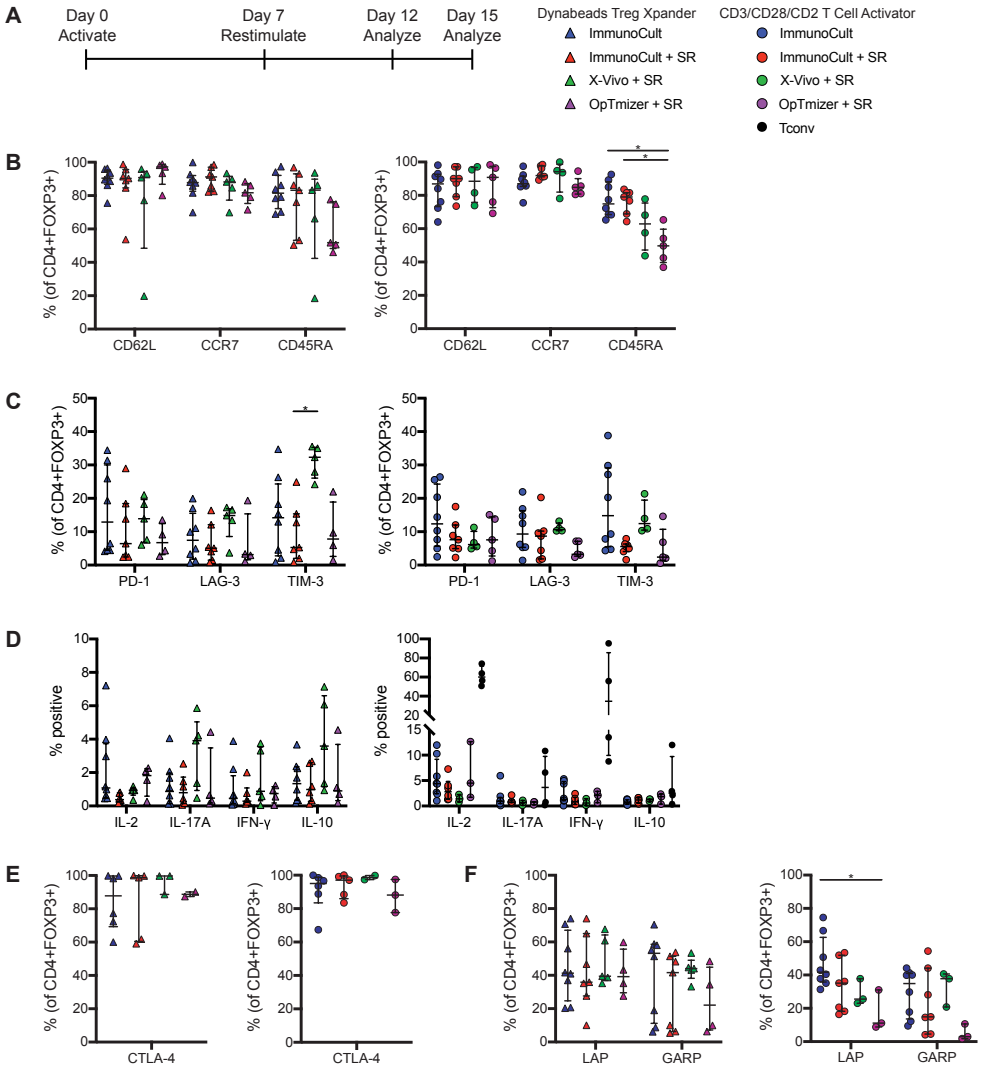
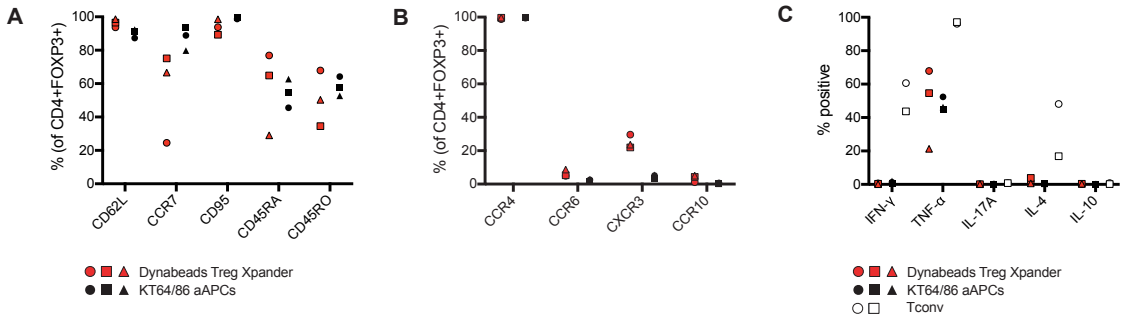
Supplemental Figures

