Cell Reports, Volume *39*

Supplemental information

Human IL-10-producing B cells have diverse states that are induced from multiple B cell subsets

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Supplemental Figure 1

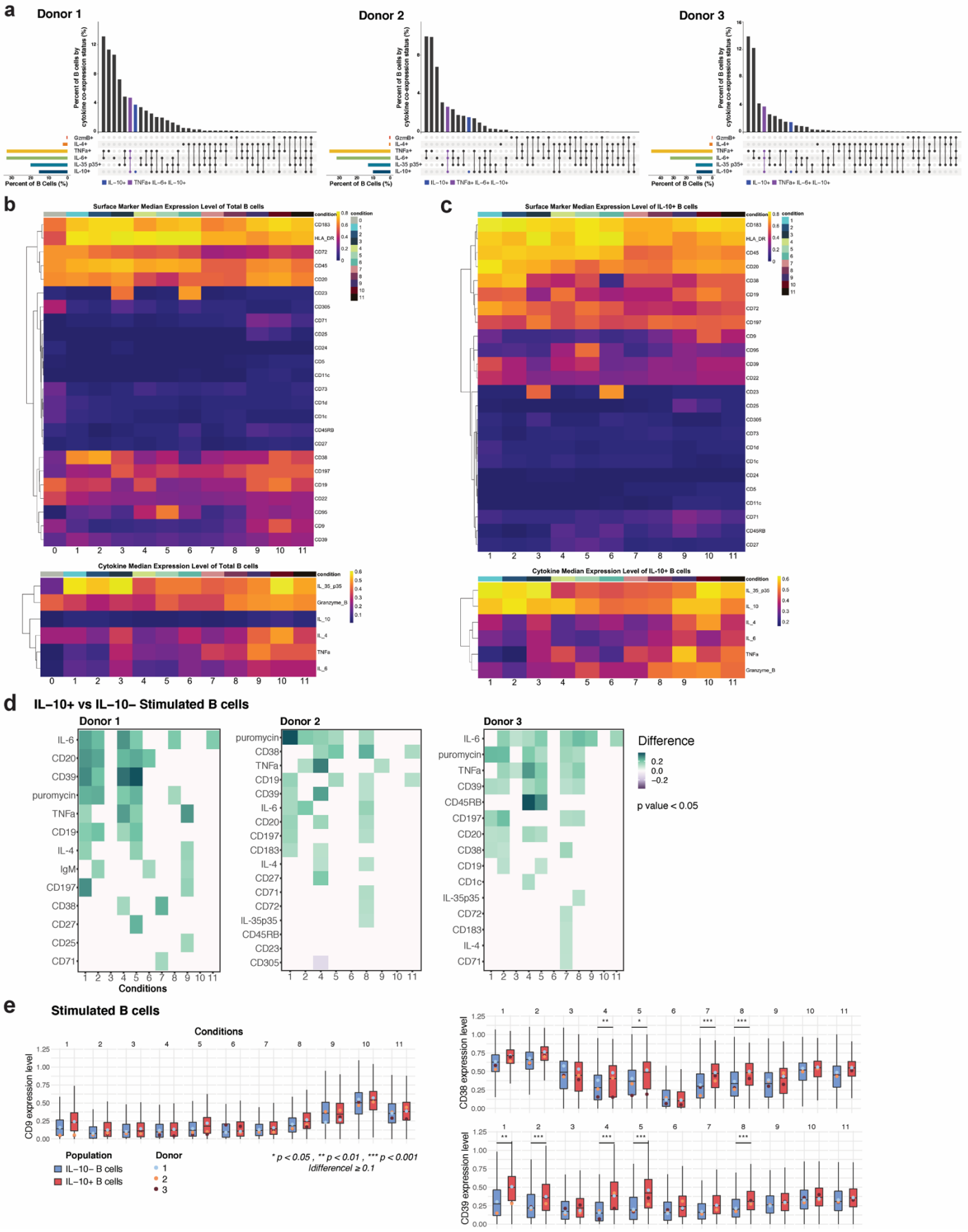

Figure S1. Surface marker and cytokine expression of unstimulated and stimulated total B cells and IL-10$^+$ B cells from stimulation screen. Related to Figure 1.

(A) Upper bar graphs indicate the percent of stimulated B cells by cytokine (IL-4, TNFα, IL-6, IL-35p35, IL-10) and GzmB expression status, as determined by Boolean gating of cytokine-positive cells, for each individual. Cytokine and GzmB co-expression status of populations are defined in the lower dot plot panels. Left lower bar graphs depict percent of cytokine/GzmB-positive B cells regardless of co-expression, across all stimulation conditions. Data was subsampled equally by condition for each individual donor.

(B) Median surface marker (top panel) and cytokine (lower panel) expression of total B cells in the unstimulated condition (0) and each stimulated condition (1-11).

(C) Median surface marker (top panel) and cytokine (lower panel) expression of IL-10$^+$ B cells from stimulated conditions 1-11.

(D) Relative difference in median expression of surface markers and cytokines in IL-10$^+$ versus IL-10$^-$ stimulated B cells by activating condition (1-11; x axis) and by individual (heatmap panels). Expression only shown for markers and cytokines with difference greater than or equal to 0.1 and a p value less than 0.05. P values denote result of Kolmogorov-Smirnov test with Bonferroni multiple hypothesis correcting.

(E) Expression levels of CD9, CD38 and CD39 for IL-10$^-$ (blue) versus IL-10$^+$ (red) B cells by stimulation condition. P values denote result of Kolmogorov-Smirnov test with Bonferroni multiple hypothesis correcting. Line depicts median, colored dots represent median expression for each individual donor, box depicts interquartile range (IQR), and whiskers depict IQR+- 1.5*IQR. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Supplemental Figure 2

