	Healthy Control 1		Healthy Control 2		Healthy Control 3	
Sample	**Productive Templates**	**TCRβ sequences**	**Productive Templates**	**TCRβ sequences**	**Productive Templates**	**TCRβ sequences**
Unstimulated CD4	170,771	151,392	159,120	120,777	167,501	93,850
Unstimulated CD8	162,864	108,291	59,266	31,246	34,497	19,975
CD4 $CFSE^{lo}$	2,211	1,923	83,140	13,624	111,812	12,023
CD8 $CFSE^{lo}$	15,419	6,028	122,387	6,078	101,356	6,486
Unstimulated Tregs	67,821	60,877	105,463	83,590	40,914	25,163
Unstimulated $CD4^{+}$nonTregs	389,387	339,442	170,440	129,766	161,868	77,775
Tregs w/ act. donor B cells	49,512	4,841	62,530	8,211	88,390	3,177
Tregs w/ B cells: 4-$1BB^{+}$ $CD40L^{-}$ cells	26,671	3,693	33,667	4,849	67,046	2,029
CTLA4Ig MLR	61,377	57,605	114,279	92,312	75,982	42,189
Restimulated CTLA4Ig MLR	58,451	25,357	115,189	18,513	145,741	22,482

Table S1. Summarized sequencing numbers for each healthy control with total number of productive templates and number of unique productive TCRβ sequences for each sample. Raw data are available through Adaptive's website (see Methods section).

		CD4 donor-reactive	Tregs w/ act. donor B cells	Tregs w/ B cells: 4-1BB$^+$ CD40L$^-$ cells	CTLA4Ig MLR	Restim. CTLA4Ig MLR
Control 1	TCRβ sequences	1,656	4,841	3,693	57,605	25,357
	Treg sequences	8	87	81	2,328	477
	CD4$^+$ nonTreg sequences	167	56	51	639	540
Control 2	TCRβ sequences	1,756	8,211	4,849	92,312	18,513
	Treg sequences	43	367	195	6,125	1,452
	CD4$^+$ nonTreg sequences	178	145	48	530	243
Control 3	TCRβ sequences	1,579	3,177	2,029	42,189	22,482
	Treg sequences	21	268	145	6,605	3,547
	CD4$^+$ nonTreg sequences	187	85	40	707	807

Table S2. Number of unique productive TCRβ sequences of each method and number of TCRβ sequences of each method that mapped to the unstimulated Treg or unstimulated CD4$^+$non-Treg repertoire in healthy controls. CD4 donor-reactive TCRβ sequences are 5-fold expanded compared to the unstimulated CD4 sample and have a minimum frequency of 10^{-4} in the CFSE^{lo} sample. Treg and non-Treg repertoires determined after removing ambiguous sequences.

		CFSE-MLR Treg	Tregs w/ act. donor B cells	Tregs w/ B cells: 4-1BB$^+$CD40L$^-$ cells	CTLA4Ig MLR	Restim. CTLA4Ig MLR
Healthy Control 1	TCRβ sequences	8	4,841	3,693	57,605	25,357
	CFSE-MLR Treg	n/a	2	2	3	3
	Tregs w/ act. donor B cells	2	n/a	2,861	74	186
	Tregs w/ B cells: 4-1BB$^+$ CD40L$^-$ cells	2	2,861	n/a	72	178
	CTLA4Ig MLR	3	74	72	n/a	504
	Restim. CTLA4Ig MLR	3	186	178	504	n/a
Healthy Control 2	TCRβ sequences	43	8,211	4,849	92,312	18,513
	CFSE-MLR Treg	n/a	23	21	17	30
	Tregs w/ act. donor B cells	23	n/a	3,781	267	232
	Tregs w/ B cells: 4-1BB$^+$ CD40L$^-$ cells	21	3,781	n/a	139	180
	CTLA4Ig MLR	17	267	139	n/a	1,639
	Restim. CTLA4Ig MLR	30	232	180	1,639	n/a
Healthy Control 3	TCRβ sequences	21	3,177	2,029	42,189	22,482
	CFSE-MLR Treg	n/a	4	3	10	13
	Tregs w/ act. donor B cells	4	n/a	1,406	320	282
	Tregs w/ B cells: 4-1BB$^+$ CD40L$^-$ cells	3	1,406	n/a	189	182
	CTLA4Ig MLR	10	320	189	n/a	4,617
	Restim. CTLA4Ig MLR	13	282	182	4,617	n/a

Table S3. Number of overlapping TCRβ sequences among the methods in healthy controls.

