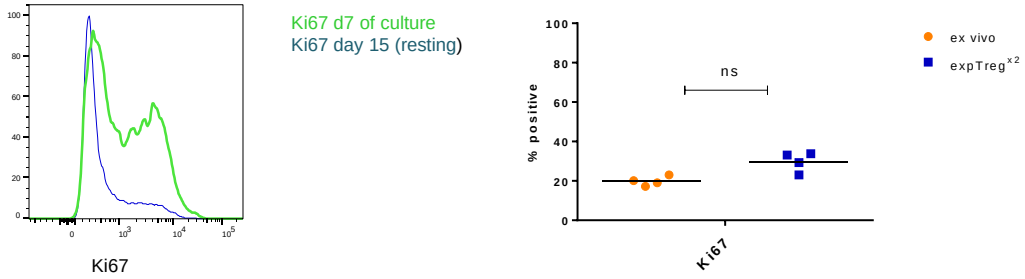
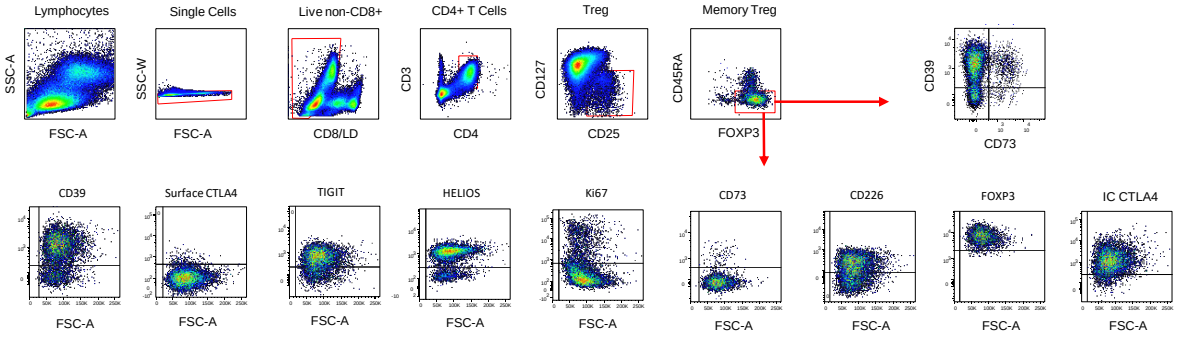
Extended Data Figures

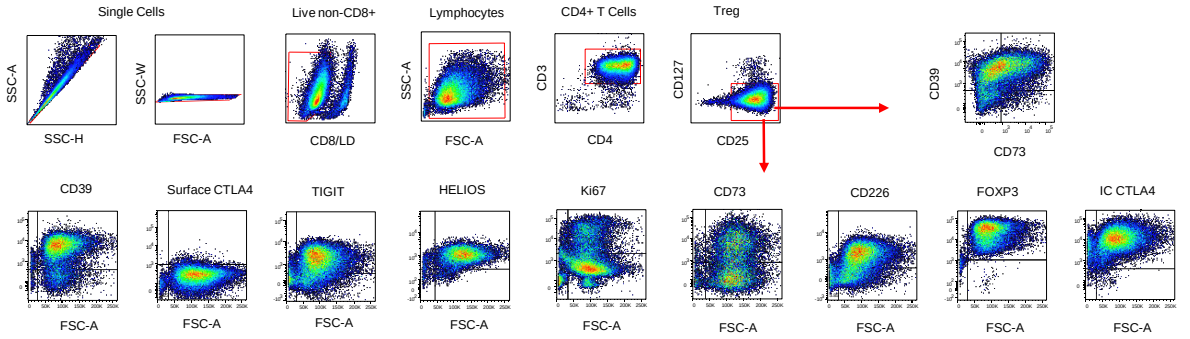


Extended data Figure 1:
ExpTregs return to resting state at end of expansion cycle
Representative example comparing Ki67^{+} proliferating expTregs mid-cycle at day 7 (green) with Tregs at the end of the expansion cycle at day 15 (blue), left hand plot. Ki67 expression was not significantly different between *ex vivo* and expTreg^{x2}, right plot (n = 4 separate donors) in a two-tailed paired Student's t-test.