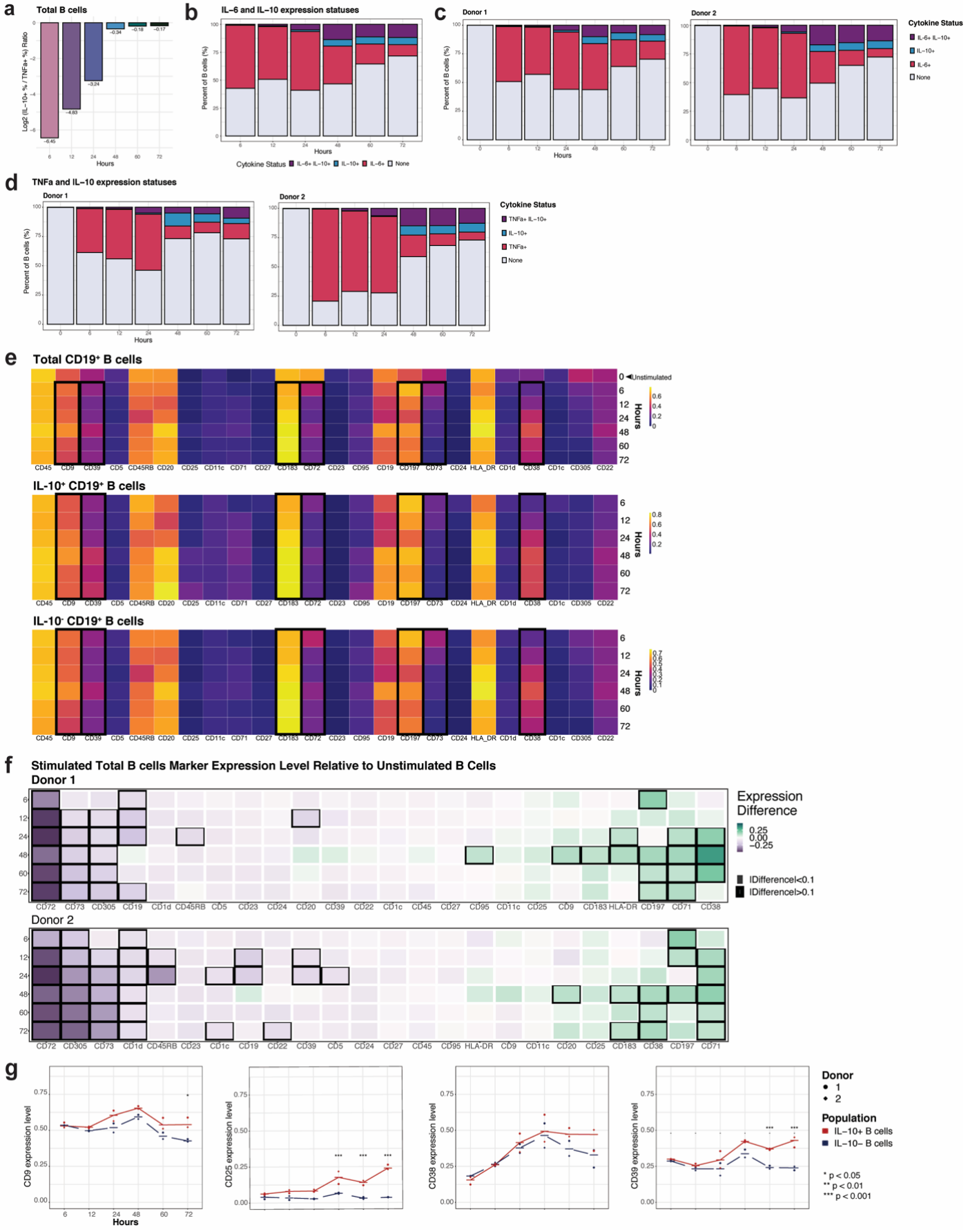

Figure S2. Surface marker and cytokine expression of unstimulated and stimulated total B cells and IL-10$^+$ B cells from stimulation timecourse. Related to Figure 2.

(A) Log2 ratio of percentage IL-10$^+$ over TNFα$^+$ donor-pooled, stimulated B cells by timepoint (hours).

(B) Percentage of cells by IL-6 and IL-10 expression for stimulated donor-pooled B cells by timepoint (hours).

(C) Percentage of cells by IL-6 and IL-10 expression for stimulated B cells by timepoint (hours; x axis) and individual donor (panels).

(D) Percentage of cells by TNFα and IL-10 expression for stimulated B cells by timepoint (hours; x axis) and individual donor (panels).

(E) Median expression heatmap of total, IL-10$^+$ and IL-10$^-$ B cells across timepoints of Breg-specific stimulation (6-72 hours).

(F) Difference in median expression of surface markers in unstimulated versus stimulated B cells by timepoint (hours; rows) and by individual donors (heatmap panels). Boxes in heatmap indicate markers with an absolute value of difference greater than 0.1. Expression difference only shown for markers and cytokines with a p value less than 0.05 (light gray fill indicates $p > 0.05$). P values denote result of Kolmogorov-Smirnov test with Bonferroni multiple hypothesis correcting.

(G) Median expression level of markers with diverging patterns between IL-10$^+$ and IL-10$^-$ B cells by timepoint (hours; x axis) and individual donors (dots). P values denote result of Kolmogorov-Smirnov test with Bonferroni multiple hypothesis correcting. * $p < 0.05$. *** $p < 0.001$.

Supplemental Figure 3

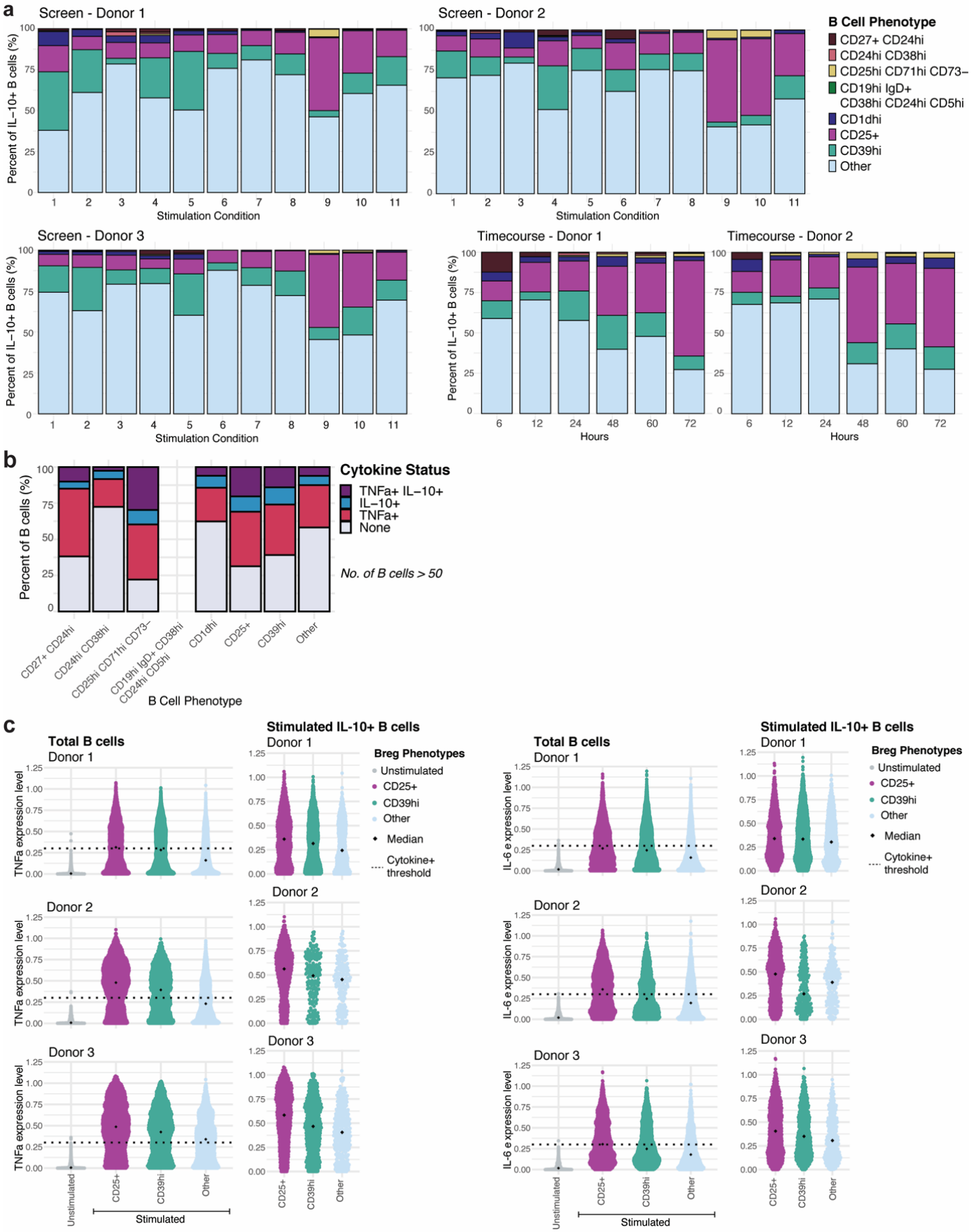

Figure S3. Cytokine expression of stimulated total and IL-10$^+$ B cells with conventional Breg immunophenotypes from stimulation screen and timecourse experiments. Related to Figure 3.