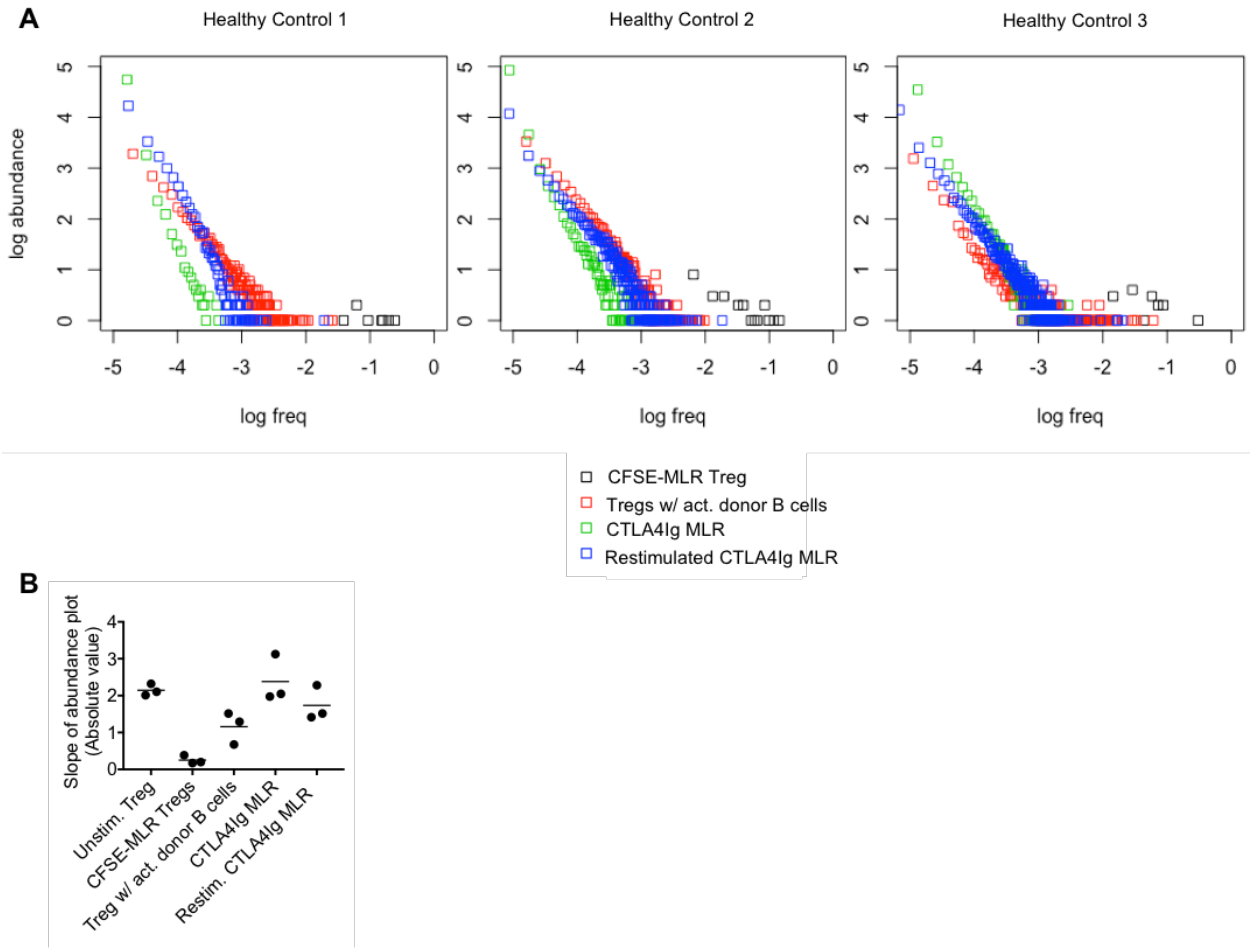


Fig. S1. Abundance plots for donor-specific Treg expansion methods in healthy controls. (A) Log_{10} of frequencies on the x-axis plotted against Log_{10} number of TCRβ sequences with the given frequency on the y-axis. An increased magnitude of the slope indicates increased repertoire diversity. (B) Absolute value of the slopes of the abundance plots from (A) calculated as described in Dewolf et al, 2018.