a **Gating strategy – Ex vivo Treg**



b **Gating strategy – Expanded Treg**



c

Panel for Treg Phenotyping:

SURFACE		Cat no	Clone	INTRACELLULAR		Cat no	Clone
CTLA-4	BV421	BL 369606	BNI3	HELIOS	FITC	BL 137214	22F6
CD3	BV570	BL 300436	UCHT1	CTLA-4	PE-Dazzle	BL 369616	BNI3
CD8	v500	BD 560774	RPA-T8	FOXP3	PE	eBio 12-4776-41	PCH101
CD45RA	BV605	BL 304134	HI100	FOXP3	PE	BL 320207	259D
CD39	BV650	BD 563681	Tu66	Ki67	BUV395	BD 564071	B56
CD73	BV786	BL 344028	AD2				
TIGIT	PerCP-ef1710	eBio 46-9500-42	MBSA43				
CD127	PE-Cy7	eBio 25-1278-42	eBioRDR5				
CD25	APC		BD 2A3				
CD25	APC		MA251				
CD4	APC-R700	BD 564976	RPA-T4				
CD226	APC-Fire750	BL 338320	11A8				

Extended data Figure 2:

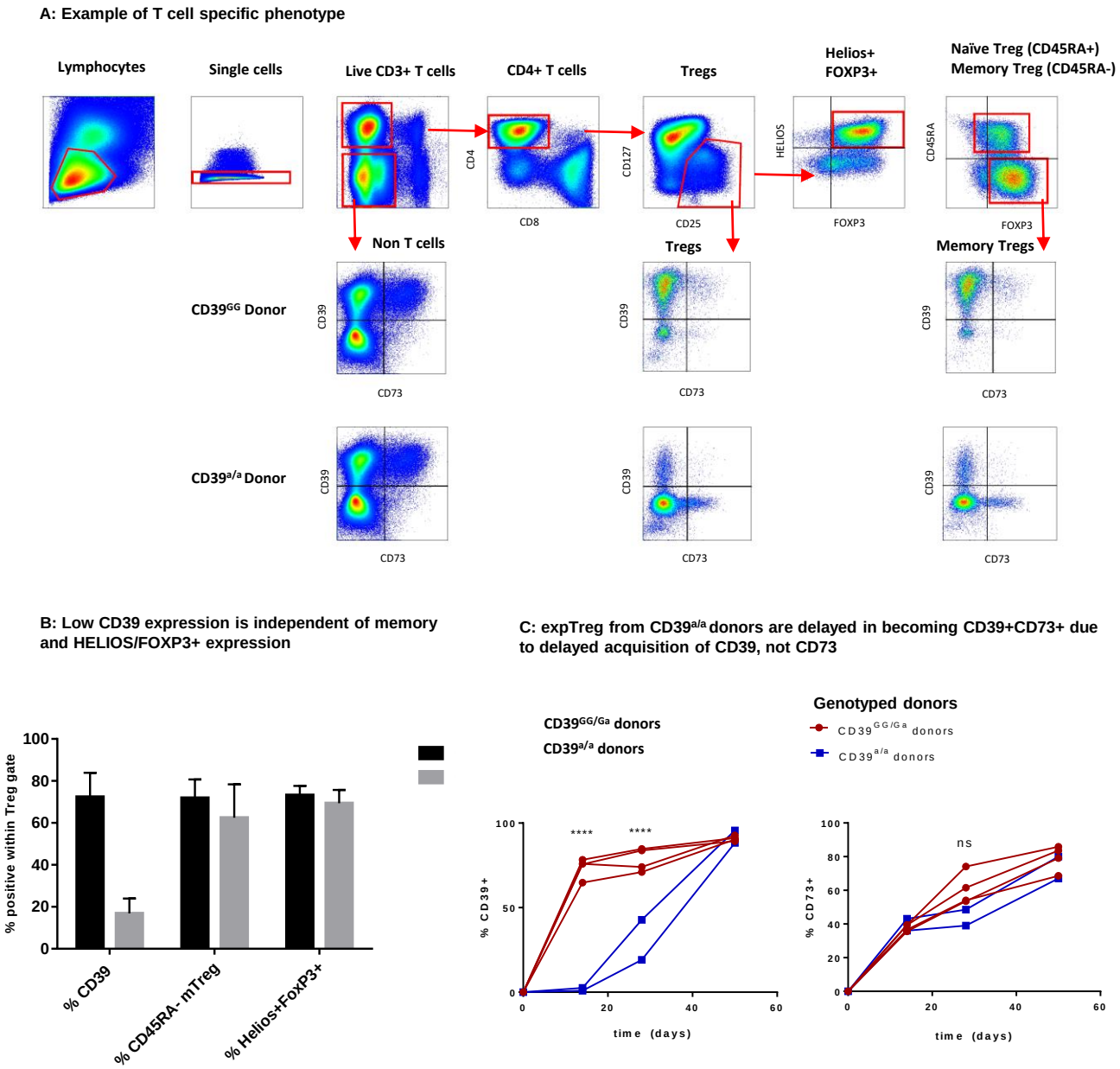
Gating strategy and antibody panel used for *ex vivo* and expTreg phenotyping

