

# Robust and cost effective expansion of human regulatory T cells highly functional in a xenograft model of graft-versus-host disease

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## Online Supplementary Design and Methods

### Phenotypic analyses

Flow cytometry analysis was performed using a FACSCalibur (BD Biosciences), and monoclonal antibodies CD4, CD8, CD25, CD62L, CD127, CCR5, CCR7 and FoxP3 (eBioscience Inc., San Diego, CA, USA) were used. Data were analyzed using CellQuest Pro software (BD Biosciences).

### Suppression assays

The *in vitro* suppressive capacity of freshly isolated nTregs and expanded nTregs was assessed using a carboxyfluorescein succinimidyl ester (CFSE)-based inhibition assay.<sup>1</sup> PBMC were labeled with 1.5 M CFSE (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and then stimulated with irradiated (40 Gy) allogeneic PBMC (at a 4:1 effector/feeders ratio) and OKT3 (0.5 µg/mL). To assess their suppressive capacity, expanded nTregs and expanded CD8<sup>+</sup> cells were added to the culture at a 1:1 ratio. As positive control, we used freshly isolated nTregs. On Day 7 of culture, cells were labeled with PE-, PerCP-, or APC-conjugated CD4 and CD8 mAbs and CFSE dilution was analyzed using a FACSCalibur to measure cell proliferation. Suppression was expressed as the percentage of inhibition of T-cell proliferation in the presence of Tregs as compared to control cells.

### Methylation specific-PCR (MS-PCR) and telomere length assay

DNA was isolated from Treg samples collected at Day 1, S1, S2 and S3. For bisulfate conversion, we used the EZ DNA Methylation kit from Zymo Research, Irvine, CA, USA.<sup>2</sup> The bisulfate-converted DNA was subjected to semi-quantitative PCR using methylation and non-methylation specific FoxP3 specific primers to detect -58, -65 and -77 CpG methylation sites in the FoxP3 promoter. Telomere length was

assessed by quantitative real-time PCR as described previously.<sup>3</sup>

### Xenogeneic GvHD model

The animal protocol was approved by the IACUC at Baylor College of Medicine. On Day -1, NOG/SCID/γc<sup>-/-</sup> mice (Jackson Lab, Bar Harbor, Maine, USA) were irradiated at 160 Gy and on Day 0, infused by tail vein injection, with PBMC (15 × 10<sup>6</sup>) alone or in combination with expanded nTregs or expanded control CD25<sup>DRP</sup> cells at a 1:1 ratio. Mice were assessed for symptoms of GvHD daily and weighed once a week.

### Tissue processing and immunohistochemistry

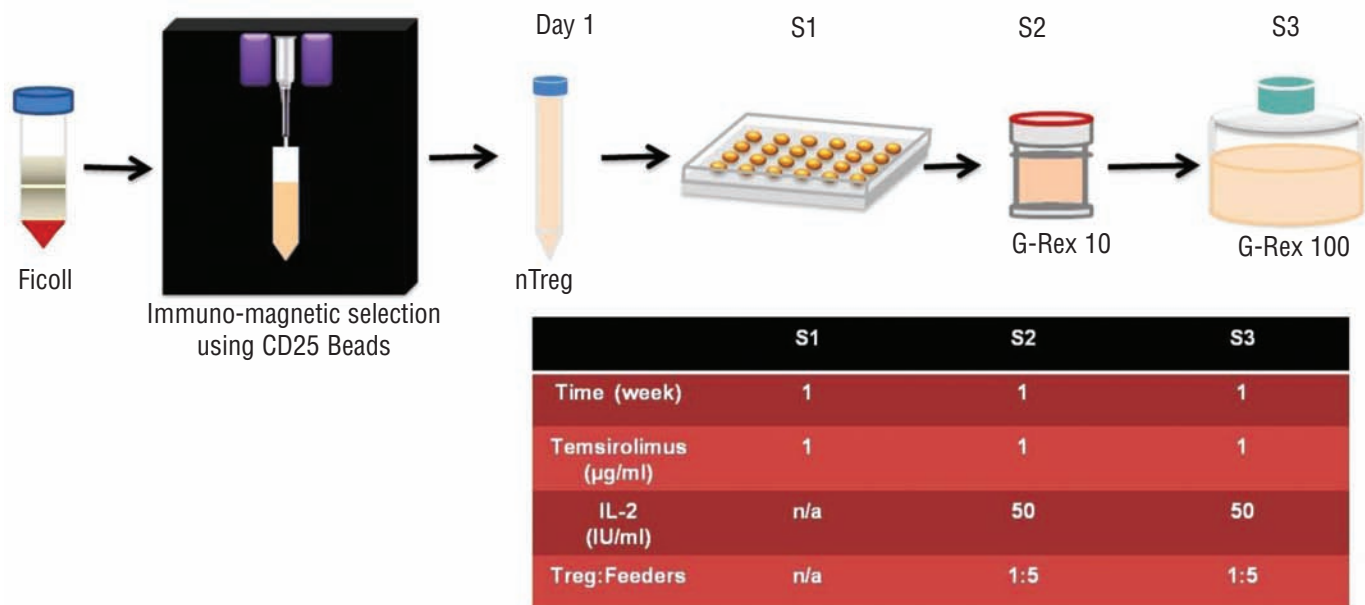
Tissue samples were fixed, processed and stained according to standard procedures. We performed H&E staining and labeling of human T cells using the CD8 mAb (clone 1C7; PharMingen, Mississauga, Ontario, Canada) and the secondary rat anti-mouse IgG HRP-conjugated mAb (Jackson ImmunoResearch, West Grove, PA, USA).

