Supplementary Information

Ex vivo-expanded human CD19^{+}TIM-1^{+} regulatory B cells suppress immune responses *in vivo* and are dependent upon the TIM-1/STAT3 axis

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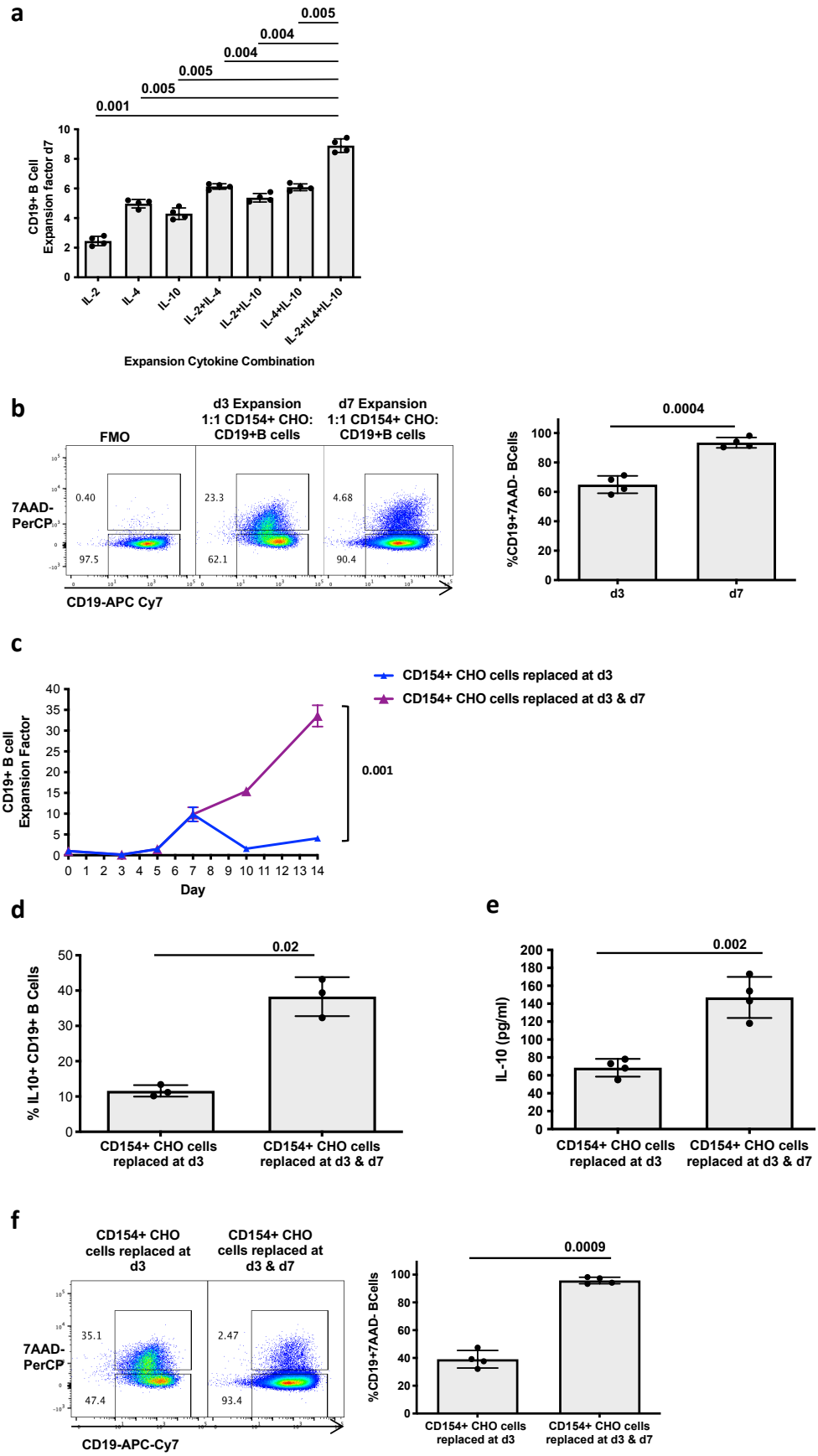
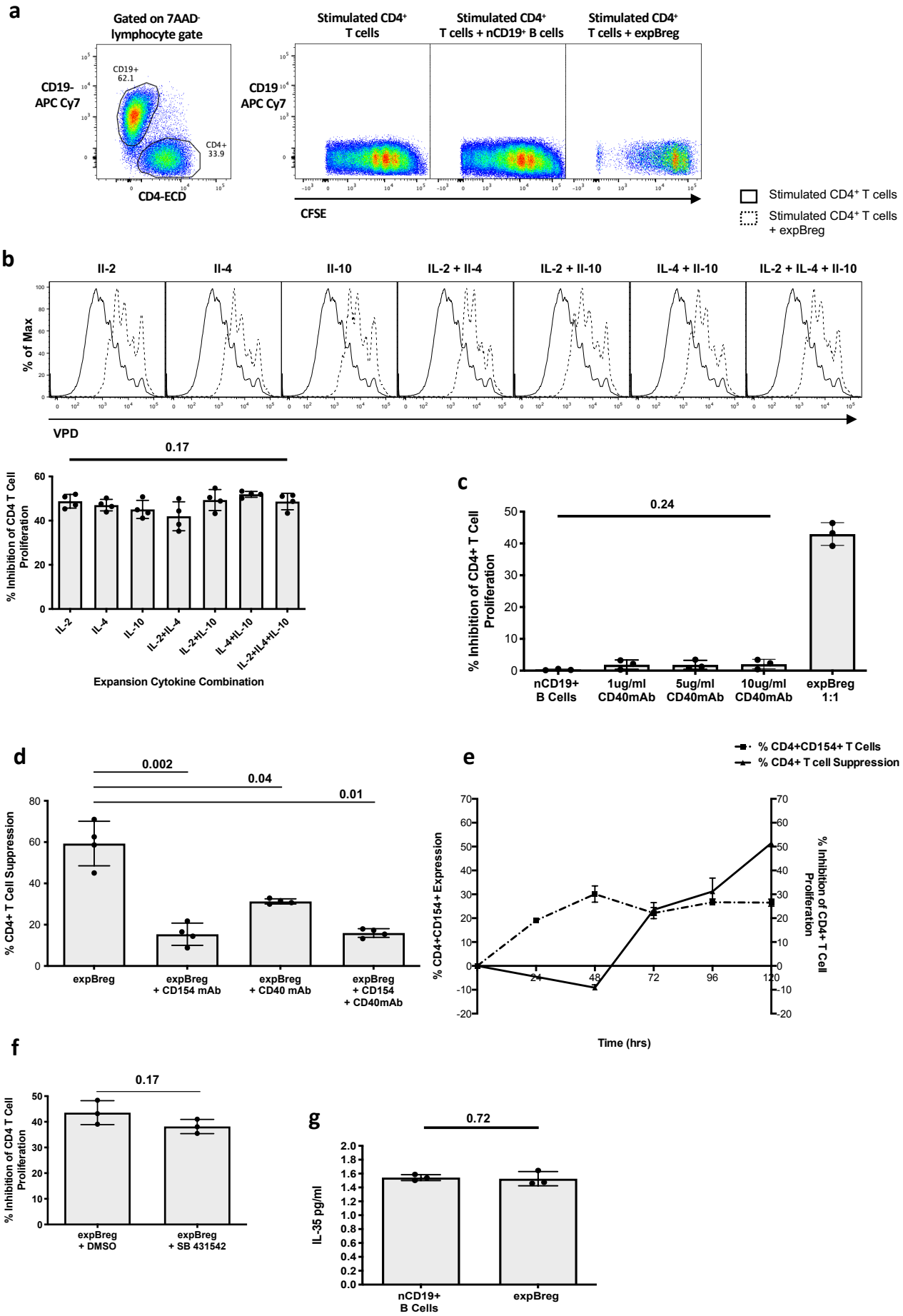
Supplementary Figure 1