(A) Percentage of B cell phenotypes among total IL-10$^+$ B cells by stimulatory condition (duration of 72 hours only; x axis) and individual donor (panels) from stimulation screen (first 3 panels) and by timepoint or hours (condition 10 only; x axis) and individual donor (panels) from stimulation timecourse (last 2 panels).

(B) Percentage of cells by TNFα and IL-10 expression and B cell phenotype from pooled data of screen and timecourse experiments. Data for those B cell phenotypes represented by less than 50 total cells were excluded.

(C) TNFα (left 6 panels) and IL-6 (right 6 panels) expression levels for total unstimulated B cells versus stimulated CD25$^+$, CD39hi and other phenotype B cells (left columns) and for stimulated IL-10$^+$ CD25$^+$, IL-10$^+$ CD39hi and IL-10$^+$ other phenotype B cells (right columns) for each individual donor (rows). Diamond indicates median; dashed line indicates threshold for cytokine positivity based on unstimulated B cell expression.

Supplemental Figure 4

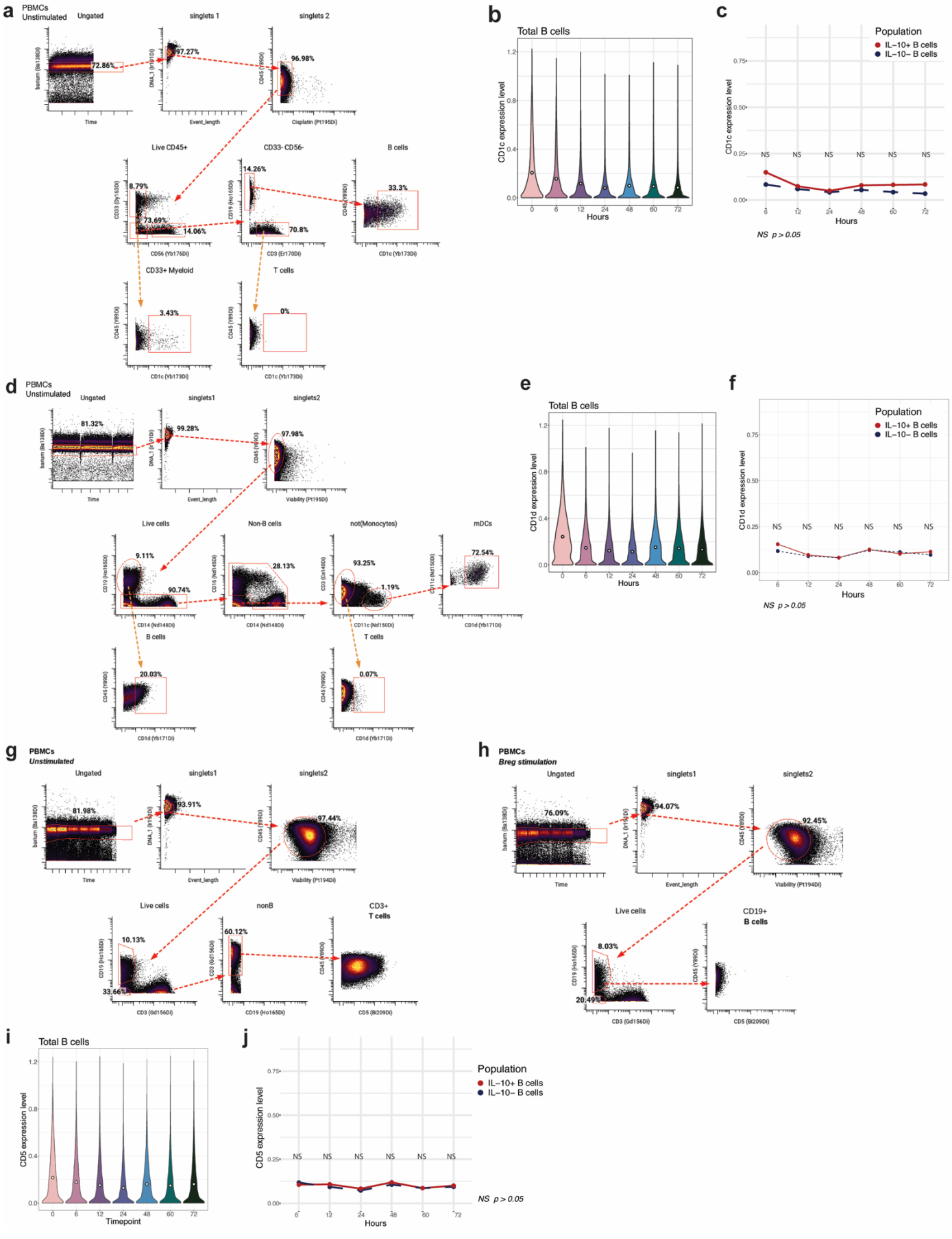

Figure S4. Mass cytometry analysis of CD1c, CD1d and CD5 expression in unstimulated immune cells and stimulated B cells of healthy individuals. Related to Figure 2 and STAR Methods.

(A) Representative gating strategy for total unstimulated peripheral B cells, T cells and $CD33^+$ myeloid cells within mass cytometry analysis of total PBMCs, with exemplary biaxial plots of CD1c expression in each gated cell population.

(B) Median CD1c expression levels in total B cells by timepoint (hours) in the timecourse experiment. Unstimulated B cell data is depicted as timepoint 0 (hours).

(C) Median CD1c expression levels in stimulated IL-10^+ B cells (red) versus IL-10- B cells (blue) by timepoint (hours) in the timecourse experiment.

(D) Representative gating strategy for total unstimulated peripheral B cells, T cells and dendritic cells (DCs) within mass cytometry analysis of total PBMCs, with exemplary biaxial plots of CD1d expression in each gated cell population.

(E) Median CD1d expression levels in total B cells by timepoint (hours) in the timecourse experiment. Unstimulated B cell data is depicted as timepoint 0 (hours).

(F) Median CD1d expression levels in stimulated IL-10^+ B cells (red) versus IL-10- B cells (blue) by timepoint (hours) in the timecourse experiment.

(G) Representative gating strategy for total unstimulated T cells within mass cytometry analysis of total PBMCs, with final biaxial plot showing positive CD5 antibody staining of T cells.

(H) Representative gating strategy for total Breg-stimulated B cells within mass cytometry analysis of total PBMCs, with final biaxial plot indicating minimal CD5 antibody staining of B cells.