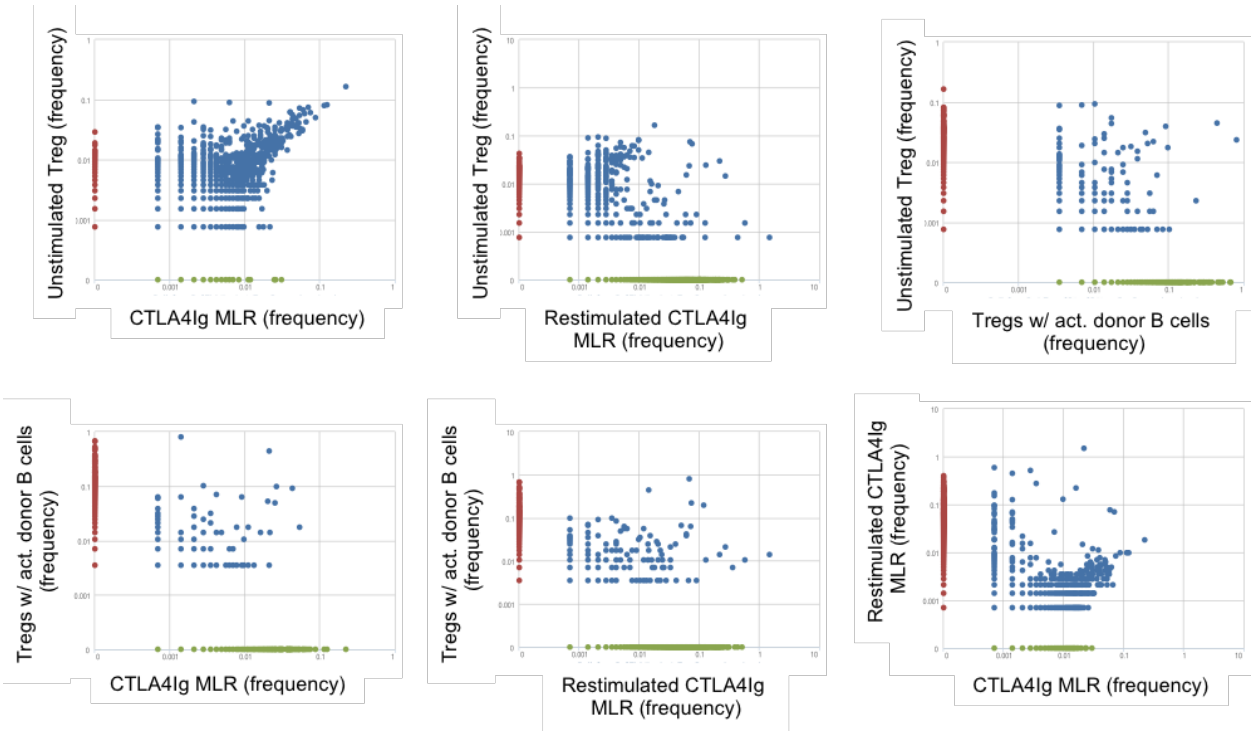
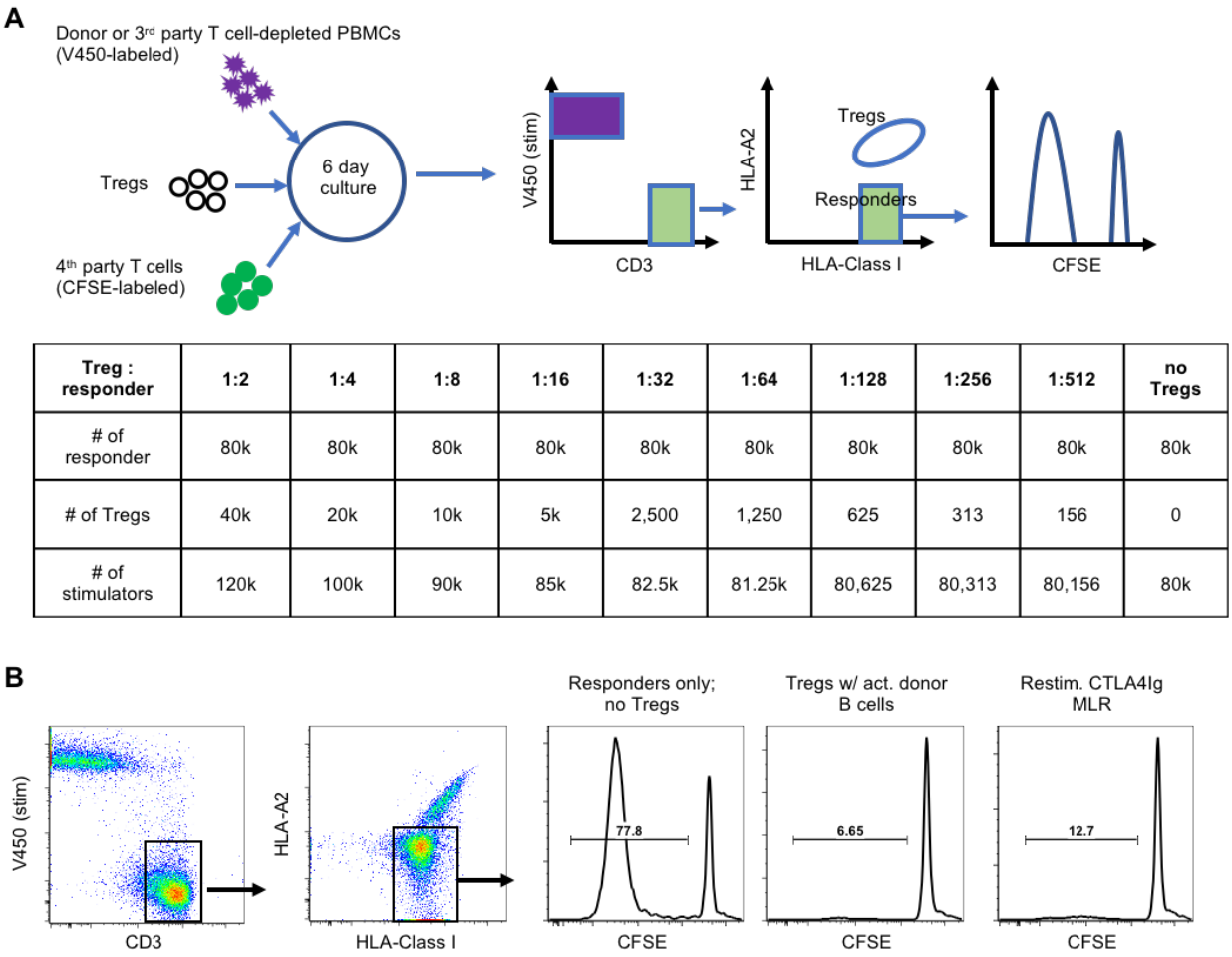