### Statistical analyses

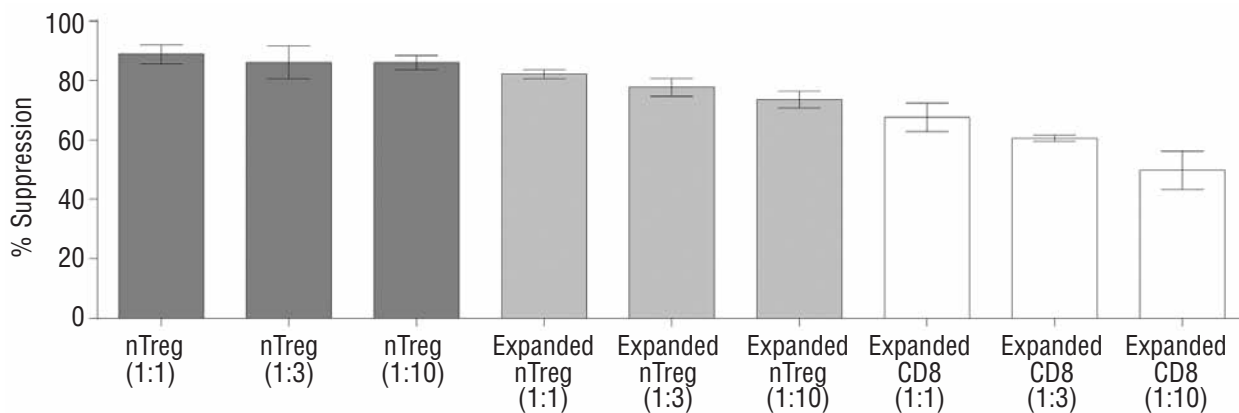
All *in vitro* experiments were summarized as mean ± SD. Student's t-test was used to determine the statistical significant differences between samples, with *P* < 0.05 indicating a statistically significant difference.