(I) Median CD5 expression levels in total B cells by timepoint (hours) in the timecourse experiment. Unstimulated B cell data is depicted as timepoint 0 (hours).

(J) Median CD5 expression levels in stimulated IL-10^+ B cells (red) versus IL-10- B cells (blue) by timepoint (hours) in the timecourse experiment.

Supplemental Figure 5

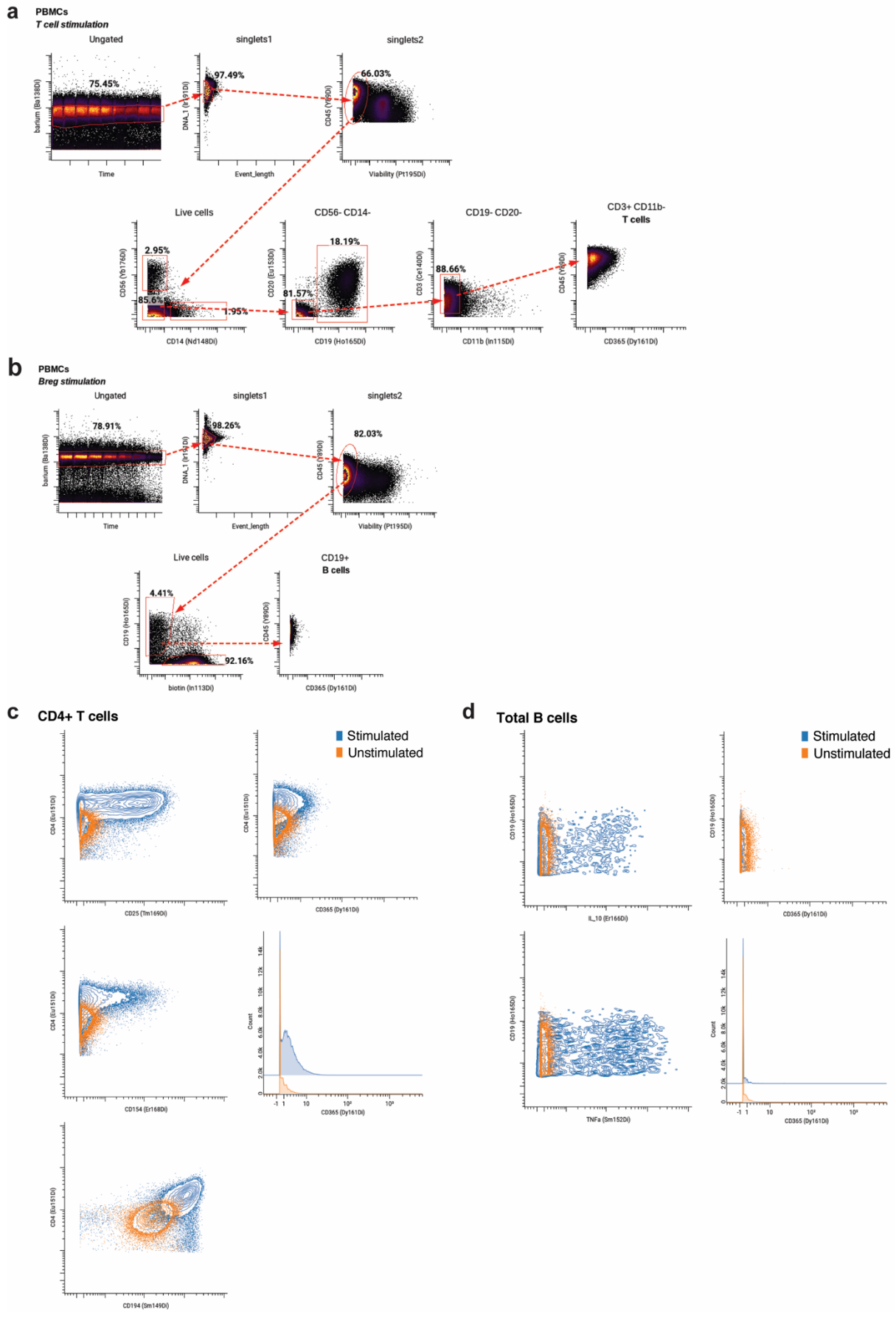

Figure S5. Mass cytometry analysis of CD365/TIM-1 expression in unstimulated and stimulated T and B cells of healthy individuals. Related to STAR Methods.

(A) Representative gating strategy for anti-CD3/anti-CD28-stimulated peripheral T cells within mass cytometry analysis of total PBMCs, with exemplary biaxial plot of CD365/TIM-1 expression in T cells.

(B) Representative gating strategy for Breg-stimulated peripheral B cells within mass cytometry analysis of total PBMCs, with exemplary biaxial plot of CD365/TIM-1 expression in B cells.

(C) Exemplarily biaxial plots depicting upregulation of activation molecules, CD25, CD154/CD40L and CD194/CCR4, and CD365/TIM-1 expression level in anti-CD3/anti-CD28-stimulated (blue) total peripheral $CD4^+$ T cells as compared to unstimulated (orange) cells from the same healthy individuals.

(D) Exemplarily biaxial plots depicting upregulation of the IL-10 and TNFα cytokines, and no change in CD365/TIM-1 expression level in Breg-stimulated (blue) total B cells as compared to unstimulated (orange) cells from the same healthy individuals.

