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Supplemental Information

Expansion of Human Tregs from Cryopreserved Umbilical Cord Blood for GMP-Compliant Autologous Adoptive Cell Transfer Therapy

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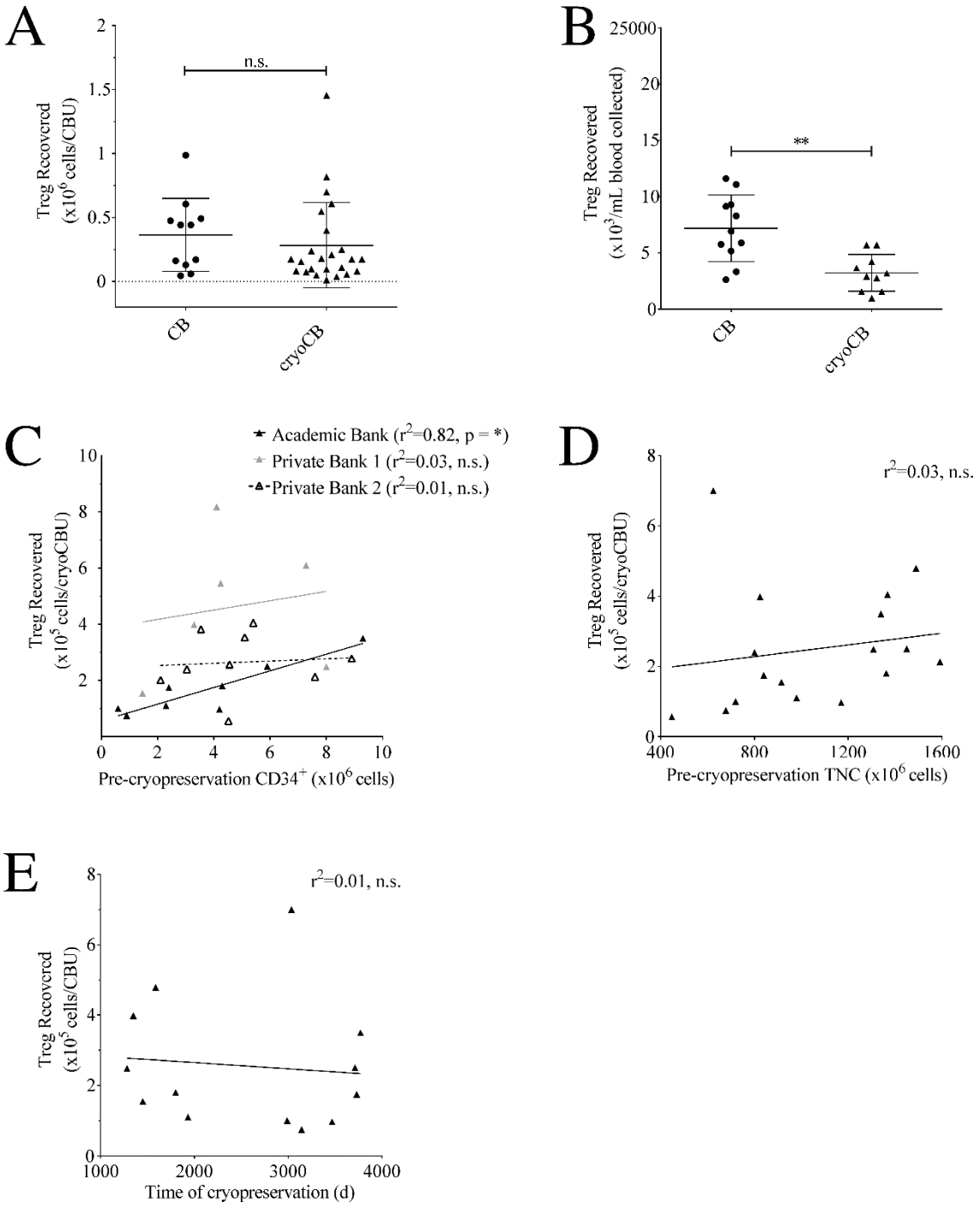
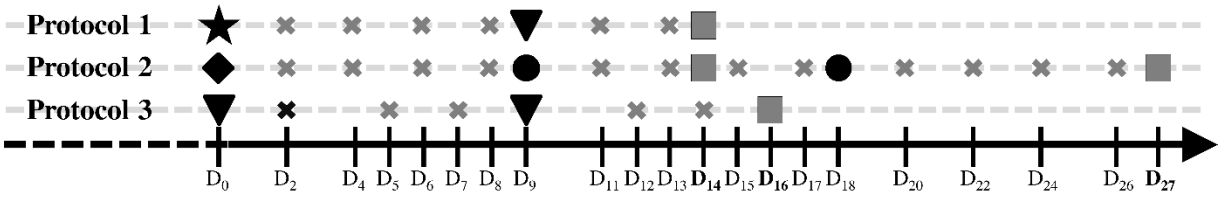
Supplement