Supplementary Figure 1

a) Summarised data of expansion factor of expBreg when expanded at a 1:1 $CD154^+$ CHO cell-$CD19^+$ B cell ratio with different cytokine combinations are presented. Data from experiments performed with cells from different healthy donors ($n = 4$) are presented. Each dot is an individual response. **(b)** Representative FACS plots and summarised data of % $CD19^+7AAD^+$ B cells at day-3 and day-7 of B cell expansion are shown. Data from experiments performed with cells from different healthy donors ($n = 4$) are presented. Each dot is an individual response. **(c)** Human $CD19^+$ B cell expansion could be maintained in culture for at least 14 days when $CD154^+$ CHO cells were replaced at day-3 and day-7 within the *ex vivo* culture system. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. **(d)** Summarised data of % $CD19^+IL\text{-}10^+$ B cells at day-14 of B cell expansion are shown, when $CD154^+$ CHO cells were replaced either at day-3 only, or at day-3 and day-7 within the *ex vivo* culture system. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. **(e)** Summarised data of IL-10 production by human $CD19^+$ B cells at day-14 of B cell expansion are shown, when $CD154^+$ CHO cells were replaced either at day-3 only, or at day-3 and day-7 within the *ex vivo* culture system. Data from experiments performed with cells from different healthy donors ($n = 4$) are presented. Each dot is an individual response. **(f)** Representative FACS plots and summarised data of % $CD19^+7AAD^+$ B cells at day-14 of B cell expansion are shown, when $CD154^+$ CHO cells were replaced either at day-3 only, or at day-3 and day-7 within the *ex vivo* culture system. Data from experiments performed with cells from different healthy donors ($n = 4$) are presented. Each dot is an individual response. Error bars in each panel represent Mean +/- SD **(a, b, d, e, f)**. One-way ANOVA with Tukey's multiple comparisons test **(a)** and 2-tailed paired t-test **(b, c, d, e, f)** were used. Source data are provided as a Source Data file.