## References

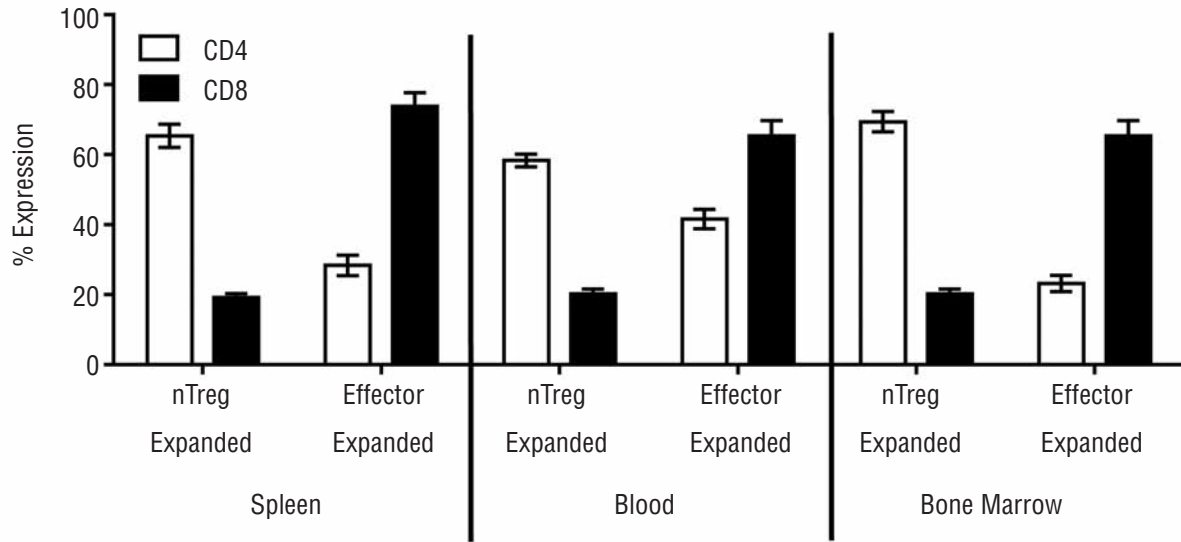
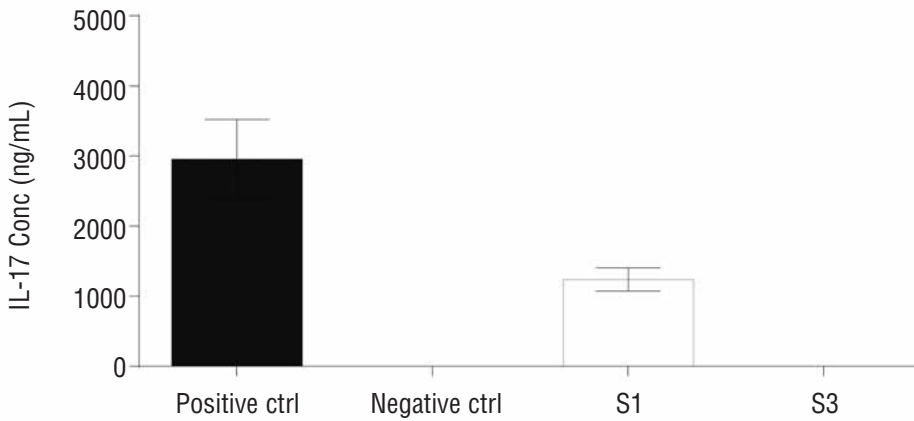
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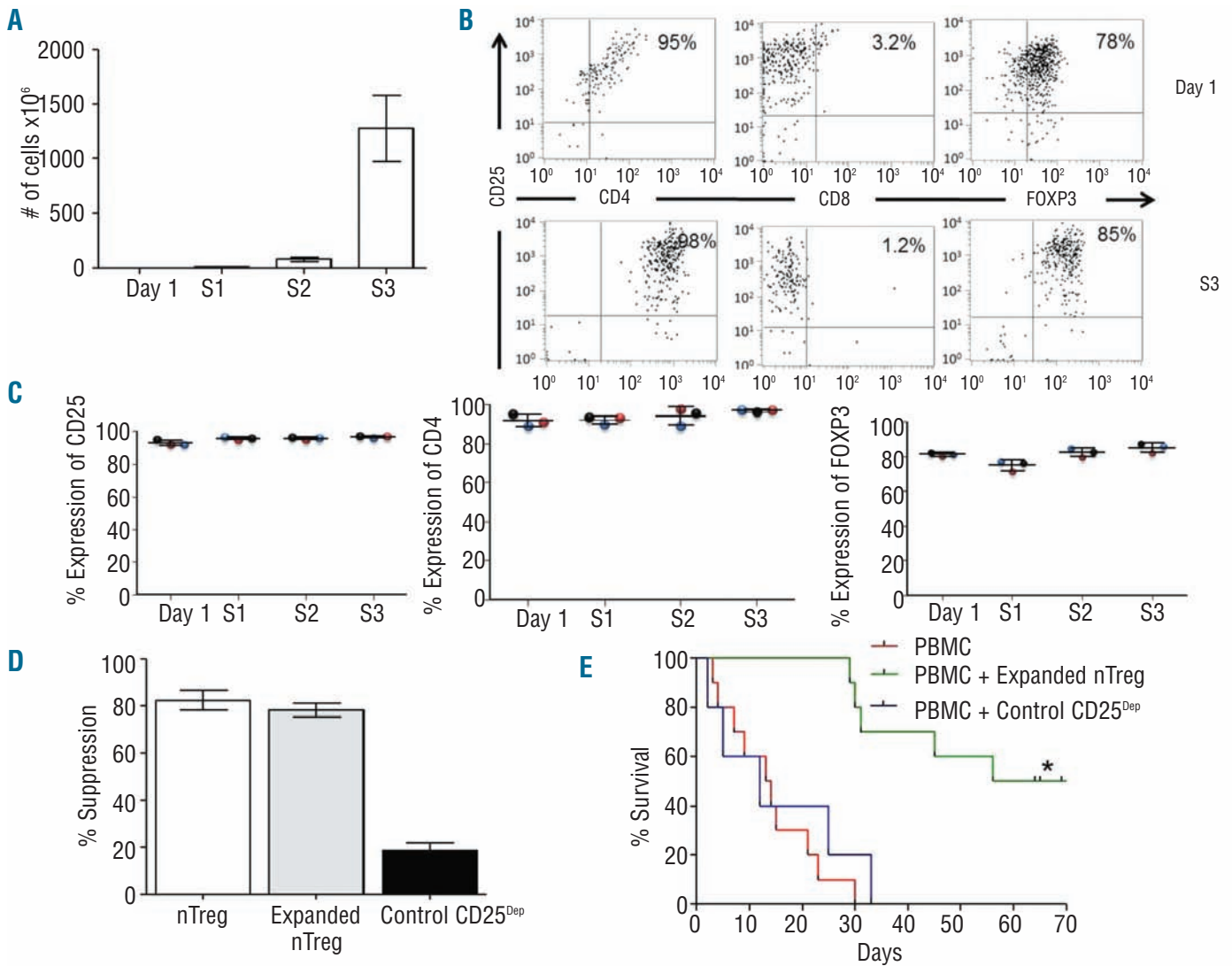
Online Supplementary Figure S1. Schema of the procedure used to select and expand nTregs. Schematic representation of the procedure used to select the nTregs using magnetic columns (small columns or CliniMACS device) and to expand the nTregs using the G-Rex device.