Fig. S2. Representative scatterplots comparing repertoires. Using the Adaptive ImmunoSEQ analyzer, scatterplots were generated to show the frequencies of sequences common to 2 repertoires. Sequences on the X- or Y-axis are found in one method but not the other. Note that samples shown were not adjusted for sorting error and that the CFSE-MLR Treg repertoire is not shown due to needed adjustments not possible in the analyzer.



Treg : responder	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	no Tregs
# of responder	80k	80k	80k	80k	80k	80k	80k	80k	80k	80k
# of Tregs	40k	20k	10k	5k	2,500	1,250	625	313	156	0
# of stimulators	120k	100k	90k	85k	82.5k	81.25k	80,625	80,313	80,156	80k

Fig. S3. Representative stain for interrogating 4th party T cell proliferation in the presence or absence of Tregs. (A) Schematic of suppression assay. Stimulators were either donor or HLA-mismatched 3rd party T cell-depleted PBMCs and were labeled with violet V450 dye. Recipient Tregs were HLA-A2$^+$ while CFSE-labeled 4th party T cells were HLA-A2$^-$. Proliferation of 4th-party T cells was measured at the end of the assay. Table details number of cells of each type in each condition. (B) Representative stain of suppression assay. 4th-party T cell CFSE dilution shown of responders in the absence of Tregs and using Tregs derived from culture of Tregs with activated donor B cells and from the restimulated CTLA4Ig MLR at a concentration of 1 Treg to 4 responders.

	ITN Subject 1		ITN Subject 2		ITN Subject 4		ITN Subject 5	
	Reads	**TCRβ sequences**	**Reads**	**TCRβ sequences**	**Reads**	**TCRβ sequences**	**Reads**	**TCRβ sequences**
Unstimulated CD4	2,104,130	72,043	999,203	62,121	2,509,697	160,586	693,591	69,694
Unstimulated CD8	3,356,790	49,316	1,468,823	39,919	1,942,118	108,065	496,400	44,296
CD4 $CFSE^{lo}$	2,728,562	8,379	325,633	479	3,062,545	25,903	2,044,441	6,121
CD8 $CFSE^{lo}$	1,357,525	4,748	497,849	495	3,493,218	13,617	2,782,223	10,073
Unstimulated Tregs	37,511 (templates)	21,412	119,829 (templates)	81,436	114,915 (templates)	110,659	144,871 (templates)	77,087
Unstimulated $CD4^+$ non-Tregs	120,414 (templates)	94,804	106,804 (templates)	86,856	136,888 (templates)	130,691	167,673 (templates)	137,372
Tregs w/ act. donor B cells	1,330,238	2,521	683,549	506	1,491,529	1,671	946,859	2,035
Tregs w/ B cells: 4-1BB^+ $CD40L^-$ cells	1,347,853	2,156	542,095	352	1,707,595	1,813	1,141,948	1,688
Restimulated CTLA4Ig MLR	3,531,618	7,351	57,007	592	911,938	14,474	503,673	5,637

Table S4. Summarized sequencing numbers for each ITN036ST subject with total number of reads or productive templates and number of unique productive TCRβ sequences for each sample.

		CD4 donor-reactive	Tregs w/ act. donor B cells	Tregs w/ B cells: 4-1BB$^+$ CD40L$^-$ cells	Restim. CTLA4Ig MLR
Subject 1	TCRβ sequences	2,200	2,521	2,156	7,351
	Treg sequences	31	159	135	442
	CD4$^+$ nonTreg sequences	117	111	70	269
Subject 2	TCRβ sequences	434	506	352	592
	Treg sequences	31	88	42	133
	CD4$^+$ nonTreg sequences	35	14	9	6
Subject 4	TCRβ sequences	1,736	1,671	1,813	14,474
	Treg sequences	11	60	54	926
	CD4$^+$ nonTreg sequences	64	9	10	52
Subject 5	TCRβ sequences	3,996	2,035	1,688	5,637
	Treg sequences	216	262	196	1,370
	CD4$^+$ nonTreg sequences	166	76	48	63

Table S5. Number of unique productive TCRβ sequences of each method and number of TCRβ sequences of each method that mapped to the unstimulated Treg or unstimulated CD4$^+$non-Treg repertoire in ITN036 subjects. CD4 donor-reactive TCRβ sequences are 5-fold expanded compared to the unstimulated CD4 sample and have a minimum frequency of 10^{-4} in the CFSE^{lo} sample. Treg and non-Treg repertoires determined after removing ambiguous sequences.