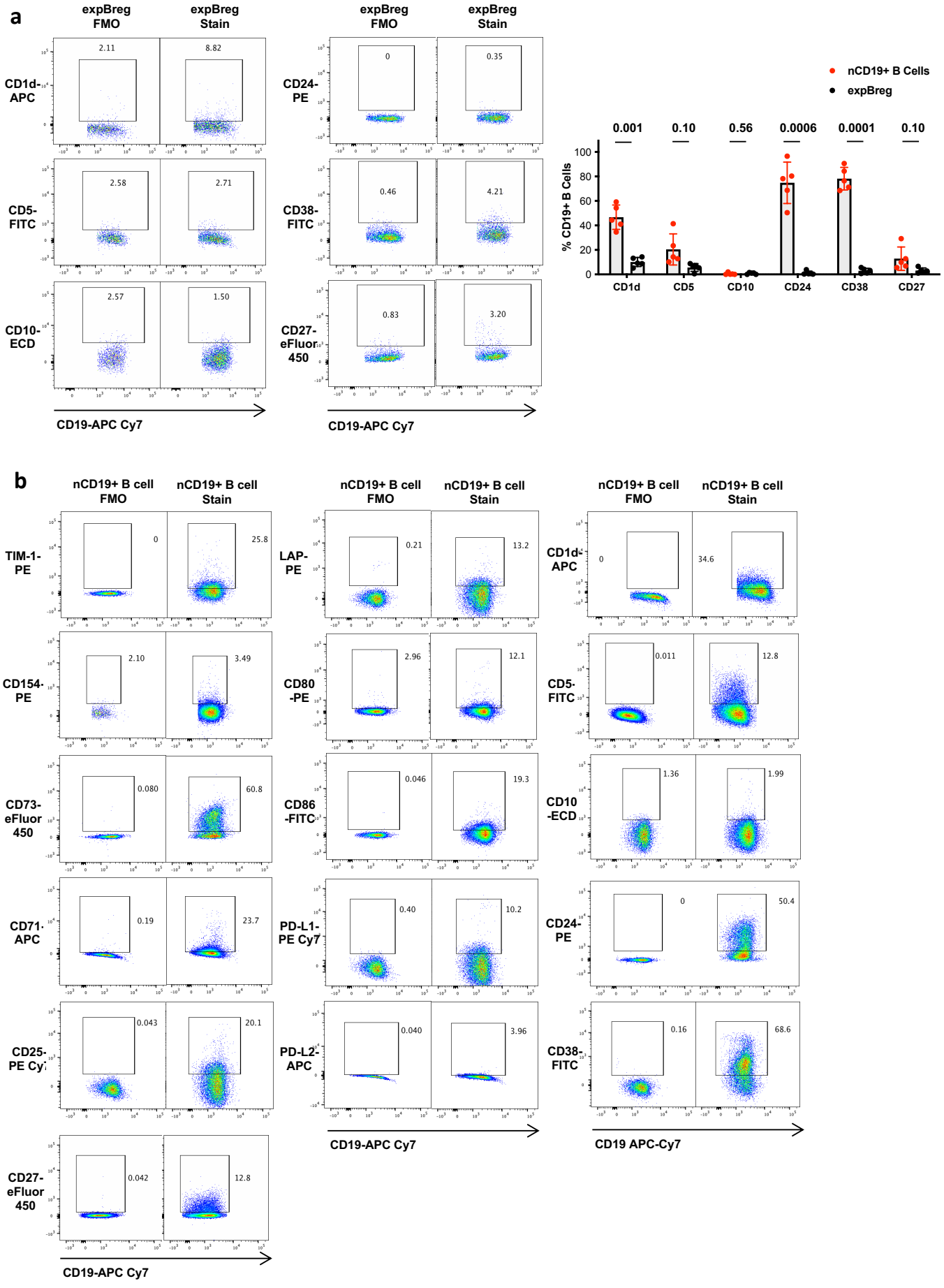
Supplementary Figure 2



Supplementary Figure 2

(a) Gating strategy & representative FACS plots of live $CD4^+CFSE^+$ T cells when autologous $CD4^+CFSE^+$ T cells were cultured with anti-CD3/CD28 beads for 5 days +/- non-expanded $CD19^+$ B cells or expBreg at 1:1 ratio of B:T cells. Live/dead exclusion was performed using 7-AAD. **(b)** Representative histograms of live $CD4^+VPD^+$ T cells and summarised data of suppressive potency of expBreg when expanded at a 1:1 $CD154^+$ CHO cell-$CD19^+$ B cell ratio with different cytokine combinations, are presented. Autologous $CD4^+VPD^+$ T cells were cultured with anti-CD3/CD28 beads for 5 days +/- expBreg. % Inhibition of $CD4^+$ T cell proliferation is an expression of Division Index of live $CD4^+CFSE^+$ T cells at day 5 relative to that of the Stimulated $CD4^+$ T cell control. Data from experiments performed with cells from different healthy donors ($n = 4$) are presented. Each dot is an individual response. **(c)** Summarised data of % inhibition of CD4+ T cell proliferation are presented, when autologous $CD4^+VPD^+$ T cells were cultured with anti-CD3/CD28 beads for 5 days +/-non-expanded $CD19^+$ B cells (n$CD19^+$ B cells), expBreg or $CD19^+$ B cells that had been stimulated with plate-bound agonistic CD40 mAb (at [1ug/ml], [5ug/ml] or [10ug/ml] concentrations) for 7 days. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. **(d)** Summarised data of % inhibition of $CD4^+$ T cell proliferation by expBreg when in the presence of blocking CD154 mAb, blocking CD40 mAb, or both. Blocking mAbs were added at day-0 of the 5-day suppression assay. Data from experiments performed with cells from different healthy donors ($n = 4$) are presented. Each dot is an individual response. **(e)** Summarised data of % Inhibition of $CD4^+$ T cell proliferation by expBreg and expression of CD154 on $CD4^+$ T cells when measured daily in the 5-day suppression assay. Data from experiments performed with cells from different healthy donors ($n = 4$) are presented. Each dot is an individual response. **(f)** Summarised data of % inhibition of $CD4^+$ T cell proliferation by expBreg when in the presence of TGFβ inhibitor SB 431542 [μM/L] or DMSO. SB 431542 or DMSO were added at day-0 of the 5-day suppression assay. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. **(g)** Summarised data of IL-35 production by n$CD19^+$ B cells or expBreg. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. Error bars in each panel represent Mean +/- SD **(b, c, e, f, g)**. One-way ANOVA with Tukey's multiple comparisons test **(b, c, d)** and 2-tailed paired t-test **(f, g)** were used. Source data are provided as a Source Data file.