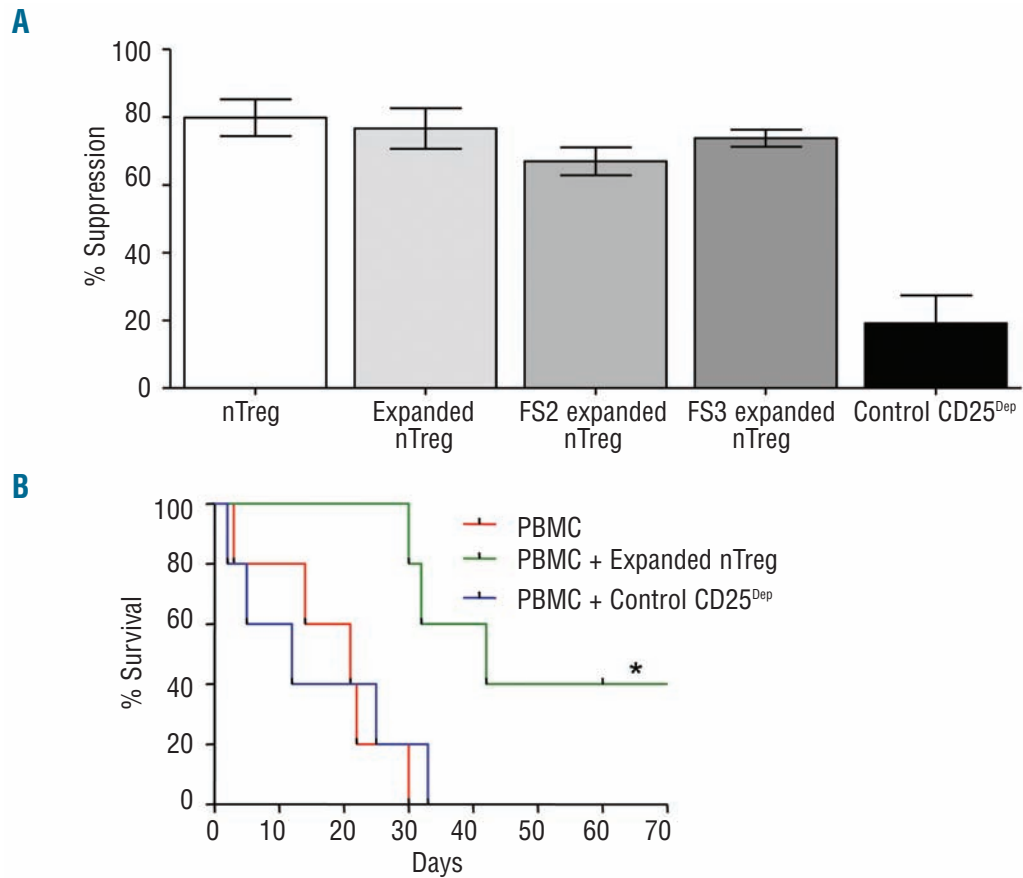
Online Supplementary Figure S2. Contaminant CD8<sup>+</sup> cells maintain potent *in vitro* suppressive function. The inhibitory activity of freshly isolated nTregs, expanded nTregs (S3), and CD8<sup>+</sup> cells remaining in the culture at S3 was assessed by using a CFSE-based suppression assay using the indicated ratio of Treg:Teff cells. The graph summarizes average and SD of the inhibitory function for 3 independent experiments.