Supplementary Figure 1



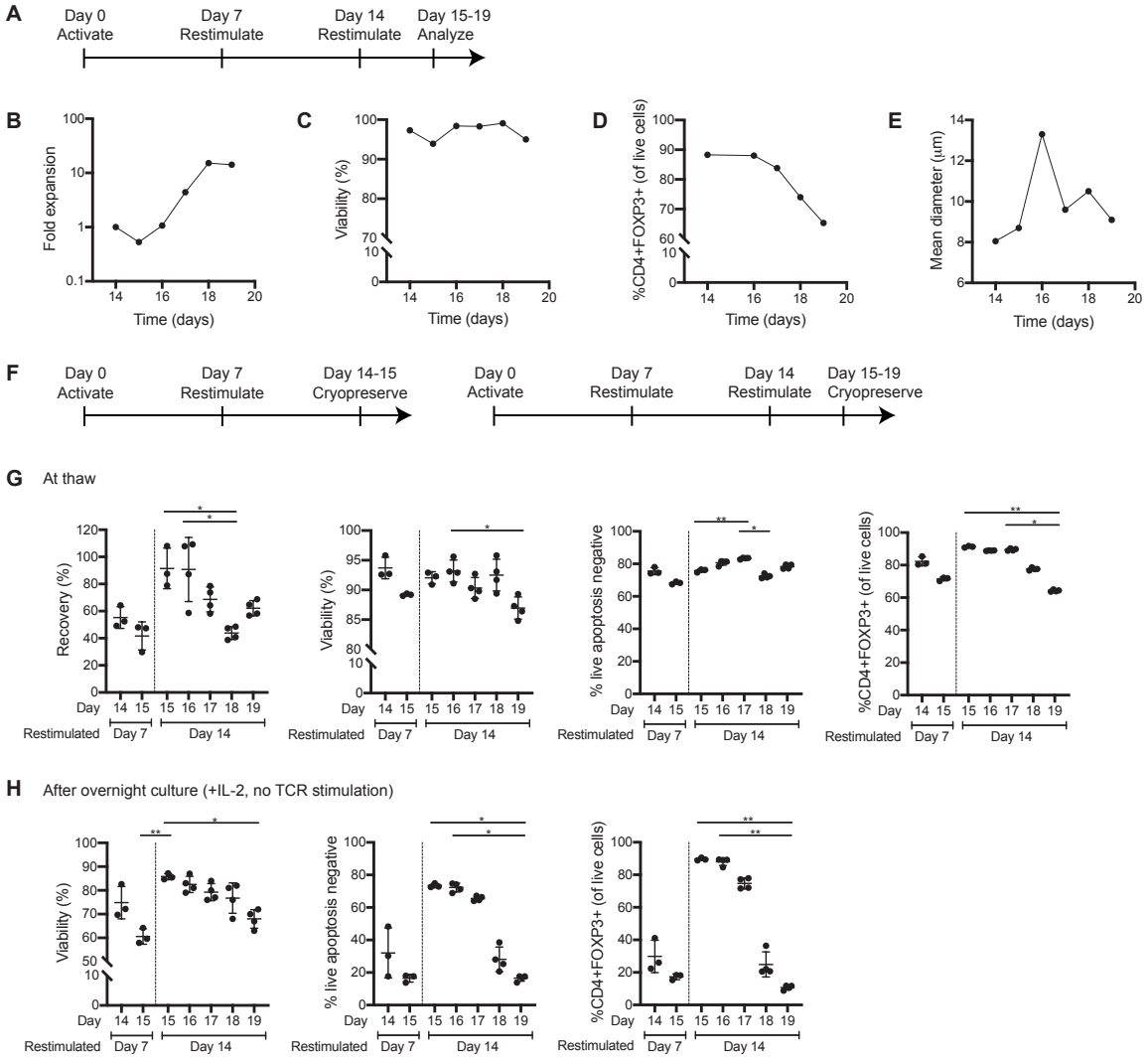
Supplemental Figure S1: *Effect of cell culture media on thymic Treg expansion with Dynabeads Treg Xpander or CD3/CD28/CD2 T Cell Activator.*
Isolated thymic Tregs were expanded and restimulated with Dynabeads Treg Xpander or CD3/CD28/CD2 T Cell Activator using different types of media as indicated in (**A**)**.** After 12 days, cells were analyzed for expression of markers of T cell (**B**) differentiation or (**C**) activation. (**D**) Expression of intracellular cytokines in Tregs (CD4+FOXP3+) and Tconv was determined after 4 hours of activation with PMA, ionomycin and brefeldin A. (**E**) Expression of CTLA-4, or (**F**) LAP and GARP after 24 hours of activation with anti-CD3/CD28 beads at a 1:16 bead to cell ratio. Within each group, each symbol represents cells from a different subject and bars indicate median ± interquartile range. n=6-8 for ImmunoCult, n=4-7 for ImmunoCult + SR, n=3-5 for X-Vivo + SR, n=2-5 for OpTmizer + SR, and n=4 for Tconv tested in 4-5 experiments. **P* < 0.05 as determined by a Kruskal-Wallis test with Dunn's multiple comparisons test to compare protein expression between conditions.