Supplemental Figure 6

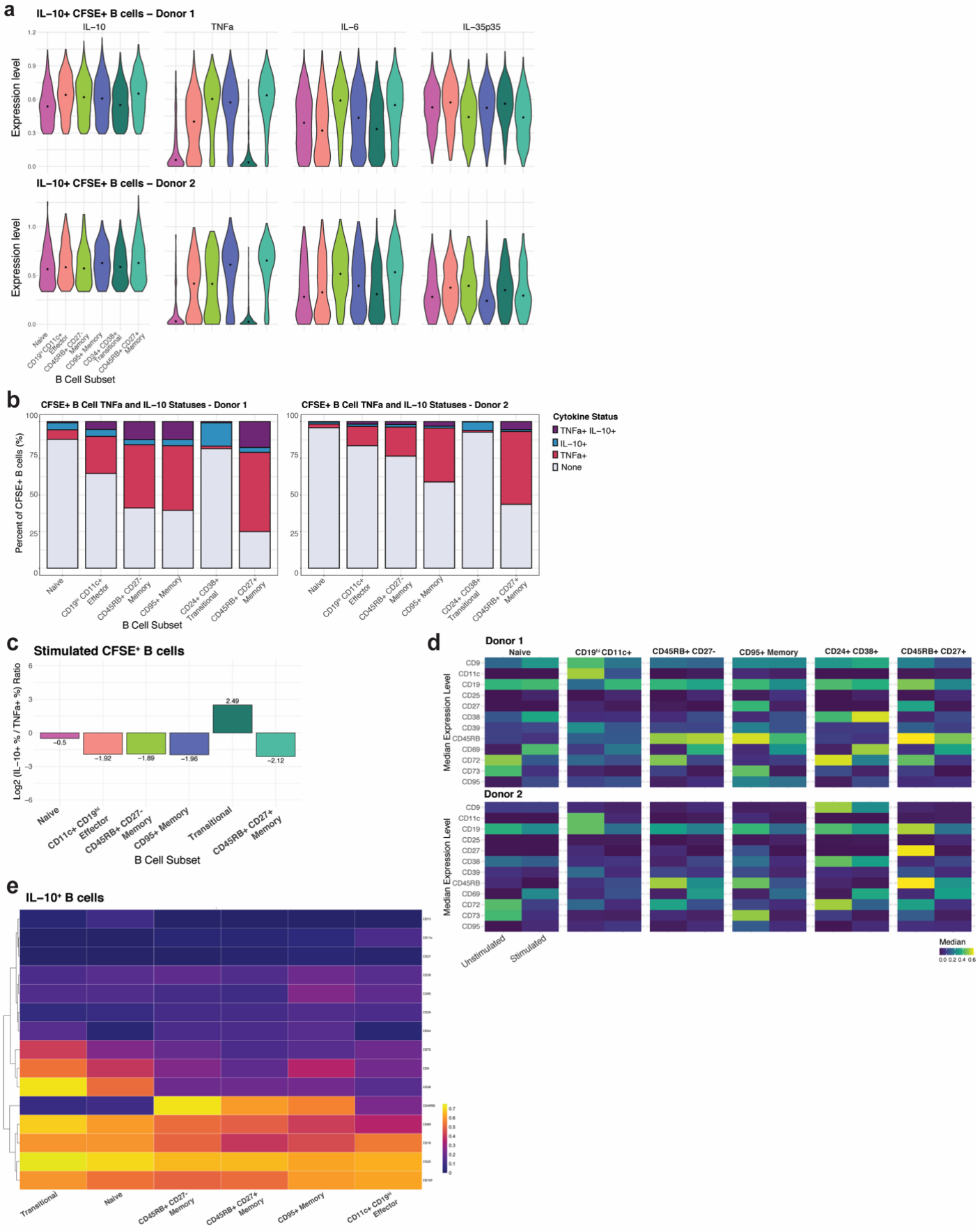

Figure S6. Surface marker and cytokine expression of stimulated total and IL-10$^+$ cells from B cell subsets within B cell sorting experiment. Related to Figure 4.

(A) Expression levels of indicated cytokines by each of CFSE$^+$ IL-10$^+$ B cell subsets for individual donors (rows of 4 plots). Diamond indicates median expression.

(B) Percentage of B cells by TNFα and IL-10 expression for stimulated CFSE$^+$ B cell subsets (x axis) of individual donors (panels).

(C) Log2 ratio of percentage IL-10$^+$:TNFα$^+$ donor-pooled stimulated B cells by sorted B cell subset.

(D) Median expression level of surface markers with differential expression in unstimulated versus stimulated B cells in each of the six B cell subsets (columns) of individual donors (top and bottom panels).

(E) Surface marker expression heatmap of IL-10$^+$ CFSE$^+$ B cell subsets.

Supplemental Figure 7

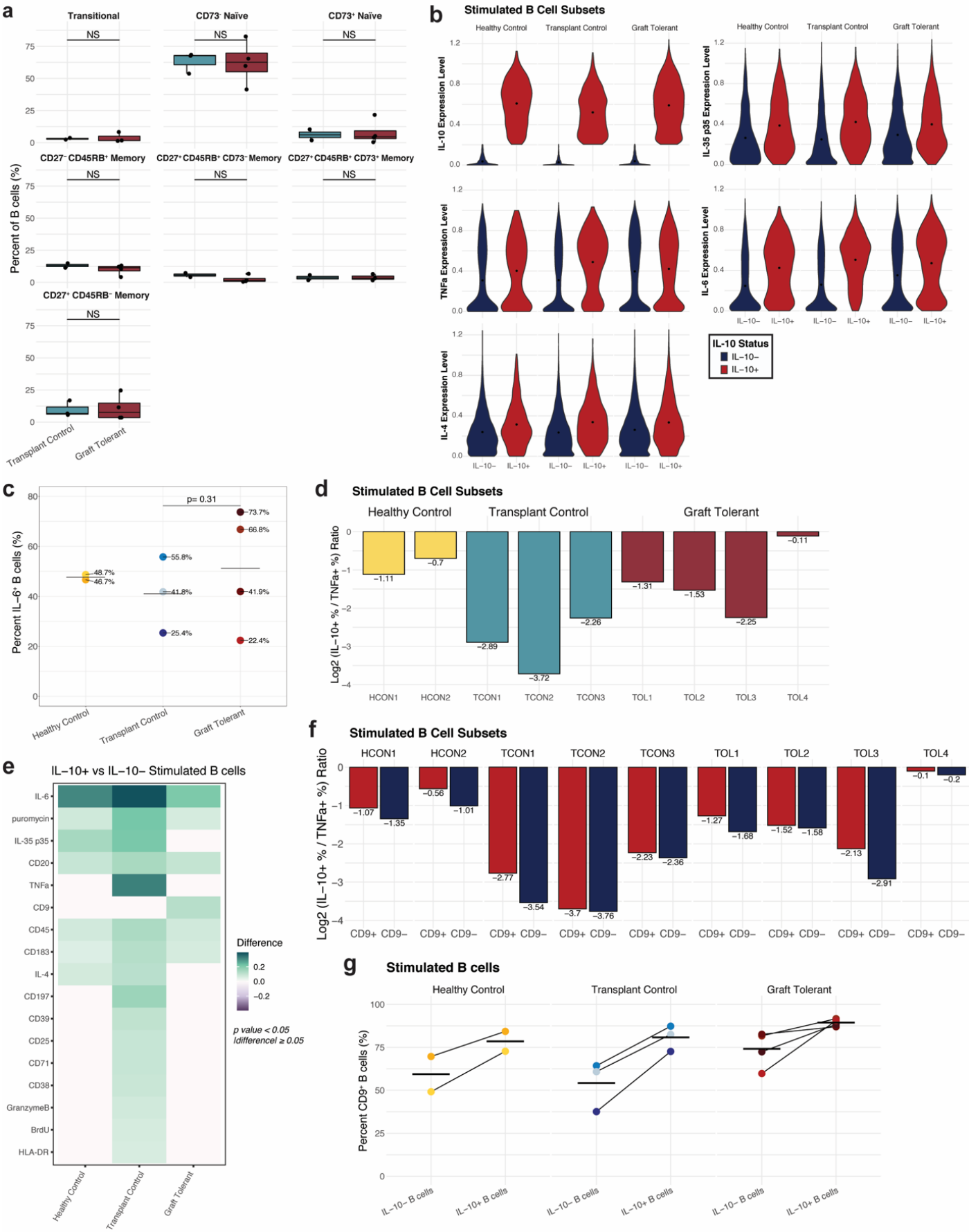

Figure S7. B cell subset proportions and surface marker and cytokine expression of stimulated B cells in clinical cohort analysis. Related to Figure 5.

(A) Percentage of B cell subsets among total unstimulated B cells by clinical group (x axis) and individual donors (dots). Data shown only for cell subsets representing >2% of total B cells. Line depicts median, box depicts interquartile range (IQR), and whiskers depict IQR+-1.5*IQR. P value denotes result of Wilcoxon signed-rank test. NS $p > 0.05$

(B) Cytokine expression levels of IL-10^+ and IL-10^- stimulated B cells in organ transplant recipient clinical cohorts and healthy control group.