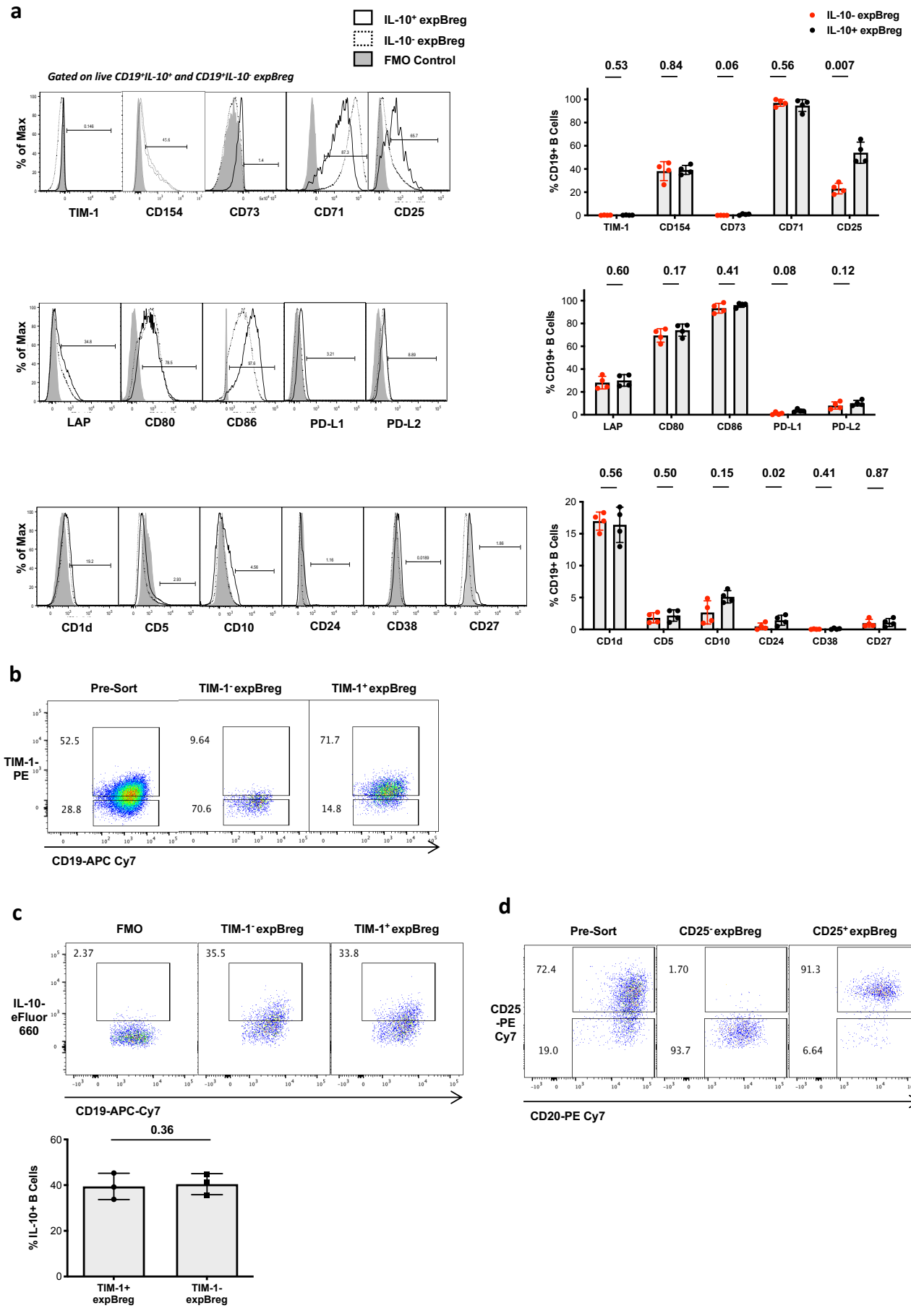
Supplementary Figure 3



Supplementary Figure 3

(a) Representative FACS plots of live $CD19^+$ B cells and summarised data demonstrating expression of cell surface markers by expBreg. Gating is on FMO controls. % Expression is compared between expBreg and autologous non-expanded $CD19^+$ B cells ($nCD19^+$ B cells). Data from experiments performed with cells from different healthy donors ($n = 5$) are presented. Each dot is an individual response. **(b)** Representative FACS plots of live $CD19^+$ B cells demonstrating expression of cell surface markers by non-expanded $CD19^+$ B cells ($nCD19^+$ B cells). Gating is on FMO controls. Data from experiments performed with cells from different healthy donors ($n = 5$) are presented. Each dot is an individual response. Error bars in **(a)** represent Mean +/- SD. 2-tailed paired t-tests **(a)** were used. Source data are provided as a Source Data file.

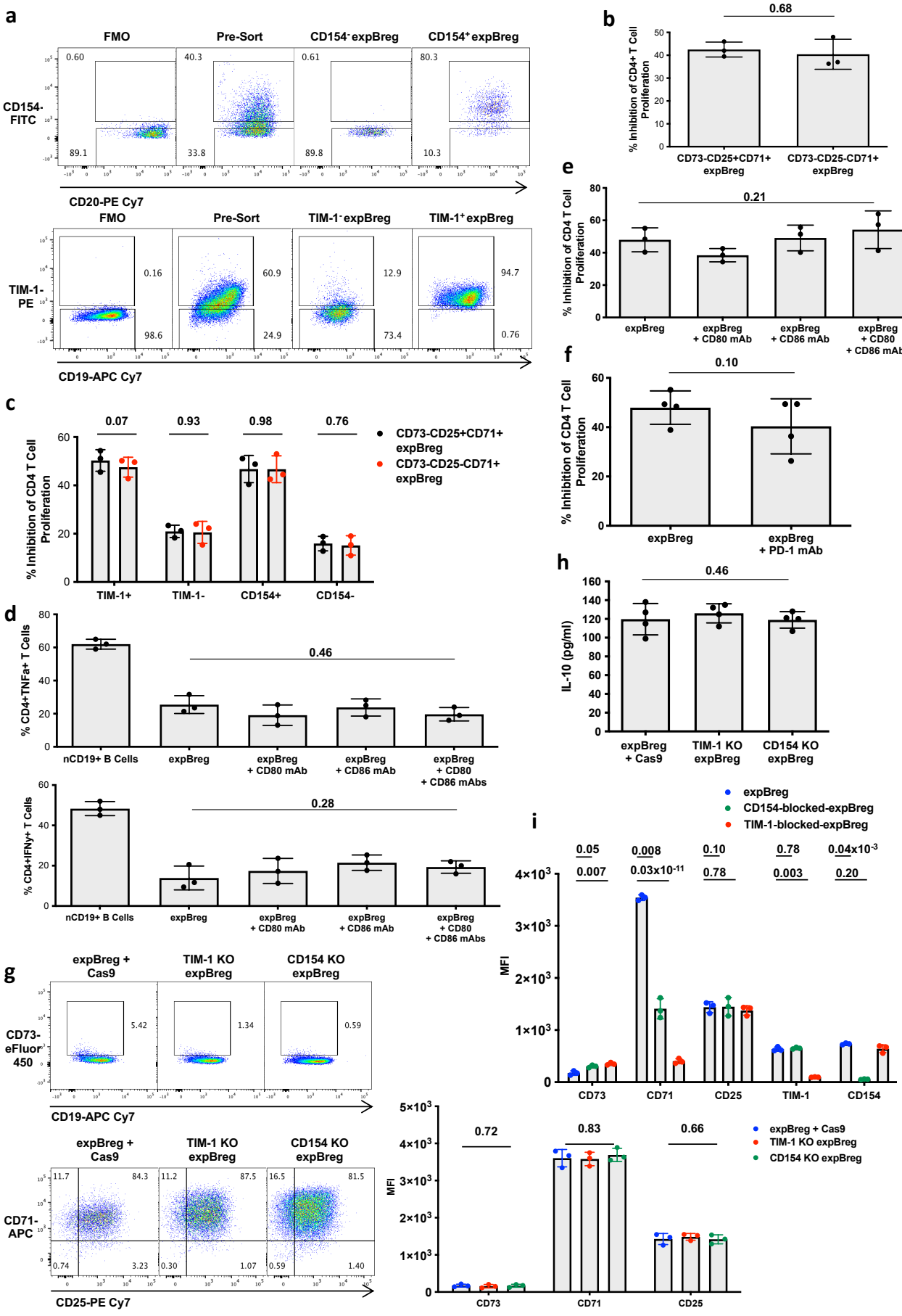
Supplementary Figure 4



Supplementary Figure 4

(a) Representative histograms and summarised data of cell surface markers expressed by IL-10^+ and IL-10^- $CD19^+$ expBreg are presented. Gating is on FMO controls. Data from experiments performed with cells from different healthy donors ($n = 4$) are presented. Each dot is an individual response. **(b)** FACS-sorting strategy to isolate expBreg based on the expression of TIM-1 **(c)** Representative FACS plots and summarised data of IL-10 expression by FACS-sorted TIM-1^+ and TIM-1^- expBreg. Gating is on fluorescence minus one controls (FMO). **(d)** FACS-sorting strategy to isolate expBreg based on the expression of CD25. Error bars in **(a, c)** represent Mean +/- SD. 2-tailed paired t-tests **(a, c)** were used. Source data are provided as a Source Data file.