**A****B**

**Online Supplementary Figure S3.** Engraftment of PBMC in the xenograft mouse model and secretion of IL-17 by expanded nTregs. (A) Tregs co-infused with PBMC did not abrogate the engraftment of PBMC. Phenotypic analysis of CD4 and CD8 expression in T cells isolated from spleen, blood, and bone marrow, respectively, of mice infused with expanded nTregs as compared to those receiving CD25<sup>DEP</sup> cells. n = 5 for each group. Data show mean ± SD. (B) IL-17 was quantified in supernatants obtained from expanded nTregs after 7 days (S1) or 21 days (S3). Serum free culture media and recombinant IL-17 served as negative and positive controls, respectively.



**Online Supplementary Figure S4.** Tregs selected and expanded using the CliniMACS and G-Rex devices, respectively maintain potent suppressive function both *in vitro* and *in vivo*. To investigate whether the expansion of fully functional nTregs can be obtained by isolating nTregs using the clinical grade CliniMACS device, we performed 3 large-scale experiments. **(A)** The graph illustrates the number of cells obtained after each round of stimulation (S1, S2 and S3) in the G-Rex device. **(B)** Representative flow-cytometry plots of expanded nTregs by Day 21 (S3) of culture. **(C)** Panels illustrate the expression of CD25, CD4 and FoxP3 by expanded nTregs after each round of stimulation (S1, S2 and S3). **(D)** Suppressive activities of freshly isolated nTregs, expanded nTregs (S3) and control CD25<sup>Depleted</sup> cells as assessed by CFSE-based assays. **(E)** Kaplan-Meier curve comparing survival of NSG mice receiving human PBMC either alone or in combination with expanded nTregs (S3) or expanded control CD25<sup>Depleted</sup> cells (n=12 animals for each group) (\*P=0.0046).



**Online Supplementary Figure S5.** Cryopreservation and thawing do not affect the functionality of expanded nTregs. (A) The suppressive activities of freshly isolated nTregs, expanded nTregs and cryopreserved expanded nTregs obtained after second (FS2) and third (FS3) stimulation were assessed using a CFSE-based suppression assay. (B) Kaplan-Meier survival curve comparing survival of NSG mice infused with PBMC alone or in combination with either cryopreserved expanded nTregs (FS3) or control expanded CD25<sup>Depleted</sup> cells (n=5 animals for each group) (\*P=0.04).