A: Gating strategy and representative example of flow cytometric analysis of Treg markers on *ex vivo* Tregs. PBMC were stained by flow cytometry and live CD3+CD4+CD25hiCD127low Tregs were gated. Values in the top right quadrants were used as % positivity.

B: Gating strategy and representative example of flow cytometric analysis of Treg markers on expTregs. Values in the top right quadrants were used as % positivity.

C: The Treg phenotyping flow panel showing all antibodies and clones.

PBMC and expTregs from the same donors were frozen, thawed and stained on the same day using the same antibody panel.



Extended data Figure 3:

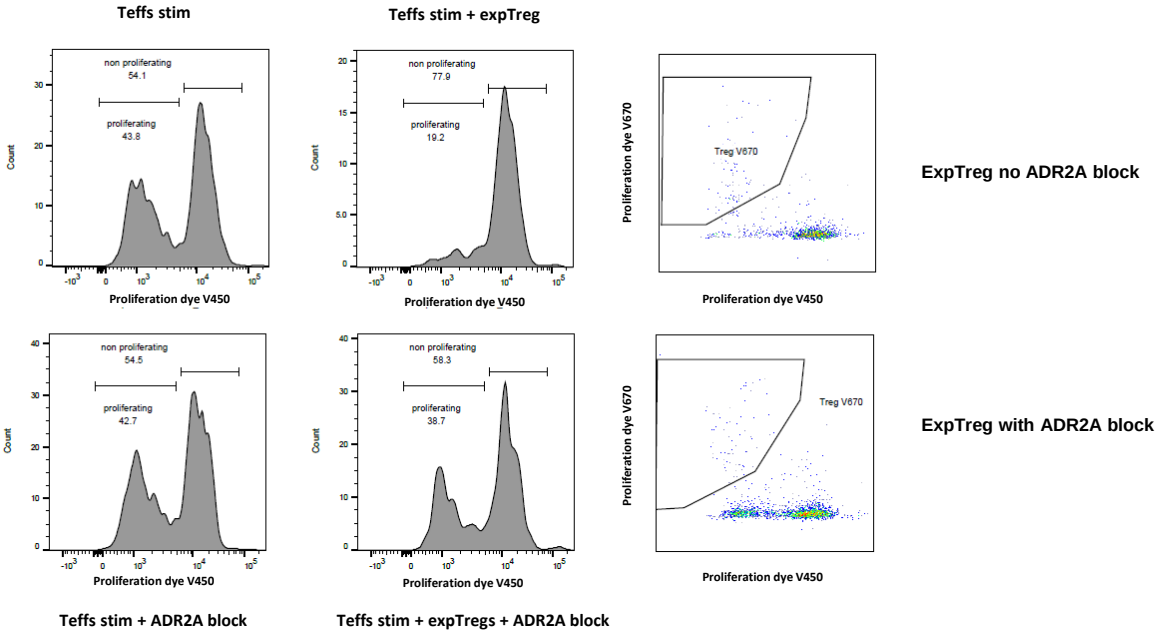
CD39lo$^{a/a}$ Individuals have inherently low T cell expression of CD39