Supplementary Figure 2



Supplemental Figure S2: *Phenotype of thymic Tregs expanded using KT64/86 aAPCs or Dynabeads Treg Xpander.*
Isolated thymic Tregs were expanded using KT64/86 aAPCs or Dynabeads Treg Xpander activation reagents. After 15 days, cells were analyzed for expression of (**A**) markers of T cell differentiation, (**B**) chemokine receptors or (**C**) intracellular cytokines in Tregs (CD4+FOXP3+) and Tconv after 4 hours of activation with PMA, ionomycin and brefeldin A. Within each group, each symbol represents cells from a different subject and matched subjects are shown with the same symbol. n=3 for Dynabeads Treg Xpander, n=3 for KT64/86 aAPCs, and n=2 for Tconv from 1-3 experiments.

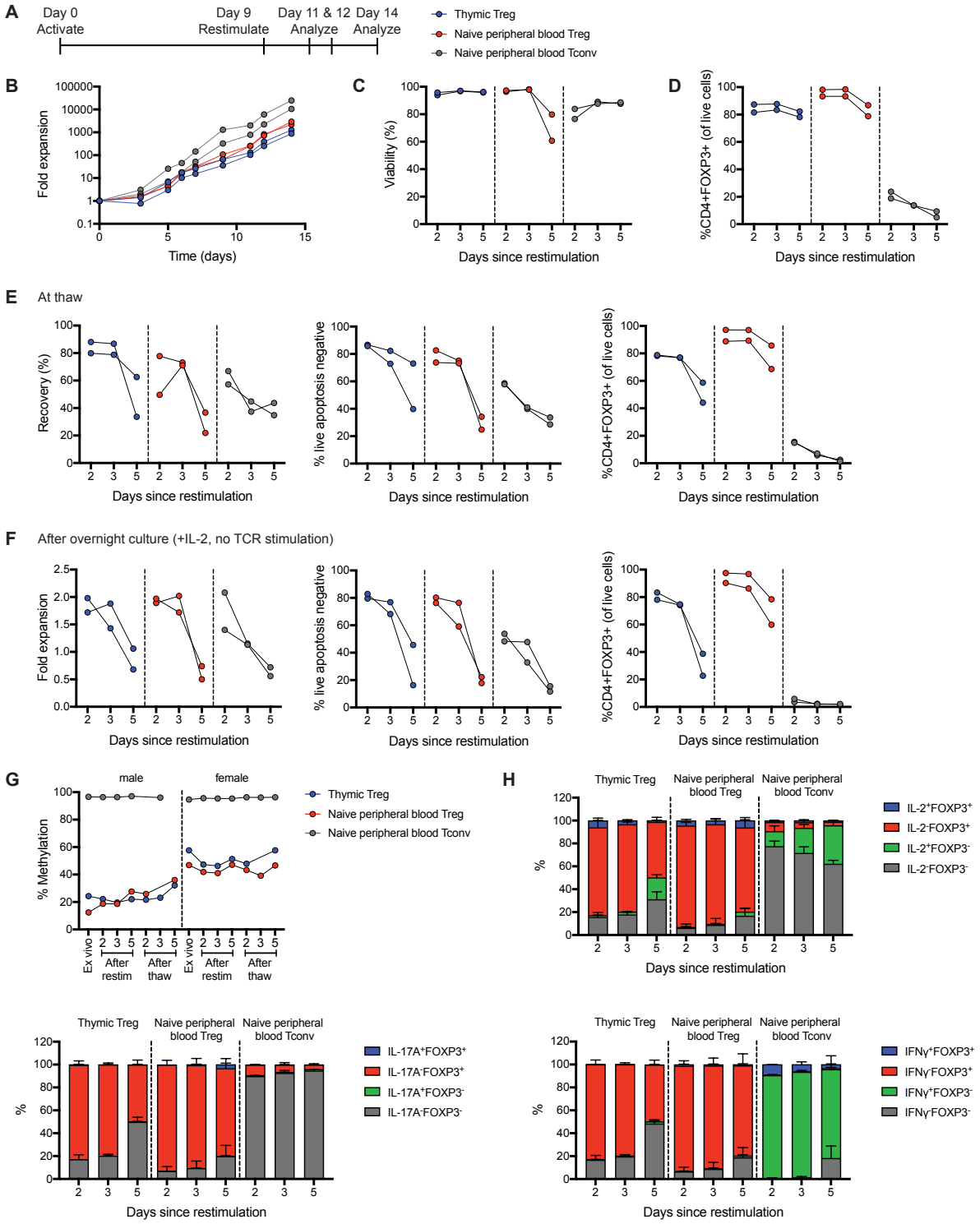
Supplementary Figure 3



Supplemental Figure S3: Cryopreservation of thymic Tregs following a second restimulation at day 14.

Isolated thymic Tregs were activated at day 0, restimulated at days 7 and 14, and analyzed at day 15-19 (**A**). (**B**) Fold expansion, (**C**) viability, (**D**) FOXP3 expression, and (**E**) mean diameter of thymic Tregs following restimulation at day 14. (**F**) Isolated thymic Tregs were activated at day 0, restimulated at days 7 and 14, and cryopreserved at day 14 or 15 following the first restimulation, or day 15-19 following the second restimulation. (**G**) Recovery (defined as the number of live apoptosis negative cells thawed relative to the number of live cells cryopreserved), viability (measured by acridine orange/propidium iodide staining or a flow cytometry-based apoptosis assay) and FOXP3 expression for cryopreserved thymic Tregs shown at thaw. (**H**) Viability (measured by acridine orange/propidium iodide staining and apoptosis assay) and FOXP3 expression for cryopreserved thymic Tregs after thaw and overnight culture with IL-2. Within each group, each symbol represents a separate vial of cells cryopreserved from the same subject and bars indicate mean ± standard deviation. n=1 from 1 experiment. *$P < 0.05$, **$P< 0.01$ as determined by a Kruskal-Wallis test with Dunn's multiple comparisons test.