(C) Percent of IL-6+ B cells by group and individuals in clinical cohort analysis.

(D) Log2 ratio of percentage IL-10^+ to TNFα^+ B cells by group and individual donor.

(E) Relative difference in median expression of surface markers and cytokines in IL-10+ versus IL-10- stimulated B cells by clinical cohort. Expression only shown for markers and cytokines with difference greater than or equal to 0.05 and a P value less than 0.05. P values denote result of Kolmogorov-Smirnov test with Bonferroni multiple hypothesis correcting.

(F) Log2 ratio of percentage IL-10^+ to TNFα^+ cells for $CD9^+$ (red) and $CD9^-$ (blue) B cells by group and individual donor.

(G) Percent of $CD9^+$ cells among IL-10^- and IL-10^+ stimulated B cells by group and individuals in clinical cohort analysis.

Supplemental Figure 8

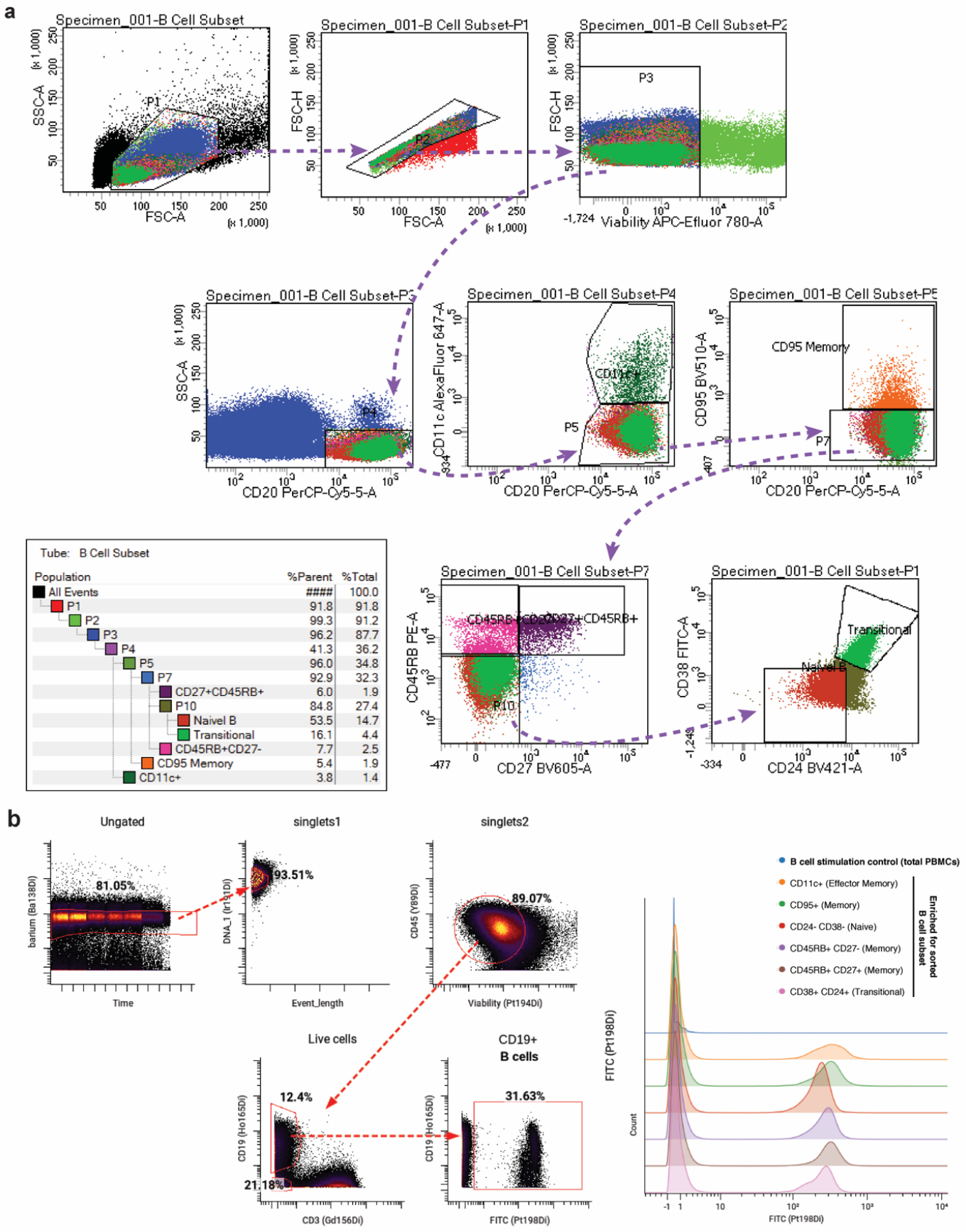

Figure S8. Representative gating strategies and CFSE labeling of B cell subsets within sorting experiments. Related to Figure 4 and STAR Methods.

(A) Sorting strategy to isolate $CD24^-$ $CD38^-$ naïve, $CD24^+$ $CD38^+$ transitional, $CD45RB^+$ $CD27^+$ memory, $CD45RB^+$ $CD27^-$ memory, $CD95^+$ memory, and $CD11^+$ effector memory subsets from live $CD20^+$ human peripheral blood B cells for CFSE labeling and *in vitro* Breg stimulation with total PBMCs.

(B) Representative gating strategy for total B cells within mass cytometry analysis of B cell subset sorting experiment (left panel). Distinct antibody staining of $CFSE^+$ B cells is indicated in the last biaxial plot of the gating strategy, representing the sorted and CFSE-labeled transitional B cell subset, and in the overlapping histograms representing all sorted and labeled B cell subsets (right panel).