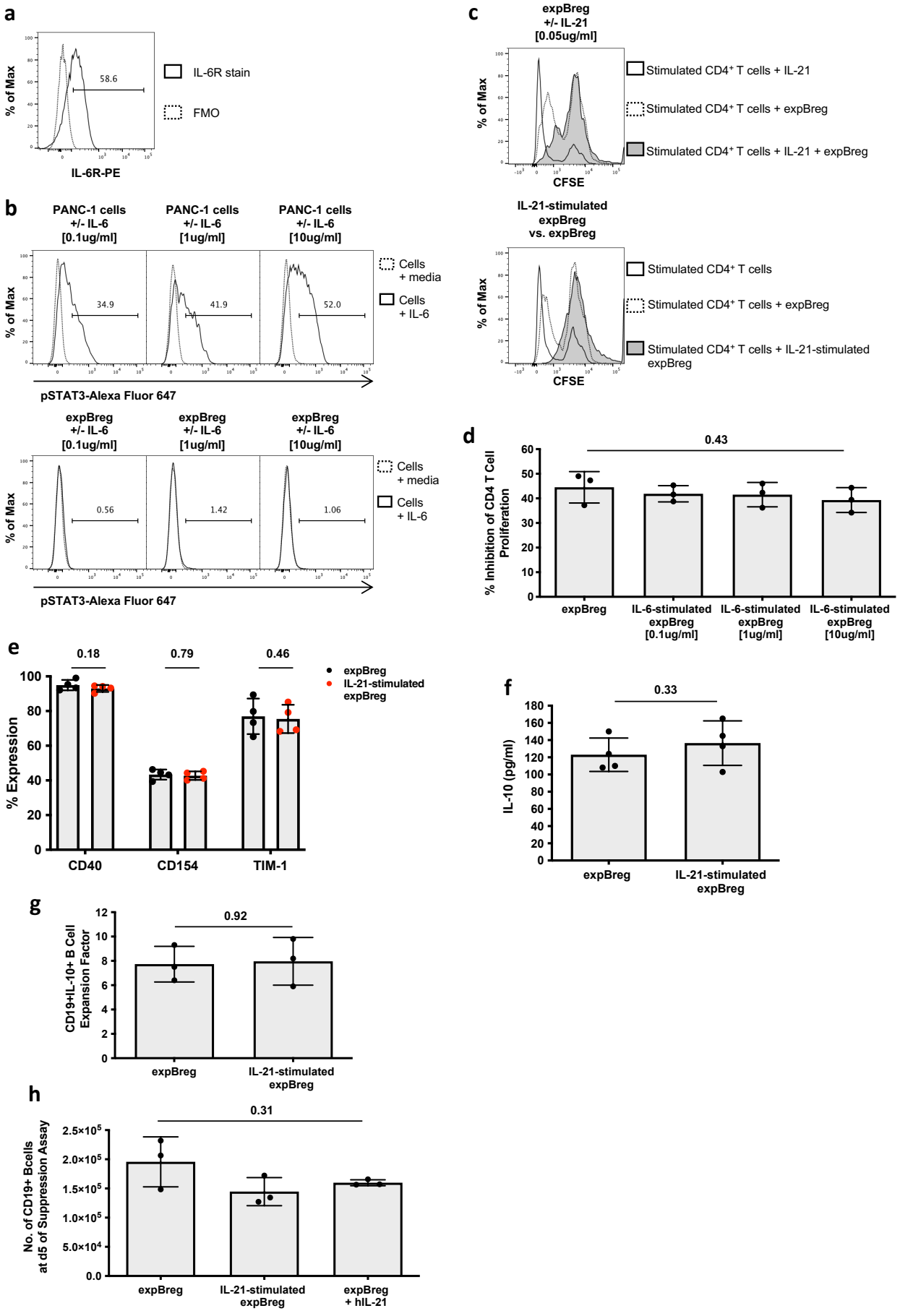
Supplementary Figure 5



Supplementary Figure 5

(a) FACS-sorting strategy to isolate expBreg based on the expression of CD154 or TIM-1. Gating is on fluorescence minus one controls (FMO). **(b)** Summarised data of % inhibition of $CD4^+$ T cell proliferation by FACS-sorted $CD73^-CD25^+CD71^+$ and $CD73^-CD25^-CD71^+$ expBreg are presented. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. **(c)** Summarised data of % inhibition of $CD4^+$ T cell proliferation by FACS-sorted $CD73^-CD25^+CD71^+$ and $CD73^-CD25^-CD71^+$ expBreg which had been further FACS-sorted based on TIM-1 and CD154 expression, are presented. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. **(d)** Summarised data of % $CD4^+TNFa^+$ and $CD4^+IFNy^+$ T cells at day-3 of suppression assay when autologous $CD4^+$ T cells were cultured with anti-CD3/CD28 beads +/- expBreg when in the presence of blocking CD80 mAb, blocking CD86 mAb, or both. Blocking mAbs was added at day-0 of suppression assay. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. **(e)** Summarised data of % inhibition of $CD4^+$ T cell proliferation by expBreg when in the presence of blocking CD80 mAb, blocking CD86 mAb, or both. Blocking mAbs were added at day-0 of suppression assay. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. **(f)** Summarised data of % inhibition of $CD4^+$ T cell proliferation by expBreg when in the presence of blocking PD-1 mAb. Blocking mAb was added at day-0 of suppression assay. Data from experiments performed with cells from different healthy donors ($n = 4$) are presented. Each dot is an individual response. **(g)** Representative FACS plots of live $CD19^+$ expBreg and summarised data are presented which demonstrate protein expression of CD73, CD25 and CD71 after electroporation with Cas9 alone +/- TIM-1 or CD154 multiguide RNAs to generate TIM-1 or CD154 knockouts (KO). **(h)** Summarised data of IL-10 production by Cas9-expBreg, TIM-1 KO or CD154 KO expBreg are presented. Data from experiments performed with cells from different healthy donor ($n = 4$) are presented. Each dot is an individual response. **(i)** Summarised data of mean fluorescence intensity (MFI) of cell surface markers expressed by expBreg which had been pre-incubated with IgG isotype control (Isotype-expBreg), anti-CD154 mAb (CD154-blocked-expBreg) or anti-TIM-1 mAb (TIM-1-blocked-expBreg) for the last 48 hours of expansion, are presented. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. Error bars in **(b, c, d, e, f, g, h, i)** represent Mean +/- SD. One-way ANOVA with Tukey's multiple comparisons test **(d, e, g, h, i)** and 2-tailed paired t-test **(b, c, f)** were used. Source data are provided as a Source Data file.