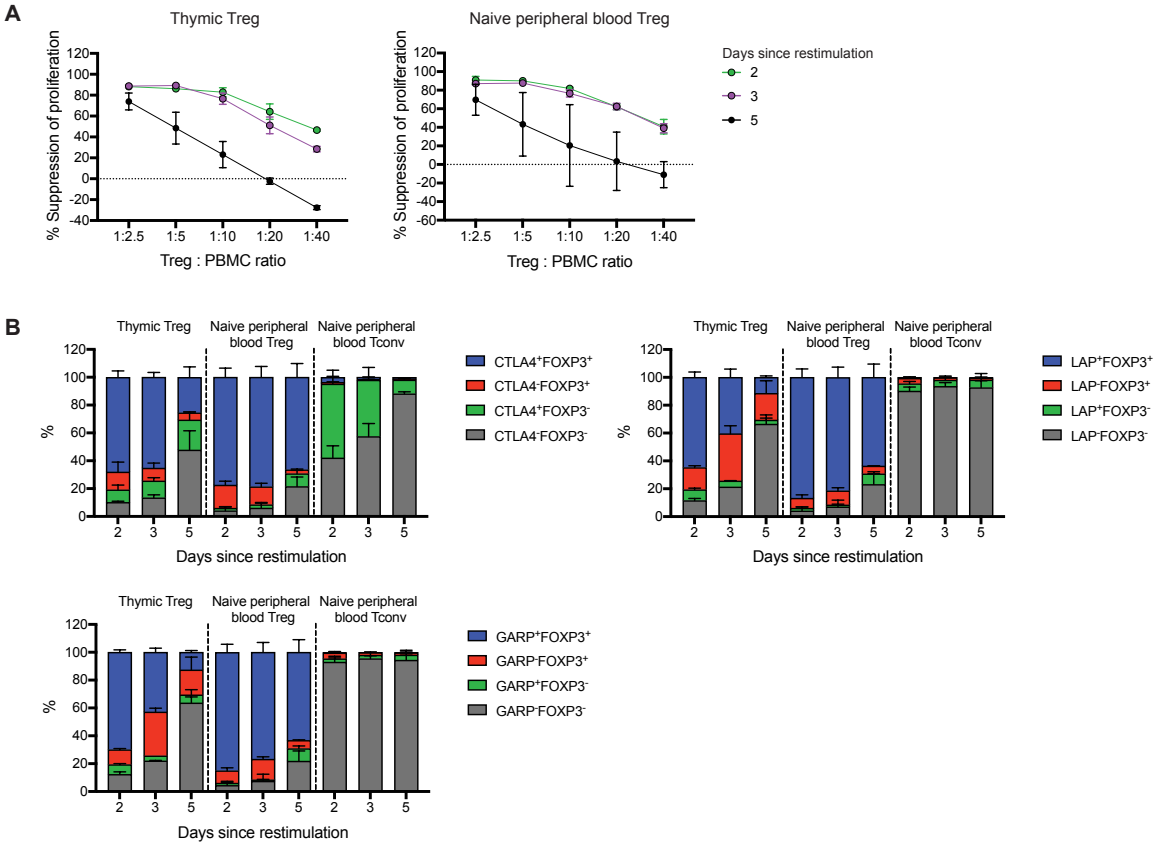
Supplemental Figure 4



Supplemental Figure S4: *Cryopreservation of thymic Tregs, naïve peripheral blood Tregs and naïve peripheral blood Tconv following restimulation at day 9.*