Supplemental Figure 9

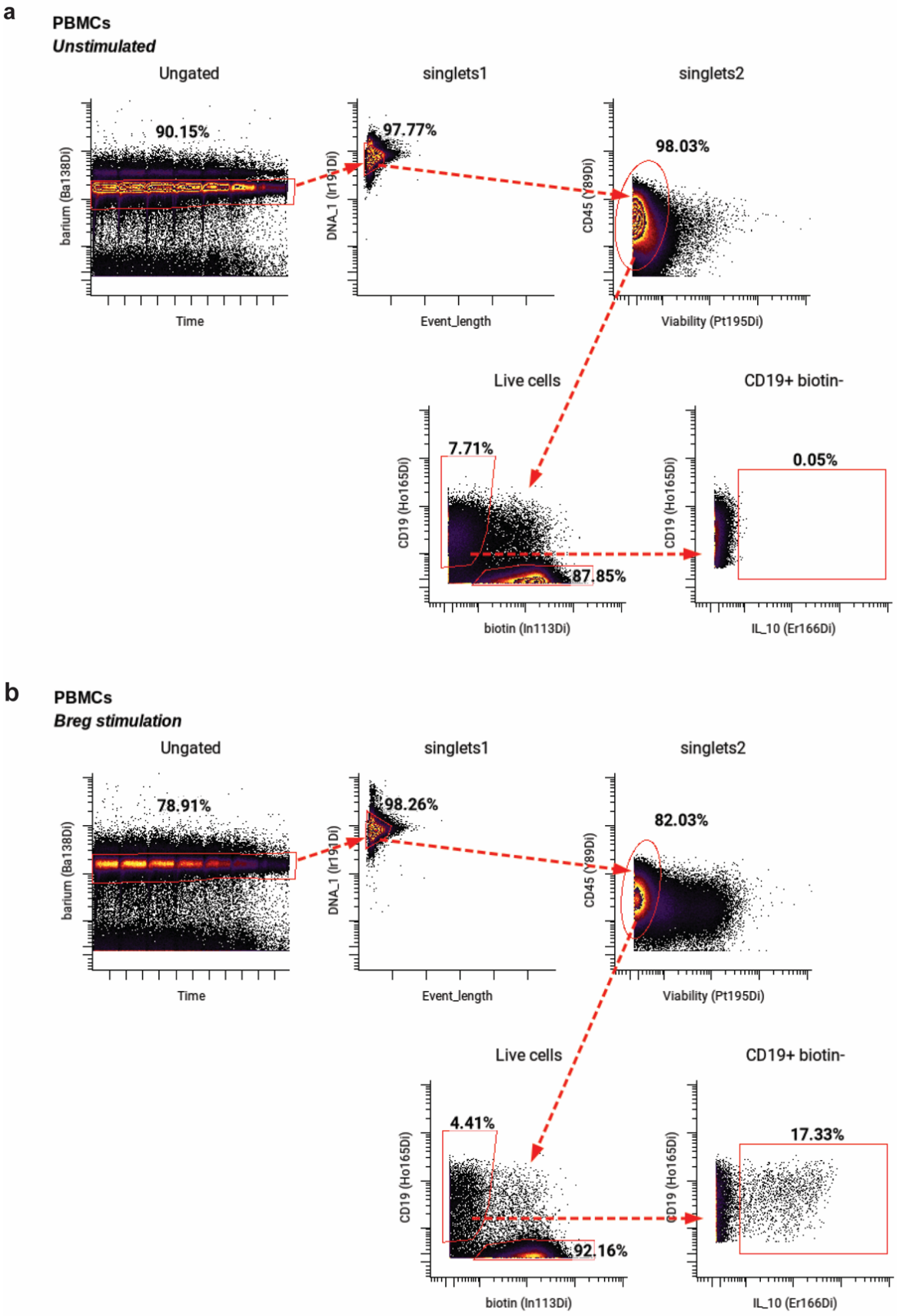

Figure S9. Representative gating strategy for unstimulated and stimulated B cells in mass cytometry analysis. Related to STAR Methods.

(A) Representative gating strategy for total unstimulated peripheral B cells within mass cytometry analysis of total PBMCs, with exemplary biaxial plot indicating absence of IL-10 expression.

(B) Representative gating strategy for total stimulated peripheral B cells within mass cytometry analysis of total PBMCs, with exemplary biaxial plot of high IL-10 expression.

Supplementary Table 1

Clinical group	n	Transplanted Organ(s)	IS at time of sampling?	Rejection episode before or at sampling?	IS	Interval wo IS before sampling range / mean (years)	Interval wo IS after sampling range / mean (years)	Sex ratio (M:F)	Age range (years)	Age mean (years)
Operational graft tolerance	4	Liver	No	No	----	0.25 - 13 / 7.1*	0.42 - 5 / 2.8*	1:1	0.42 - 21	13.6
Transplant recipient control	3	Liver	Yes	No	*Prograf*	----	----	1:2	7 - 19	14.8
Healthy control	2	None	No	----	----	----	----	1:1	19 - 32	25.5

** Data not available for one patient*

Table S1. Clinical characteristics of transplant patients and healthy controls. Related to Figure 5 and STAR Methods.

Abbreviations: *IS, Immunosuppression*

Supplementary Table 2

Donor	Status	Condition	Proportion live CD45+ cells among singlets (%)	Proportion B cells among live CD45+ cells (%)	Proportion IL-10+ B cells among total B cells (%)
HCON1	Healthy Control	Unstimulated	98.03	8.05	NA
HCON1	Healthy Control	Stimulated	72.05	1.33	25.42
HCON2	Healthy Control	Unstimulated	98.61	7.62	NA
HCON2	Healthy Control	Stimulated	81.82	1.37	28.89
TCON1	Transplant Control	Unstimulated	97.40	7.97	NA
TCON1	Transplant Control	Stimulated	84.32	4.41	8.09
TCON2	Transplant Control	Unstimulated	39.41	1.38	NA
TCON2	Transplant Control	Stimulated	66.76	2.88	2.57
TCON3	Transplant Control	Unstimulated	86.44	7.15	NA
TCON3	Transplant Control	Stimulated	90.65	2.02	9.31
TOL1	Graft Tolerant	Unstimulated	79.91	17.28	NA
TOL1	Graft Tolerant	Stimulated	70.63	12.23	23.70
TOL2	Graft Tolerant	Unstimulated	67.29	5.50	NA
TOL2	Graft Tolerant	Stimulated	57.81	3.14	24.43
TOL3	Graft Tolerant	Unstimulated	67.22	6.84	NA
TOL3	Graft Tolerant	Stimulated	82.03	4.64	16.74
TOL4	Graft Tolerant	Unstimulated	95.20	7.69	NA
TOL4	Graft Tolerant	Stimulated	36.76	8.69	28.03

Table S2. Frequency of live cells and total and IL-10^{+} B cells in clinical groups and healthy control individuals. Related to Figure 5 and STAR Methods.

Supplementary Table 3

Experiment	Condition	Reagent	Final concentration	Vendor	Catalogue number
Cytokine and phenotypic screen of B cell stimulations (Fig 1 and 3)	1 - 3	R848	1 ug/mL	Mabtech	3660-1
	4 - 6	Lipopolysaccharides (LPS) from Escherichia coli O127:B8	10 ug/mL	Sigma	L4516-1MG
	7 - 11	CpG ODN 2006	10 ug/mL	Invivogen	tlrl-2006
		Soluble human anti-CD40 clone 82111	500 ng/mL	RD Systems	MAB6321-100
	3, 6	Recombinant human IL-4	20 ng/mL	RD Systems	204-IL-020
	3, 6, 9, 11	Recombinant human IL-10	25 ng/mL	Biolegend	571002
	9 - 11	Recombinant human IL-2	600 IU/mL	Mabtech	3660-1
	9 - 10	Recombinant human IL-21	100 ng/mL	RD Systems	8879-IL-010
	10 - 11	Recombinant human IL-35	20 ng/mL	Enzo Life	ALX-522-140-C010
	All	Phorbol 12-myristate 13-acetate	50 ng/mL	Sigma	P8139-1MG
	All	Ionomycin calcium salt from Streptomyces conglobatus	1 ug/mL	Sigma	I0634-1MG
	All	Brefeldin A Solution	1x	eBioscience	00-4506-51
Timecourse of Breg stimulation (Fig 2-3), B cell subset sorting and stimulation (Fig 4), and clnical cohort and control B cell analysis (Fig 5)	All	CpG ODN 2006	10 ug/mL	Invivogen	tlrl-2006
	All	Soluble human anti-CD40 clone 82111	500 ng/mL	RD Systems	MAB6321-100
	All	Recombinant human IL-2	600 IU/mL	Mabtech	3660-1
	All	Recombinant human IL-21	100 ng/mL	RD Systems	8879-IL-010
	All	Recombinant human IL-35	20 ng/mL	Enzo Life	ALX-522-140-C010
	All	Phorbol 12-myristate 13-acetate	50 ng/mL	Sigma	P8139-1MG
	All	Ionomycin calcium salt from Streptomyces conglobatus	1 ug/mL	Sigma	I0634-1MG
	All	Brefeldin A Solution	1x	eBioscience	00-4506-51
T cell stimulation to validate antibody staining (Fig S5)	All	Plate-bound human anti-CD3	10 ug/mL	Biolegend	302933
	All	Plate-bound human anti-CD28	5 ug/mL	Biolegend	317325
	All	Recombinant human IL-4	12.5 ng/mL	RD Systems	204-IL-020
	All	Recombinant human IL-2	50 IU/mL	Biolegend	589102
	All	Brefeldin A Solution	1x	eBioscience	00-4506-51