Supplementary Figure 1



Supplementary Figure 1. Regulatory T cell (Tregs) recovery from fresh umbilical cord blood (CB) and cryopreserved umbilical cord blood (cryoCB). The number of Tregs isolated (A) per umbilical cord blood units (CBU) was not significantly different for CB versus cryoCB (Welsh's unpaired t-test, NS = $P \geq 0.05$), but (B) Treg per mL of cord was significantly less is cryoCB (Welsh's unpaired t-test; $^{**}P < 0.01$). (C) $CD34^{+}$ cell count prior to cryopreservation was significantly correlated with Treg recovery from cryoCB units obtained from an academic CB bank [University of Florida Stem Cell Lab (UFSCL), black triangles] ($^{*}P < 0.05$, $r^2 = 0.823$) but there was no correlation for cryoCB units obtained from either private bank institution (Private Bank 1 and 2, gray and open triangles, respectively) (NS = $P \geq 0.05$, $r^2 = 0.028$). (D) Total nucleated cell (TNC) count prior to cryopreservation and (E) duration of cryopreservation (d=days) were not significantly correlated with Treg recovery from cryoCB (NS = $P \geq 0.05$; $r^2 = 0.001$ and $r^2 = 0.009$, respectively). (C-E) Statistical tests applied were linear regression with Pearson correlation.

Supplementary Figure 2



Stimulation Methods

- Artificial APC (1:1)
- αCD3/αCD28 GMP ExpAct Beads (4:1)
- αCD3/αCD28 GMP Dynal Beads (1:1)
- αCD3/αCD28 GMP ExpAct Beads (1:1)
- Addition of media and IL-2 (300U/mL)
- Addition of media and IL-2 (600U/mL)
- FOXP3 and Functional Analysis

Figure S1. Timeline for *in vitro* expansion of Tregs isolated from fresh or cryopreserved umbilical cord blood according to three unique protocols. Days (D_0-D_{27}) are indicated along the x-axis, and the manipulations to the culture are indicated using symbols defined in the figure. Protocol 1 follows a standard laboratory procedure for expanding Tregs for experimental purposes using artificial antigen-presenting cells (aAPC) loaded with anti-CD3 (OKT3). Protocols 2 and 3 involve Treg stimulation with anti-CD3 and anti-CD28 conjugated beads.

Supplementary Table 1.

Culture Flask/Plate	Minimum	Suggested	Maximum*
96-well, round-bottom plate	25µL	50-200µL	360µL
96-well, flat-bottom plate	50µL	100-200µL	360µL
48-well plate	100µL	190-285µL	1300µL
24-well plate	200µL	380-570µL	2.6mL
12-well plate	400µL	0.76-1.14mL	6mL
6-well plate	800µL	1.9-2.9mL	15mL
T-12.5	1mL	2.5-3.75mL	8mL
T-12.5 (upright)	250µL	500µL	20mL
T-25	2mL	5-7.5mL	15mL
T-25 (upright)	500µL	1mL	40mL
T-75	8mL	15-22.5mL	75mL
T-75 (upright)	2mL	4mL	175mL
T-175	20mL	35-52.5mL	80mL
T-175 (upright)	5mL	10mL	400mL
T-225	28mL	45-67.5mL	125mL
T-225 (upright)	7mL	14mL	1000mL

*Some wide-mouth flasks with have a lower maximum volume.

Table S1. Cryopreserved cord blood Treg (cryoCB Treg) culture plating volume recommendations. A key technical aspect of ensuring the expansion of CB Treg is plating the cells for stimulation and restimulation at 5.0 x 10^5 cells per mL of culture media in a vessel that will allows for doubling the volume at day 2 of culture without disturbing the cells. To that end, we made a reference table to assist those trying to reproduce the protocol.