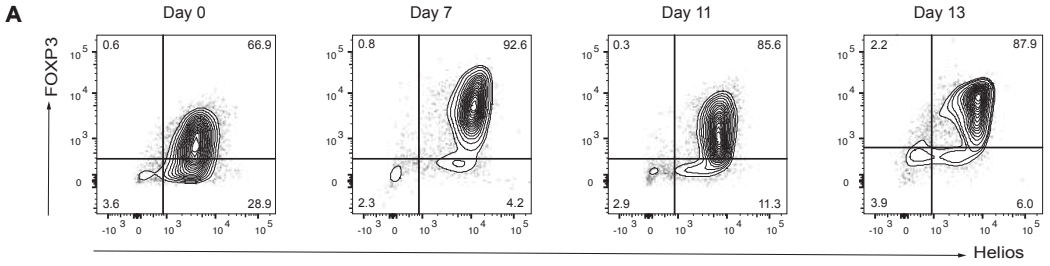
Thymic Tregs, naïve peripheral blood Tregs and naïve peripheral blood Tconv were expanded with Dynabeads Treg Xpander (Treg) or Dynabeads Human T-Expander (Tconv), restimulated at day 9 and cryopreserved 2, 3 or 5 days following restimulation **(A)**. **(B)** Fold expansion of cells over the course of culture. **(C)** Viability and **(D)** FOXP3 expression were measured 2, 3 or 5 days following restimulation. **(E)** Recovery (defined as the number of live apoptosis negative cells thawed relative to the number of live cells cryopreserved), viability (measured by apoptosis assay) and FOXP3 expression for cells upon thawing. Cryopreserved cells were cultured overnight with IL-2 and (**F**) fold expansion, viability (measured by apoptosis assay) and FOXP3 expression were determined. **(G)** TSDR analysis of ex vivo and expanded thymic Tregs and Tconv after restimulation and after thawing and overnight culture. Average data from male and female donors shown is the average methylation for 7 CpGs within the TSDR. **(H)** Intracellular cytokines after 4 hours of activation with PMA, ionomycin and brefeldin A. For B-D&G, each symbol represents cells from a different subject and matched subjects are linked. For E&F, each symbol represents the mean of 3 technical replicates of cryopreserved cells from a different subject. For H, each bar represents the median and range of data from 2 subjects, using the mean of 3 technical replicates of cryopreserved cells per subject. n=2 from 1 experiment.

Supplemental Figure 5



Supplemental Figure S5: *Function of cryopreserved thymic Tregs, naïve peripheral blood Tregs and naïve peripheral blood Tconv.*
Thymic Tregs, naïve peripheral blood Tregs and naïve peripheral blood Tconv were expanded with Dynabeads Treg Xpander (Treg) or Dynabeads Human T-Expander (Tconv), restimulated at day 9 and cryopreserved 2, 3 or 5 days following restimulation. Cells were thawed and cultured overnight prior to testing. (**A**) Tregs were cocultured with CPD-labeled PBMC at the indicated ratios and stimulated 1:16 with anti-CD3/CD28 beads for 4 days. Suppression of $CD8^+$ T cells within PBMC was determined by division index. (**B**) Tregs and Tconv were analyzed for expression of CTLA-4, LAP and GARP. For A, median ± range of subjects is shown. For B, each bar represents the median and range of data from 2 subjects, using the mean of 3 technical replicates of cryopreserved cells per subject. n=2 from 1 experiment.

Supplemental Figure 6



Supplemental Figure S6: FOXP3 expression of thymic Tregs over the course of expansion. Isolated thymic Tregs were expanded with Dynabeads Treg Xpander, restimulated on day 11 and analyzed 2 days following restimulation. Representative flow cytometry data for FOXP3 expression of expanded thymic Tregs, measured at day 0, 7, 11 (prior to restimulation) and 13.