A: Representative gating strategy used for analysing CD39^{+} (CD39+$^{GG/Ga}$) and CD39low (CD39lo$^{a/a}$) donors, demonstrating T cell specific loss of CD39 expression. CD39+$^{GG/Ga}$ and CD39lo$^{a/a}$ donors express normal levels of CD39 on non-T cells (left lower dot plots) but CD39lo$^{a/a}$ donors have reduced levels on Tregs (middle dot plot). Low CD39 is not due to differences in proportion of memory Tregs, since memory (CD45RA^{-}FOXP3^{+}) Tregs also lack CD39 in CD39lo$^{a/a}$ donors (right lower dot plots).

B: CD39 expression in CD39lo$^{a/a}$ donors is independent of memory and CD39lo$^{a/a}$ donors have similar proportion of HELIOS^{+}FOXP3^{+} double positive Tregs as shown by summary data of 3 CD39+$^{GG/Ga}$ and 3 CD39lo$^{a/a}$ donors, analysed by flow cytometry (bar chart shows mean % positivity within CD3^{+}CD4^{+}CD25^{hi}CD127^{lo} Treg gate +/- 1 SD).

C: Comparison of CD39 expression (left) and CD73 expression (right) over time on expanded CD39^{-} CD73^{-} DN Tregs from CD39+$^{GG/Ga}$ and CD39lo$^{a/a}$ donors, demonstrating delayed acquisition of CD39 but not CD73. (Two way ANOVA with Bonferroni multiple testing correction).

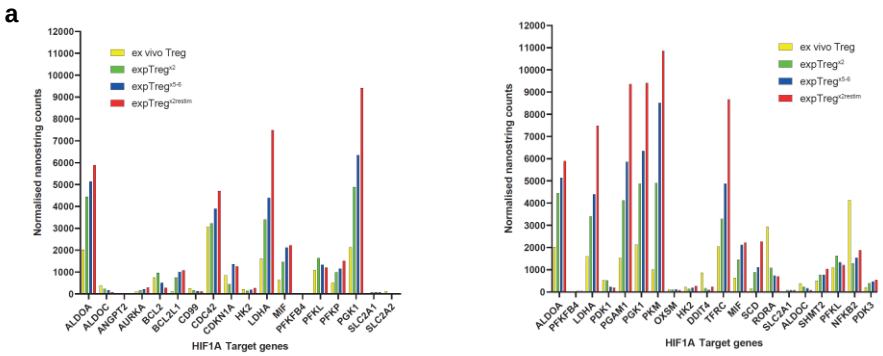
*P <0.05, **P <0.01, ***P <0.001, ****P <0.0001.