		CFSE-MLR Treg	Tregs w/ act. donor B cells	Tregs w/ B cells: 4-1BB$^+$CD40L$^-$ cells	Restim. CTLA4 Ig MLR
ITN Subject 1	TCRβ sequences	31	2,521	2,156	7,351
	CFSE-MLR Treg	n/a	14	15	15
	Tregs w/ act. donor B cells	14	n/a	1,375	123
	Tregs w/ B cells: 4-1BB$^+$ CD40L$^-$ cells	15	1,375	n/a	112
	Restim. CTLA4Ig MLR	15	123	112	n/a
ITN Subject 2	TCRβ sequences	31	506	352	592
	CFSE-MLR Treg	n/a	0	0	3
	Tregs w/ act. donor B cells	0	n/a	175	1
	Tregs w/ B cells: 4-1BB$^+$ CD40L$^-$ cells	0	175	n/a	1
	Restim. CTLA4Ig MLR	3	1	1	n/a
ITN Subject 4	TCRβ sequences	11	1,671	1,813	14,474
	CFSE-MLR Treg	n/a	1	2	5
	Tregs w/ act. donor B cells	1	n/a	618	24
	Tregs w/ B cells: 4-1BB$^+$ CD40L$^-$ cells	2	618	n/a	27
	Restim. CTLA4Ig MLR	5	24	27	n/a
ITN Subject 5	TCRβ sequences	216	2,035	1,688	5,637
	CFSE-MLR Treg	n/a	21	11	60
	Tregs w/ act. donor B cells	21	n/a	999	27
	Tregs w/ B cells: 4-1BB$^+$ CD40L$^-$ cells	11	999	n/a	27
	Restim. CTLA4Ig MLR	60	27	27	n/a

Table S6. Number of overlapping TCRβ sequences among the methods in ITN036ST subjects 1, 2, 4, and 5.