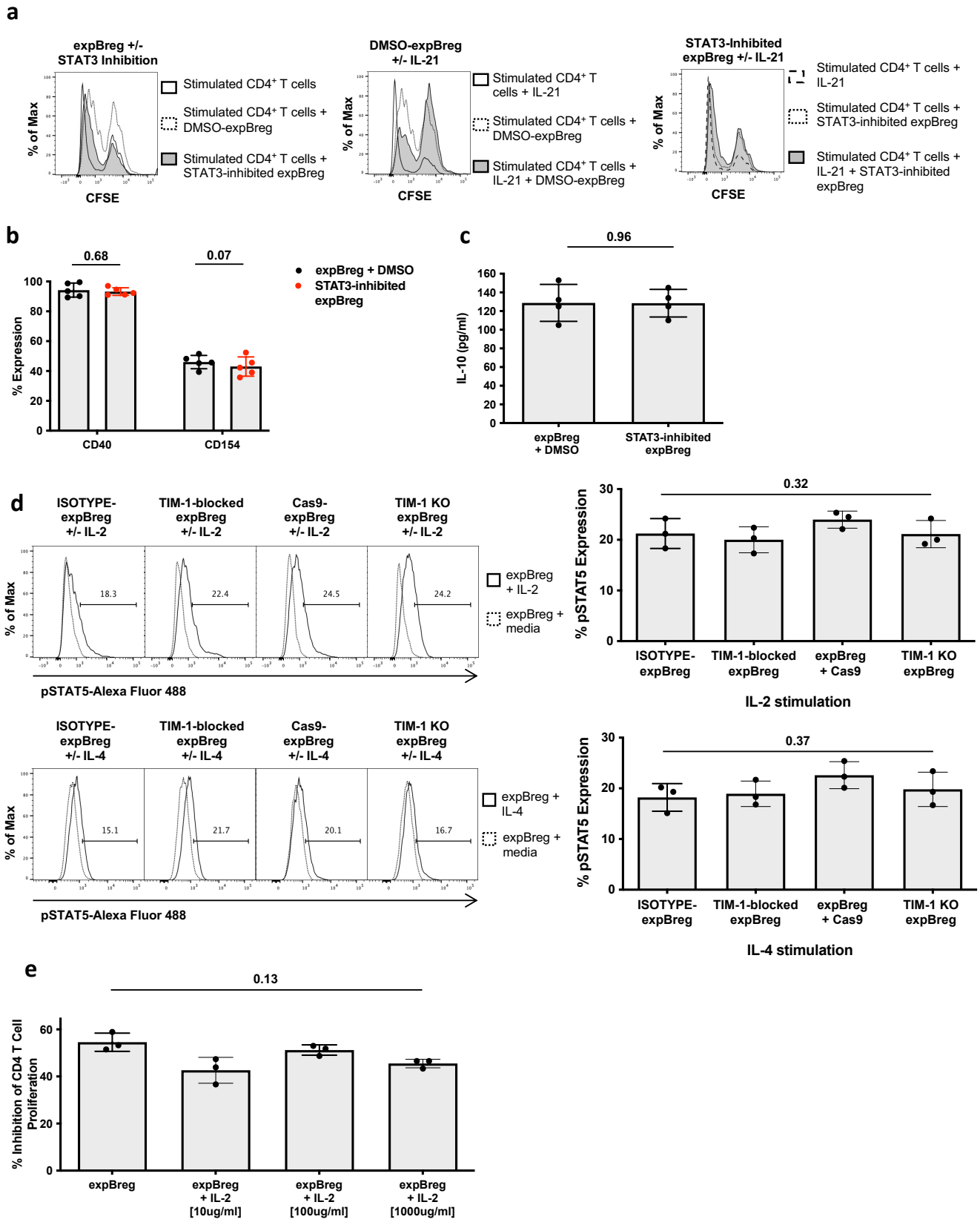
Supplementary Figure 6



Supplementary Figure 6

(a) Representative histograms of live $CD19^+$ expBreg are shown to demonstrate extracellular surface expression of IL-6 receptor (IL-6R). Gating is on fluorescence minus one controls (FMO). **(b)** Representative histograms of % STAT3 phosphorylation (pSTAT3) are presented when expBreg or an immortalised PANC-1 cell line were incubated with media or IL-6 ([0.01µg/ml], [1ug/ml] or [10ug/ml]) for 10 minutes and stained for pSTAT3. The PANC-1 cell line served as a positive control for IL-6-induced STAT3 phosphorylation. **(c)** Representative histograms of live $CD4^+CFSE^+$ T cells are presented when autologous $CD4^+CFSE^+$ T cells and anti-CD3/CD28 beads were co-cultured with expBreg +/- IL-21. expBreg were either pre-incubated with IL-21 [0.05µg/ml] for the last 48hrs of the 7-day expansion co-culture (IL-21-stimulated expBreg) prior to addition to the suppression assay at day-0, or IL-21 [0.05µg/ml] was directly added at day-0 of the suppression assay +/- expBreg (expBreg + hIL-21). **(d)** Summarised data of % inhibition of $CD4^+$ T cell proliferation are presented when autologous $CD4^+VPD^+$ T cells and anti-CD3/CD28 beads were co-cultured alone or with expBreg. expBreg had been pre-incubated with IL-6 ([0.01µg/ml], [1ug/ml] or [10ug/ml]) for the last 48hrs of the 7-day expansion co-culture (IL-6-stimulated expBreg). Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. **(e)** Summarised data are presented which demonstrate % expression of CD40, CD154 and TIM-1 by expBreg and IL-21-stimulated expBreg. Data from experiments performed with cells from different healthy donors ($n = 4$) are presented. Each dot is an individual response. **(f)** Summarised data of IL-10 production by expBreg and IL-21-stimulated expBreg. Data from experiments performed with cells from different healthy donors ($n = 4$) are presented. Each dot is an individual response. **(g)** Summarised data of expansion factor of expBreg +/- IL-21 for the last 48hrs of the 7-day expansion co-culture. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. **(h)** Summarised data of number of $CD19^+$ B cells at day-5 of suppression assay when expBreg, IL-21-stimulated expBreg or expBreg + IL-21 had been added at day-0 of suppression assay. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Error bars in **(d, e, f, g, h)** represent Mean +/- SD. One-way ANOVA with Tukey's multiple comparisons test **(d, h)** and 2-tailed paired t-test **(e, f, g)** were used. Source data are provided as a Source Data file.

