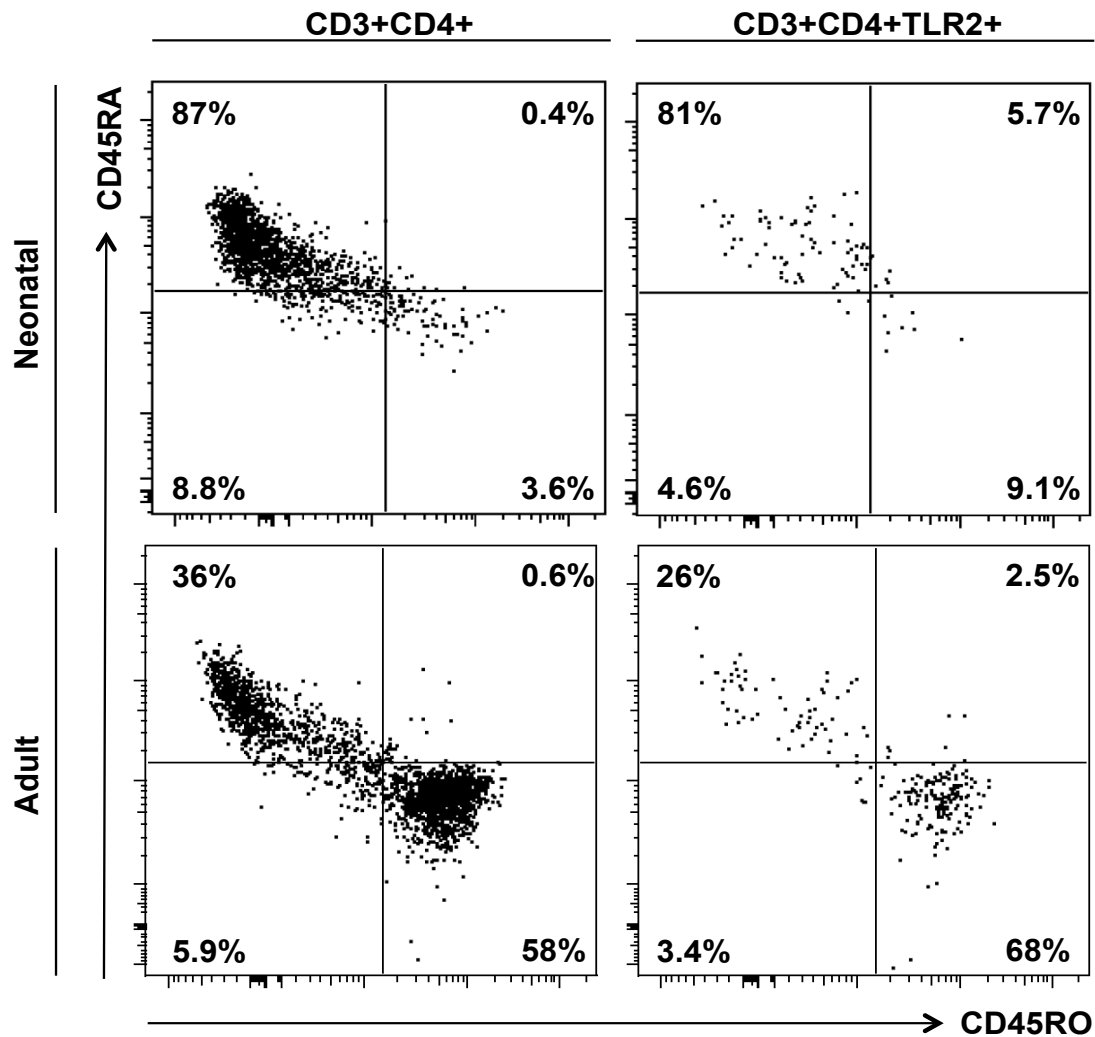
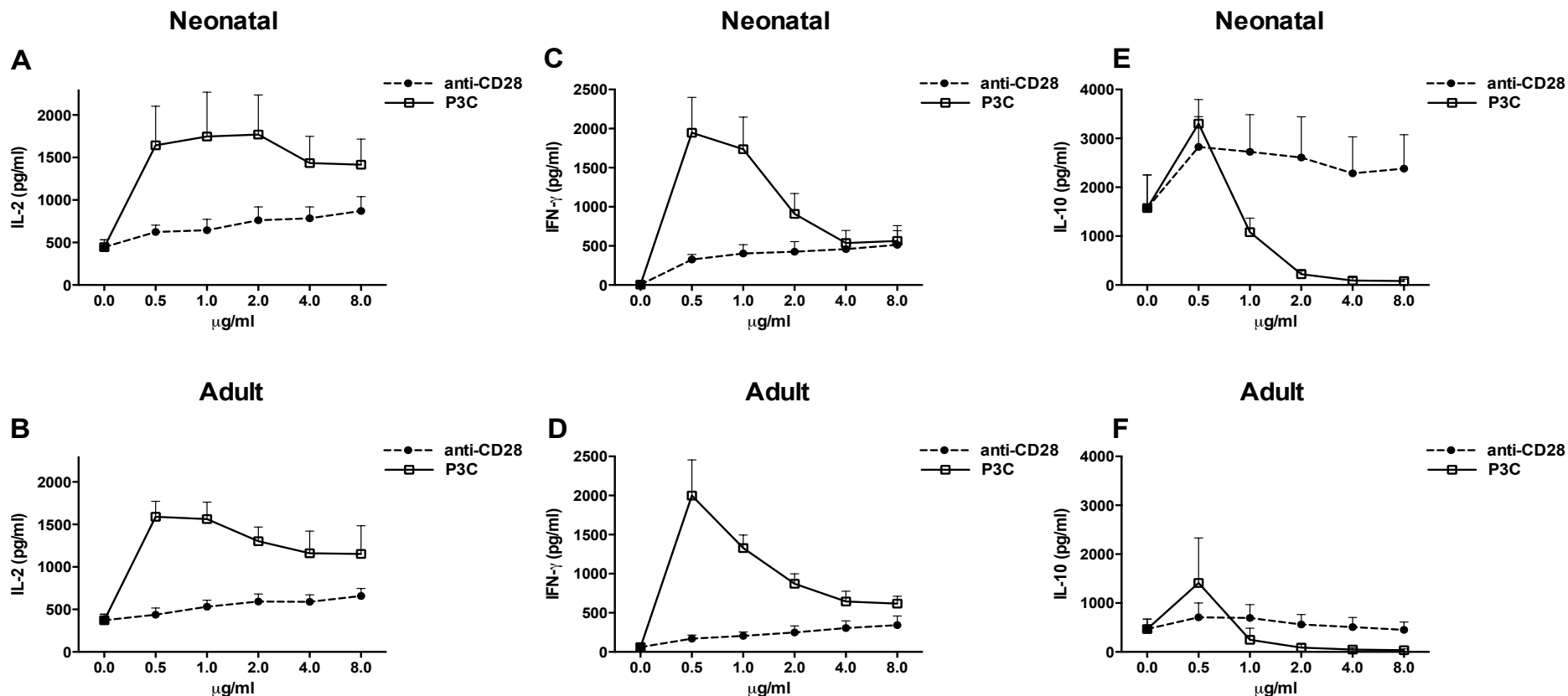


Supplemental Figure 1. Highly purified naive CD4+CD45RA+ T cell subsets are obtained from CBMC and PBMC. To determine the optimal method to obtain naive CD4+ T cell subsets, CD4+ T cells were isolated from PBMC by two, parallel approaches: 1) negative magnetic selection of CD4+ T cells followed by FACS purification for CD4+/CD45RA+/CD14, CD19, CD123, CD1c, CD56<sup>negative</sup> T cells; versus 2) negative magnetic selection of CD4+ T cells followed by FACS purification for CD4+/CD45RA+/CD45RO-/CD14, CD19, CD123, CD1c, CD56<sup>negative</sup> T cells. Naive CD4+ T cells purified by these two methods were then cultured separately in 96-well flat bottom plates (10<sup>5</sup> cells/well) in serum free medium (XVNS-15) and recombinant IL-2 (30 U/ml) with and without: plate-bound  $\alpha$ CD3 (10  $\mu$ g/ml; clone Hit3a), soluble  $\alpha$ CD28 (1  $\mu$ g/ml; clone 28.2), the synthetic TLR-1/2 ligand Pam3Cys4 (P3C; 2  $\mu$ g/ml and 10  $\mu$ g/ml). Cell-free culture supernatants were obtained at 18h and 72h and assessed by ELISA for IL-2 (A) and IFN- $\gamma$  (B), respectively. Shown in A, B are mean values and SD from 4 adult donors. In all subsequent experiments, naive CD4+ T cells are obtained using Method 1 as described above. Purity of CD4+CD45RA+ T cell separation is assessed by post-FACS staining of relevant markers (representative data from an adult donor shown in C). Absence of accessory cell contamination in naive CD4+ T cell preparations is demonstrated by absence of IFN- $\gamma$  production in response to the mitogen PHA as measured by ELISA of 72h culture supernatants (n=9 cord and n=9 adult donors; D). IFN- $\gamma$  production in response to PHA by unfractionated PBMC was significantly greater than that observed by unfractionated CBMC ( $p < 0.001$ ; t-test).

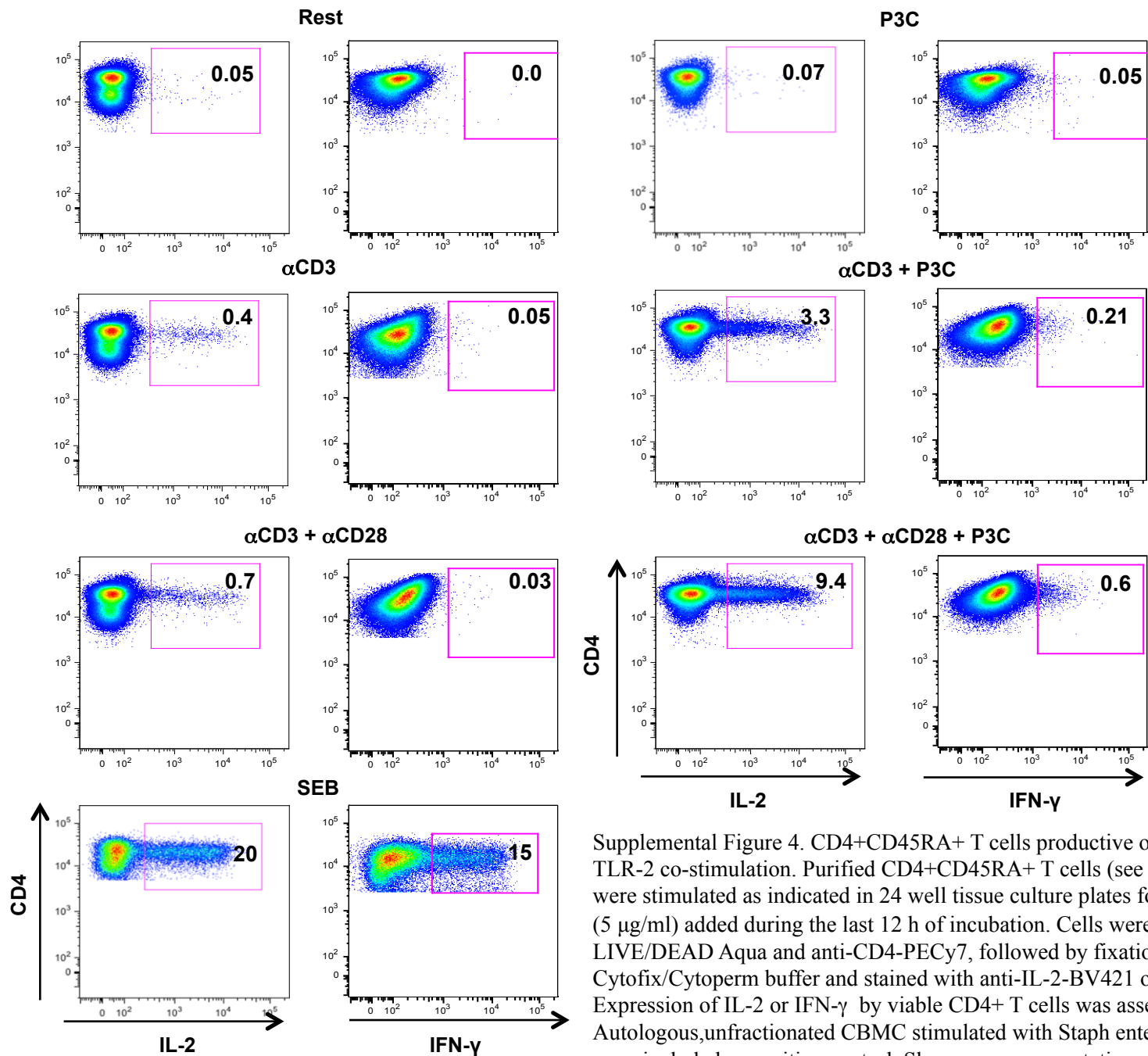