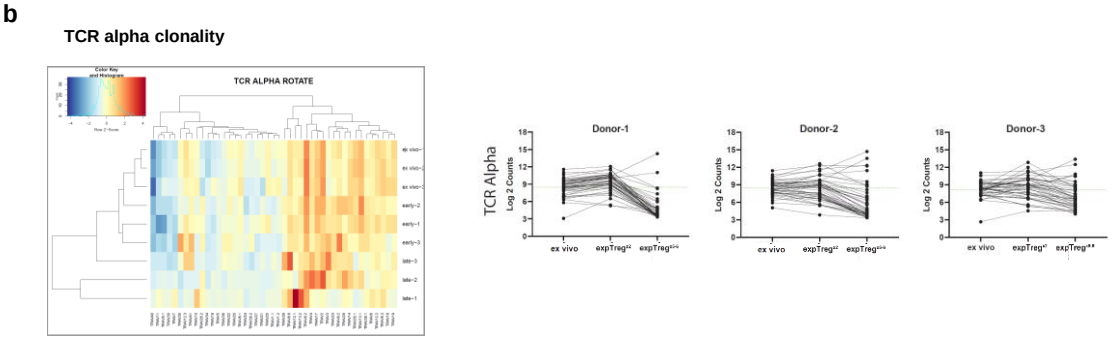


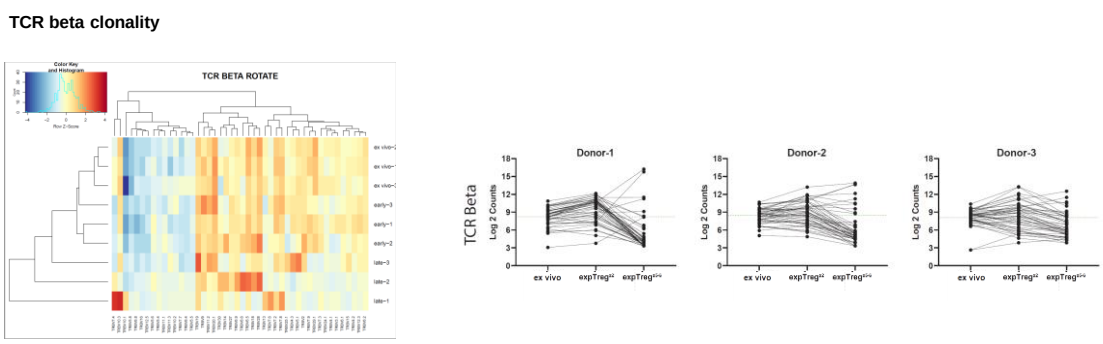
Extended data Figure 4:

Example ADR2A blocking assay

Representative flow plot of AD2RA blocking suppression assay. V450 proliferation dye labelled Teffector cells (Teffs) were stimulated for 5 days using anti-CD3/CD28 Treg suppression assay beads with (middle histograms) and without (left histograms) expTregs at a ratio of 8:1 Teffs:Tregs and with (bottom plots) and without (top plots) ADR2A block. Histograms show V450 dye dilution of the Teffs (V670- non Treg) cells as demonstrated by the gating in right hand dot plots.







Extended data Figure 5:
Additional Nanostring data

A: Expression levels of HIF1A responsive genes captured by the CAR-T Nanostring panel. Data shows Nanostring normalised counts for the top 20 HIF1A responsive genes reported by Smelc *et al*[27] (left plot) and by Oki *et al*[26] (right plot). Data from donor matched *ex vivo* Tregs, expTreg^{x2} and expTreg^{x5-6} from 3 donors and expTreg^{x2} (expTreg^{x2restim}) from 2 donors are shown.

B-C: The expression of 45 TCR alpha and 46 TCR beta variable chains was measured in *ex vivo* Tregs, expTreg^{x2} and expTreg^{x5-6} from 3 donors. Log2 normalised counts measured by Nanostring are displayed and the green dotted line represents the mean of the *ex vivo* dataset.

Supplementary Table 1

Supplementary table 2 contains the raw and normalised gene expression counts measured by Nanostring for the *ex vivo* Tregs, expTregs^{x2}, expTregs^{x5} and expTregs^{x2restim}.

See: Supplemental table 1 Nanostring.xls

Supplementary Table 2

Gene Set Enrichment Analysis was performed using the GSEA tool, and enrichment in the genesets from Hallmark, Reactome and GO Biological Processes.

See: Supplemental table 2 Gene Set Enrichment Analysis.xls