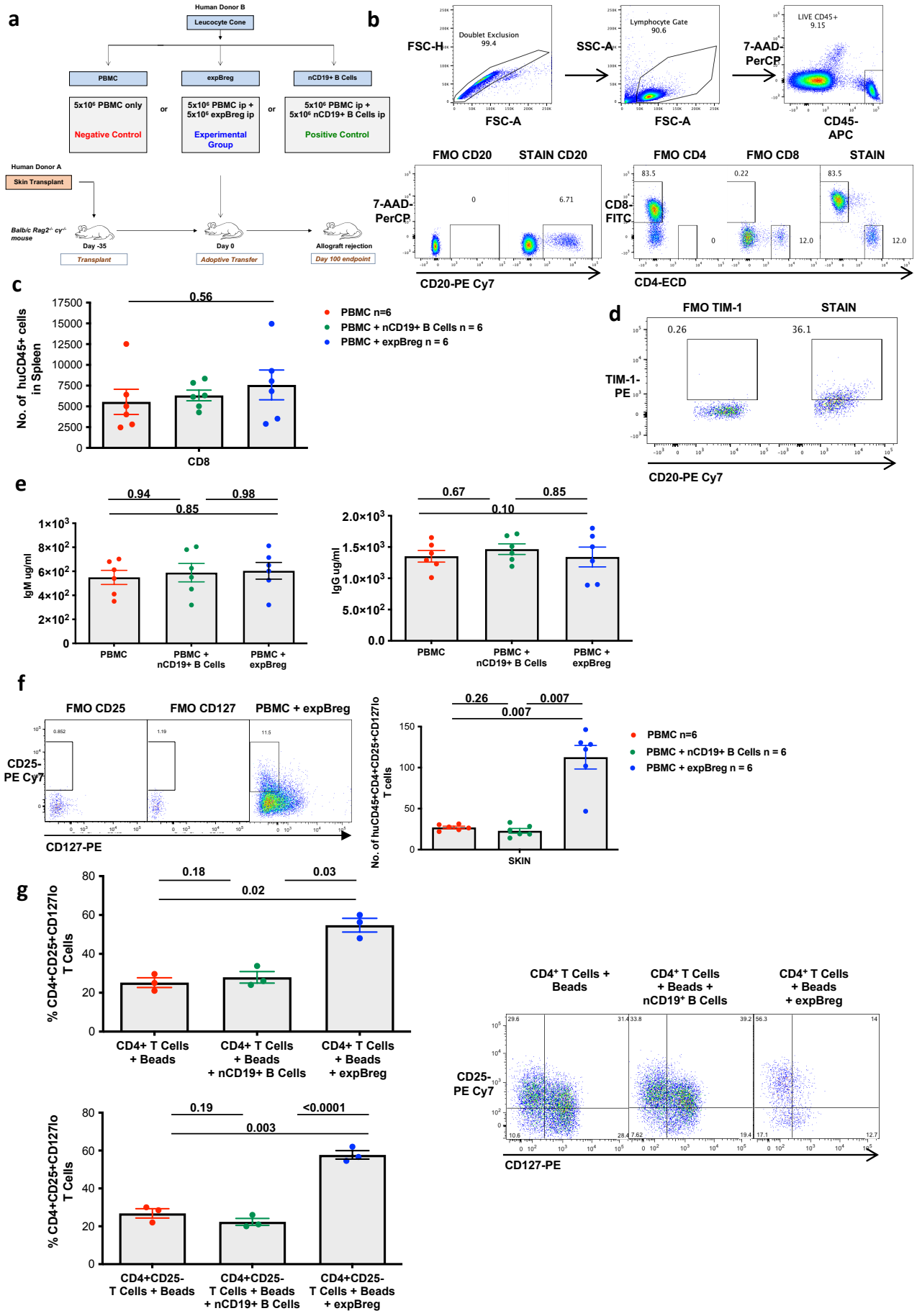
Supplementary Figure 7



Supplementary Figure 7

(a) Representative histograms of live $CD4^+CFSE^+$ T cells are presented when autologous $CD4^+CFSE^+$ T cells and anti-CD3/CD28 beads were co-cultured with expBreg +/- STAT3 inhibitor +/- IL-21 [0.05µg/ml]. expBreg were incubated for 2 hours with the STAT3 inhibitor WP 1066 [10µM] +/- hIL-21 [0.05µg/ml], or DMSO +/- hIL-21 [0.05µg/ml], washed and suppressive function analysed. **(b)** Summarised data are presented which demonstrate expression of CD40 and CD154 in the presence of STAT3 inhibition or DMSO. Data from experiments performed with cells from different healthy donors ($n = 5$) are presented. Each dot is an individual response. **(c)** Summarised data of IL-10 production by expBreg are presented when in the presence of STAT3 inhibition or DMSO. Data from experiments performed with cells from different healthy donors ($n = 4$) are presented. Each dot is an individual response. **(d)** Representative histograms of $CD19^+$ B cells and summarised data of % STAT5 phosphorylation (pSTAT5) are presented when Isotype-expBreg, TIM-blocked-expBreg, Cas9-expBreg or TIM-1 KO expBreg were incubated with media, IL-2 [10^4 U/ml] or IL-4 [1µg/ml] for 10 minutes and stained for pSTAT5. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. **(e)** Summarised data of % inhibition of $CD4^+$ T cell proliferation are presented when autologous $CD4^+CFSE^+$ T cells and anti-CD3/CD28 beads were co-cultured with expBreg +/- IL-2. IL-2 was added at day-0 of the suppression assay, at different concentrations ([10ug/ml], [100ug/ml], 1000ug/ml]). Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. Error bars in **(b, c, d, e)** represent Mean +/- SD. One-way ANOVA with Tukey's multiple comparisons test **(d, e)** and 2-tailed paired t-test **(b, c)** were used. Source data are provided as a Source Data file.