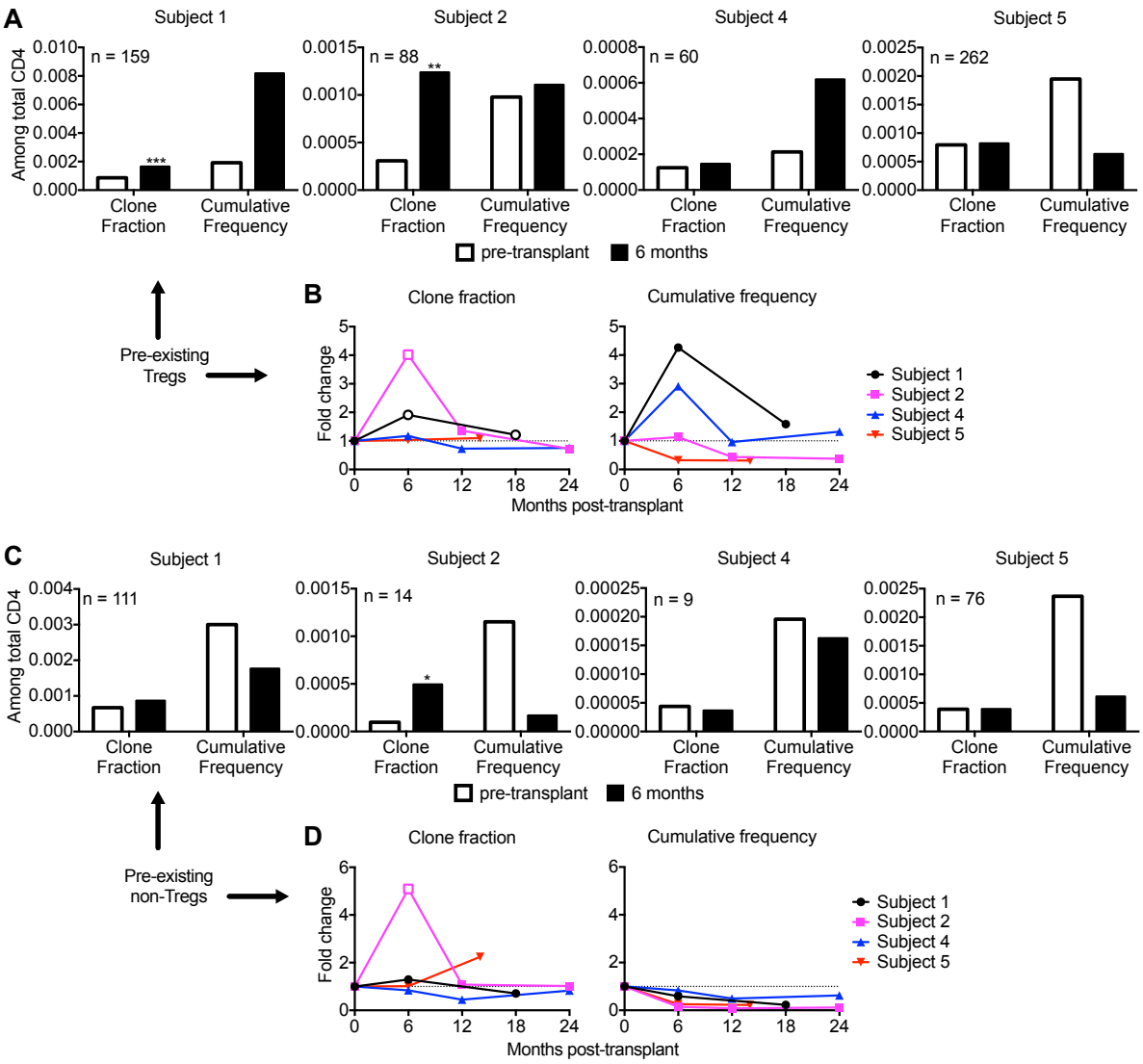


Fig. S4. Pre-existing donor-specific Tregs expand at 6 months in tolerant patients. (A-B) Pre-existing Tregs (mapped to the pre-transplant unstimulated Treg repertoire) and (C-D) pre-existing non-Tregs (mapped to the pre-transplant unstimulated non-Treg repertoire) identified in the culture of Tregs with activated donor B cells tracked among total CD4 repertoire. (A, C): *p<0.05, **p<0.01, ***p<0.001 reduction or increase in clone fraction compared to pre-transplant (two-sided Fisher's exact test). (B, D) Open symbols, statistically significant reduction or increase by clone fraction compared to pre-transplant (P < 0.05, two-sided Fisher's exact test).