Supplemental Figure 2. CD45RA and CD45RO expression on total CD3+CD4+ T cells and CD3+CD4+TLR2+ T cells from neonatal and adult donors. Freshly thawed, unfractionated CBMC and PBMC specimens were stained with anti-CD3-PE, anti-CD4-PECy7, anti-CD45RA APC H7, anti-CD45RO BV 421, and anti-TLR-2-Alexa 647. Shown are representative dot plots from one neonatal and one adult donor.



Supplemental Figure 3. Dose response to P3C versus anti-CD28 by naïve neonatal and adult CD4+ T cells.

Naïve CD4<sup>+</sup>CD45RA<sup>+</sup> T cells purified from cryopreserved CBMC/PBMC by negative magnetic selection of CD4<sup>+</sup> T cells followed by FACS purification for CD4<sup>+</sup>/CD45RA<sup>+</sup>/CD14<sup>-</sup>, CD19<sup>-</sup>, CD123<sup>-</sup>, CD1c<sup>-</sup>, CD56<sup>negative</sup> subsets, were cultured in 96-well flat bottom plates (10<sup>5</sup> cells/well) in serum free medium (XVNS-15) and recombinant IL-2 (30 U/ml) with plate-bound αCD3 (10 µg/ml; clone Hit3a) and with or without increasing concentration of: soluble αCD28 (0.5-8 µg/ml; clone 28.2), or the synthetic TLR-1/2 ligand P3C (0.5-8 µg/ml). Cell-free culture supernatants were obtained at 18h and 72h and assessed by individual ELISAs for IL-2 (A,B) and IFN-γ (C,D) plus IL-10 (E,F), respectively. Shown are mean values ± SEM from 5 neonatal and 5 adult donors.



Supplemental Figure 4. CD4<sup>+</sup>CD45RA<sup>+</sup> T cells productive of cytokine following direct TLR-2 co-stimulation. Purified CD4<sup>+</sup>CD45RA<sup>+</sup> T cells (see T cell stimulation assays) were stimulated as indicated in 24 well tissue culture plates for 18 - 72 h, with Brefeldin A (5  $\mu$ g/ml) added during the last 12 h of incubation. Cells were harvested and stained with LIVE/DEAD Aqua and anti-CD4-PECy7, followed by fixation and permeabilization in Cytofix/Cytoperm buffer and stained with anti-IL-2-BV421 or anti-IFN- $\gamma$  FITC. Expression of IL-2 or IFN- $\gamma$  by viable CD4<sup>+</sup> T cells was assessed by flow cytometry. Autologous, unfractionated CBMC stimulated with Staph enterotoxin B (SEB; 5  $\mu$ g/ml) were included as positive control. Shown are representative plots from two neonatal donors.