Table S3. Reagents for PBMC *in vitro* stimulations. Related to STAR Methods.

Supplementary Table 4

Experiment	Antigen	Clone	Tag	Vendor
Panel 1: Mass cytometry analysis of healthy human peripheral B cells (Fig 1-3) and organ transplant recipient peripheral B cells (Fig 5)	CD45	HI30	89 Y	Biolegend
	CD9	HI9a	141 Pr	Biolegend
	CD39/ENTPD1	A1	142 Nd	Biolegend
	CD5	UCHT2	143 Nd	Biolegend
	CD45RB	MEM-55	145 Nd	Fluidigm
	CD20	2H7	147 Sm	Fluidigm
	CD25	BC96	149 Sm	Biolegend
	CD11c	Bu15	150 Nd	Biolegend
	CD71	CY1G4	151 Eu	Biolegend
	IgK	A8B5	153 Eu	Invitrogen
	CD27	M-T271	155 Gd	Biolegend
	CD183	G025H7	156 Gd	Fluidigm
	CD72	3F3	157 Gd	Biolegend
	CD23	EBVCS-5	160 Gd	Biolegend
	CD365/TIM-1	1D12	161 Dy	Biolegend
	CD95/FASR	DX2	164 Dy	Biolegend
	CD19	HIB19	165 Ho	Biolegend
	CD197/CCR7	G043H7	167 Er	Fluidigm
	CD73/NT5E1	AD2	168 Er	Biolegend
	CD24	ML5	169 Tm	Biolegend
	HLA-DR	L243	170 Er	Fluidigm
	CD1d	51.1	171 Yb	Biolegend
	CD38	HIT2	172 Yb	Biolegend
	CD1c	L161	173 Yb	Biolegend
	IgL	MHL-38	174 Yb	BD Biosciences
	CD305/LAIR1	NKTA255	176 Yb	Genetex
	CD22	HIB22	209 Bi	Biolegend
	Biotin	1D4-C5	113 In	Biolegend
	IgM	polyclonal	140 Ce	Invitrogen
	IL-4	MP4-25D2	144 Nd	Fluidigm
	IgD	IA6-2	146 Nd	Biolegend
	IgA	polyclonal	148 Nd	Fluidigm
	TNFa	Mab11	152 Sm	Fluidigm
	IL-6	MQ2-13A5	154 Sm	Biolegend
	Puromycin	12D10	158 Gd	Miltenyi Biotec
	IL-35/IL-12 p35	27537	159 Tb	RD Systems
	IgG	M1310G05	162 Dy	Biolegend
	Granzyme B	351927	163 Dy	RD Systems
	IL-10	JES3-9D7	166 Er	Biolegend
	BrU	3D4	175 Lu	BD Biosciences
	CD3	OKT3	Biotin	Biolegend
	CD7	CD7-6B6	Biotin	Miltenyi Biotec
	CD15	HI98	Biotin	Biolegend
	CD33	WM53	Biotin	Biolegend
	CD56	5.1H11	Biotin	Biolegend
	CD61	Y2/51	Biotin	Miltenyi Biotec
	CD235ab	HIR2	Biotin	Biolegend

Panel 2: Sorting B cell subsets (Fig 4)	CD20	2H7	PerCP-Cy5.5	Biolegend
	CD38	HIT2	FITC	Biolegend
	CD24	ML5	Brilliant Violet 421	Biolegend
	CD45RB	MEM-55	PE	Biolegend
	CD27	O323	Brilliant Violet 605	Biolegend
	CD95	DX2	Brilliant Violet 510	Biolegend
	CD11c	Bu15	Alexa Fluor 647	Biolegend
Panel 3: Mass cytometry analysis of healthy donor peripheral B cells in sorting experiment (Fig 4)	CD45	HI30	89 Y	Biolegend
	CD14	M5E2	113 In	Biolegend
	CD11b	ICRF44	115 In	Biolegend
	CD9	HI9a	141 Pr	Biolegend
	CD39/ENTPD1	A1	142 Nd	Biolegend
	CD16	3G8	143 Nd	Biolegend
	CD69	FN50	144 Nd	Fluidigm
	CD45RB	MEM-55	145 Nd	Fluidigm
	CD20	2H7	147 Sm	Fluidigm
	CD25	BC96	149 Sm	Biolegend
	CD11c	Bu15	150 Nd	Biolegend
	CD4	RPA-T4	151 Eu	Biolegend
	TIGIT	MAB7898	153 Eu	Fluidigm
	CD27	M-T271	155 Gd	Biolegend
	CD3	UCHT1	156 Gd	Biolegend
	CD72	3F3	157 Gd	Biolegend
	CD95/FASR	DX2	164 Dy	Biolegend
	CD19	HIB19	165 Ho	Biolegend
	CD197/CCR7	G043H7	167 Er	Fluidigm
	CD24	ML5	169 Tm	Biolegend
	HLA-DR	L243	170 Er	Fluidigm
	CD8a	RPA-T8	171 Yb	Biolegend
	CD38	HIT2	172 Yb	Biolegend
	CD73/NT5E	AD2	173 Yb	Biolegend
	CD279/PD1	EH12.2H7	174 Yb	Fluidigm
	CD127	A019D5	176 Yb	Biolegend
	CD5	UCHT2	209 Bi	Biolegend
	IgM	polyclonal	140 Ce	Invitrogen
	IgD	IA6-2	146 Nd	Biolegend
	IgA	polyclonal	148 Nd	Fluidigm
	TNFa	Mab11	152 Sm	Fluidigm
	IL-6	MQ2-13A5	154 Sm	Biolegend
	Puromycin	12D10	158 Gd	Miltenyi Biotec
	IL35/IL12 p35	27537	159 Tb	RD Systems
	CD152/CTLA4	14D3	160 Gd	Invitrogen
	IL17A	BL168	161 Dy	Fluidigm
	IgG	M1310G05	162 Dy	Biolegend
	IL-10	JES3-9D7	166 Er	Biolegend
	IFNg	B27	168 Er	Fluidigm
	BrU	3D4	175 Lu	BD Biosciences
	CFSE/FITC	polyclonal	198 Pt	Biolegend

Table S4. Antibody panel used for mass cytometry analysis. Related to STAR Methods.