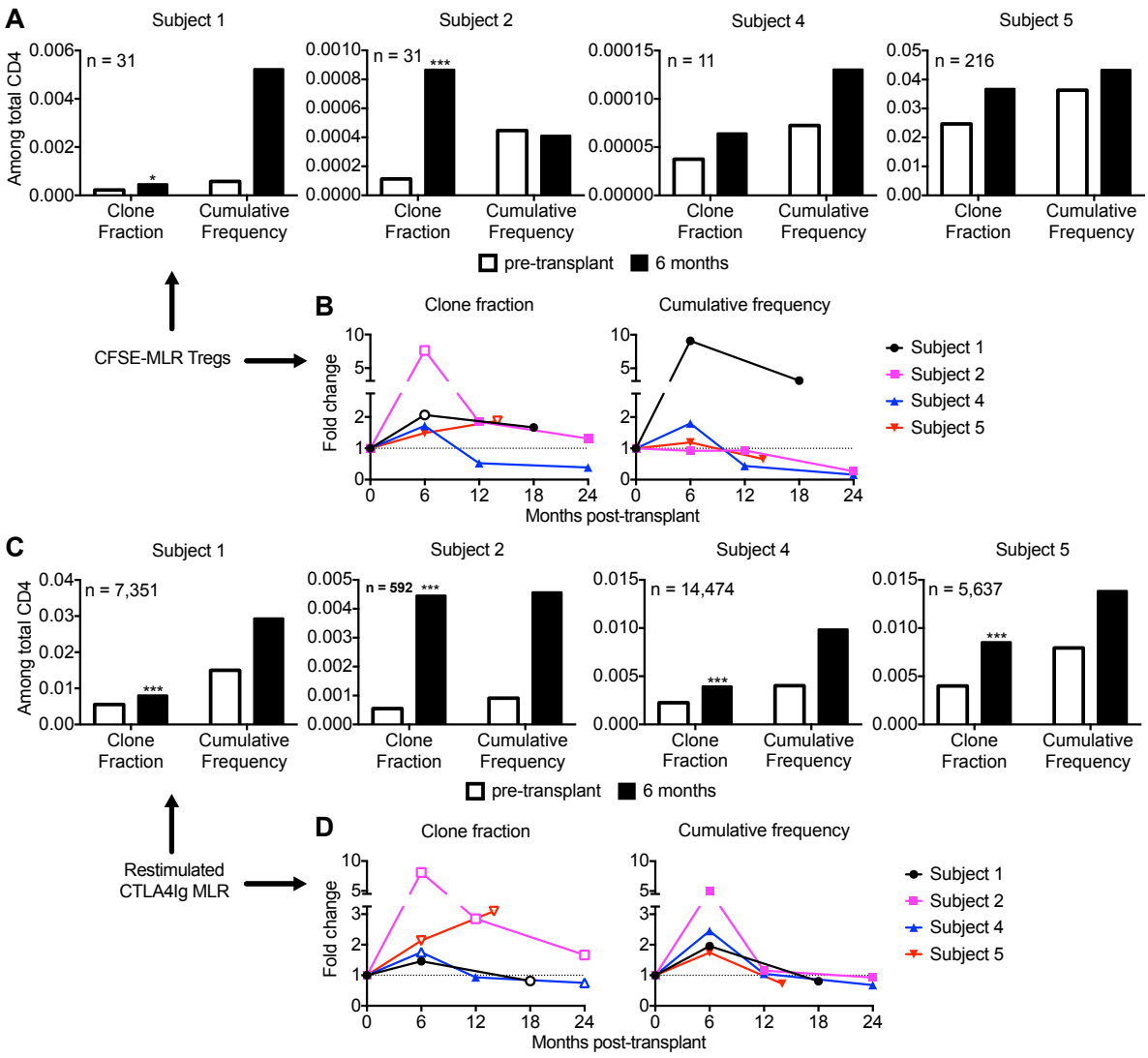


Fig. S5. CFSE-MLR Tregs and restimulated CTLA4Ig MLR repertoires following CKBMT. CFSE-MLR Treg and restimulated CTLA4Ig MLR repertories among total CD4 repertoires at 6 months (A) and (C), respectively, and long-term change relative to pre-transplant among the total CD4 repertoire, (B) and (D), respectively. (A, C) *p<0.05, **p<0.01, ***p<0.001 reduction or increase in clone fraction compared to pre-transplant (two-sided Fisher's exact test). (B, D) Open symbols, statistically significant reduction or increase compared to pre-transplant ($P < 0.05$, two-sided Fisher's exact test).

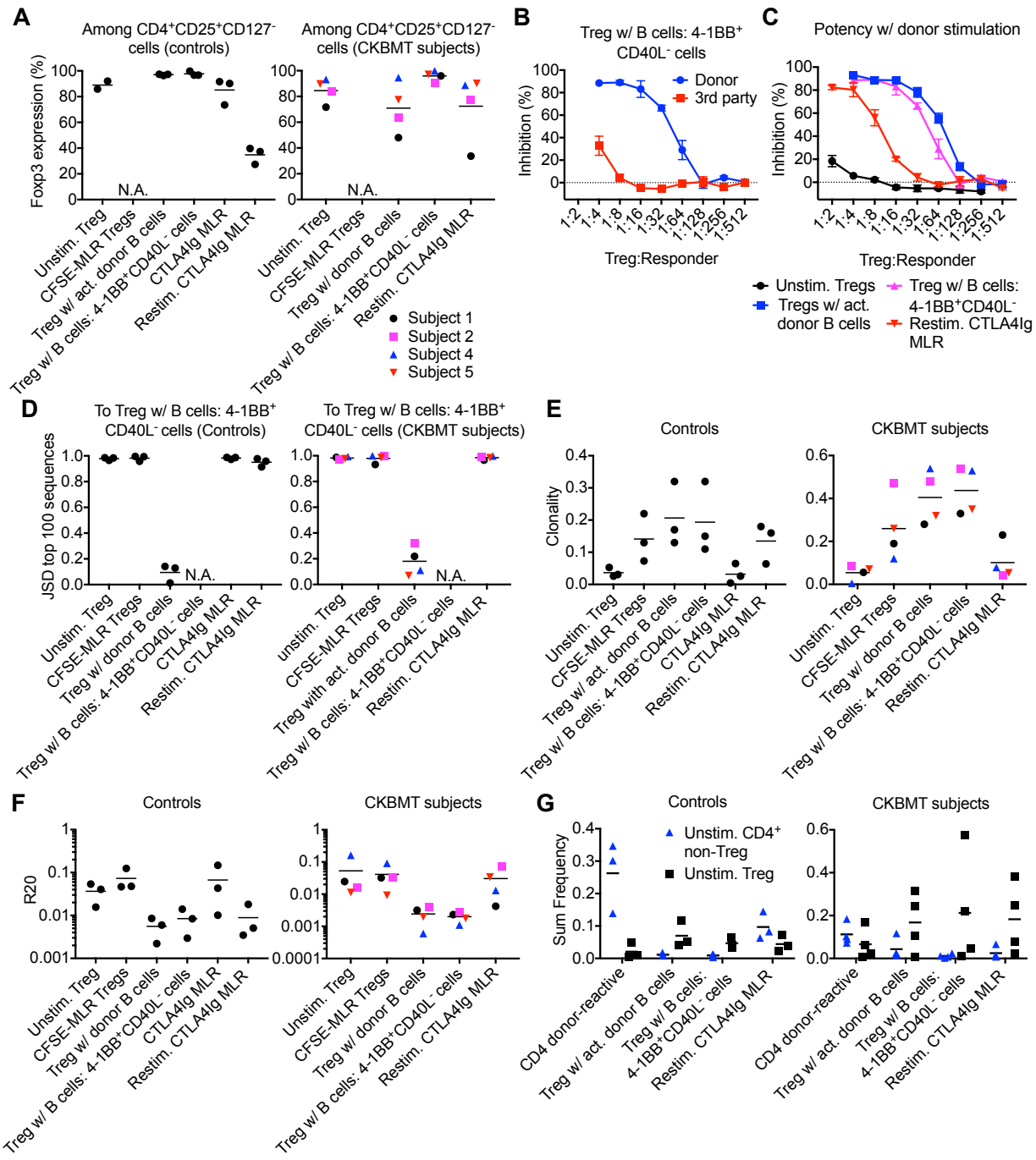


Fig. S6. Comparisons of CD4$^+$4-1BB$^+$CD40L$^-$ population to other methods. (A) Foxp3 expression among methods in healthy controls (left) and CKBMT subjects (right). Specificity (B) and potency (C) in a suppression assay using cells from healthy control 1. (D) JSD comparing the CD4$^+$4-1BB$^+$CD40L$^-$ repertoire to each other method. (E) Clonality and (F) R20 in each method. (G) Sum frequency of sequences mapping to unstimulated Tregs and non-Tregs populations in each expanded Treg population.

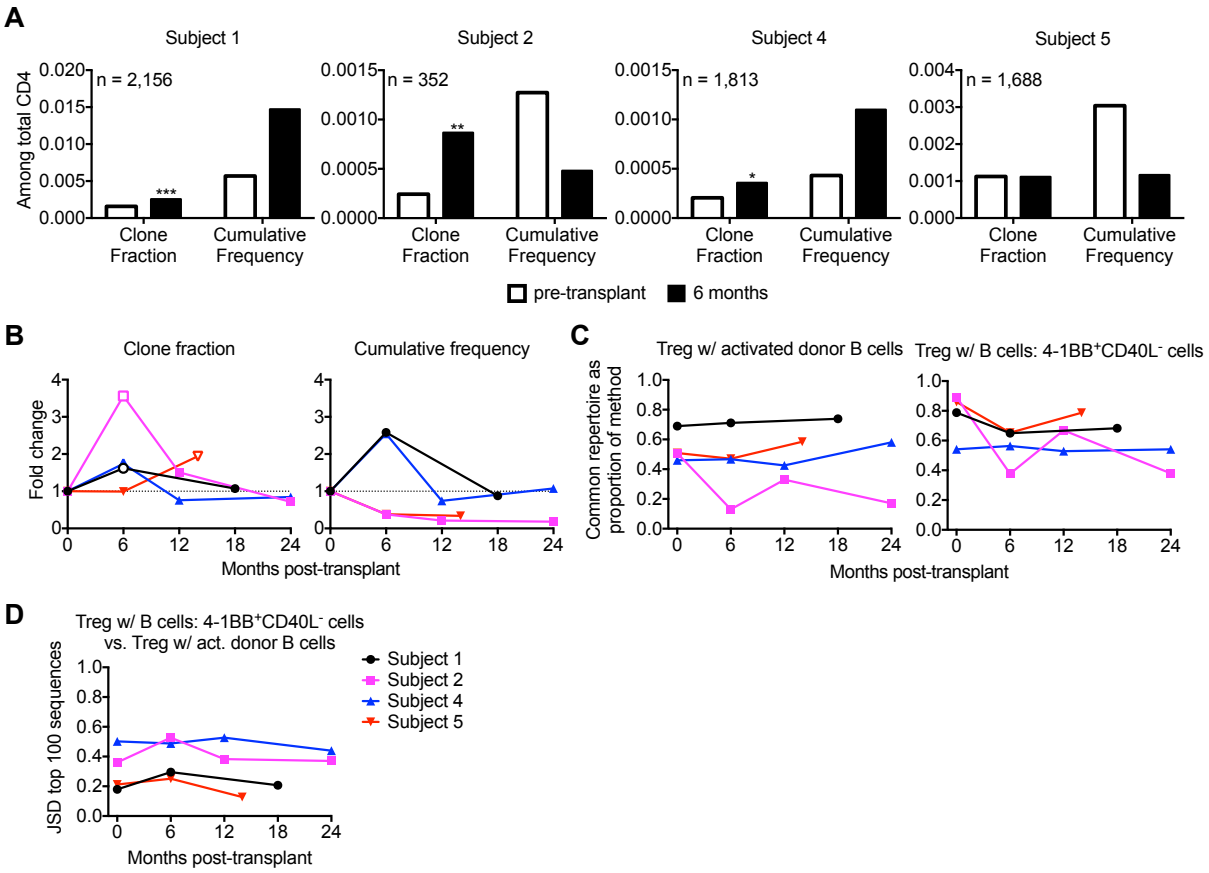


Fig. S7. Tracking of CD4$^+$4-1BB$^+$CD40L$^-$ population provides similar information as bulk population from Treg with activated donor B cell culture. CD4$^+$4-1BB$^+$CD40L$^-$ repertoire among total CD4 repertoire (A) pre-transplant and at 6 months, and (B) long-term relative to pre-transplant. (A) *p<0.05, **p<0.01, ***p<0.001 reduction or increase in clone fraction compared to pre-transplant (two-sided Fisher's exact test). (B) Open symbols, statistically significant reduction or increase by clone fraction compared to pre-transplant ($p < 0.05$, two-sided Fisher's exact test). (C) Sequences common to the bulk Treg with activated B cell repertoire and the CD4$^+$4-1BB$^+$CD40L$^-$ repertoire as a proportion of the cumulative frequency of bulk repertoire (left) and the CD4$^+$4-1BB$^+$CD40L$^-$ repertoire (right). (D) Divergence of sequences in the circulation mapping to the bulk Treg with activated B cell and those mapping to the CD4$^+$4-1BB$^+$CD40L$^-$ repertoires, quantified by JSD.