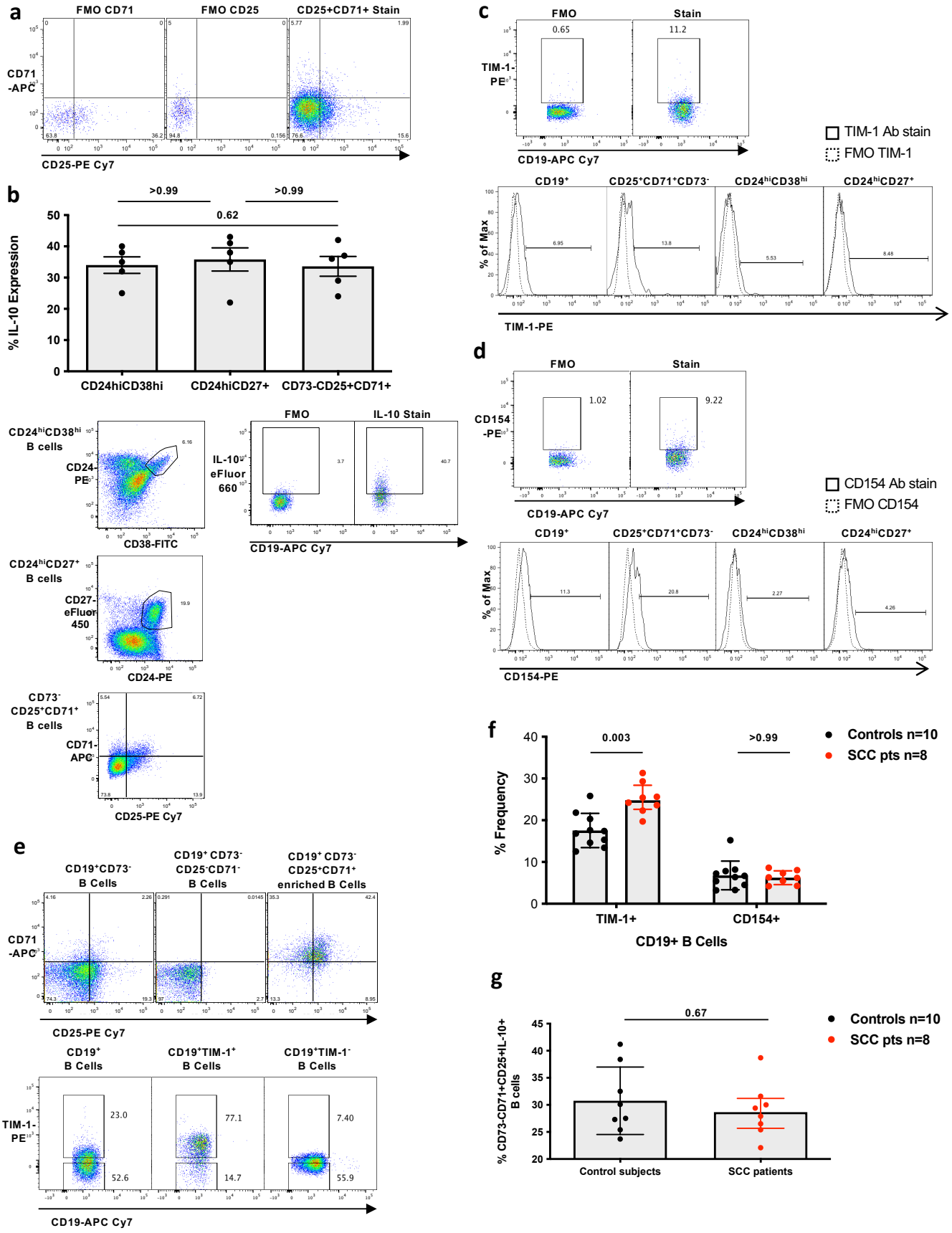
Supplementary Figure 8



Supplementary Figure 8

(a) Humanised mouse model of human skin transplantation experimental set-up. **(b)** Gating strategy and representative FACS plots of live huCD45$^+$ cells in human skin allograft are presented. Gating is on fluorescence minus one controls (FMO). **(c)** Summarised data of the number of huCD45$^+$CD8$^+$ T cells in spleen of mice receiving PBMC +/- nCD19$^+$ B cells or expBreg are presented. n=6 per group per experiment, one of three independent experiments is shown. Each dot is an individual mouse response. Each experiment used a different HLA-mismatched human donor pair. **(d)** Representative FACS plots of live huCD45$^+$CD20$^+$TIM-1$^+$ B cells in human skin allograft are presented. Gating is on FMO controls. **(e)** Summarised data of serum levels of human IgM and IgG in peripheral blood of mice receiving PBMC +/- nCD19$^+$ B cells or expBreg are presented. n=6 per group per experiment, one of three independent experiments is shown. Each dot is an individual mouse response. Each experiment used a different HLA-mismatched human donor pair. **(f)** Representative FACS plots and summarised data of the number of live huCD45$^+$CD4$^+$CD25$^+$CD127^{lo} putative Treg in human skin allograft of mice receiving PBMC +/- nCD19$^+$ B cells or expBreg are presented. n=6 per group per experiment, one of three independent experiments is shown. Each dot is an individual mouse response. Each experiment used a different HLA-mismatched human donor pair. **(g)** Representative FACS plots and summarised data of live CD4$^+$ T cells demonstrating % of putative Treg when expBreg and anti CD3/CD28 beads are co-cultured with CD4+ T cells or CD4$^+$CD25$^-$ T cells *in vitro* for 5 days, when compared to controls. Data from experiments performed with cells from different healthy donors ($n = 3$) are presented. Each dot is an individual response. Error bars in **(c, e, f)** represent Mean +/- SEM. Error bars in **(g)** represent Mean +/- SD. One-way ANOVA with Tukey's multiple comparisons test **(c, e, f, g)** were used. Source data are provided as a Source Data file.

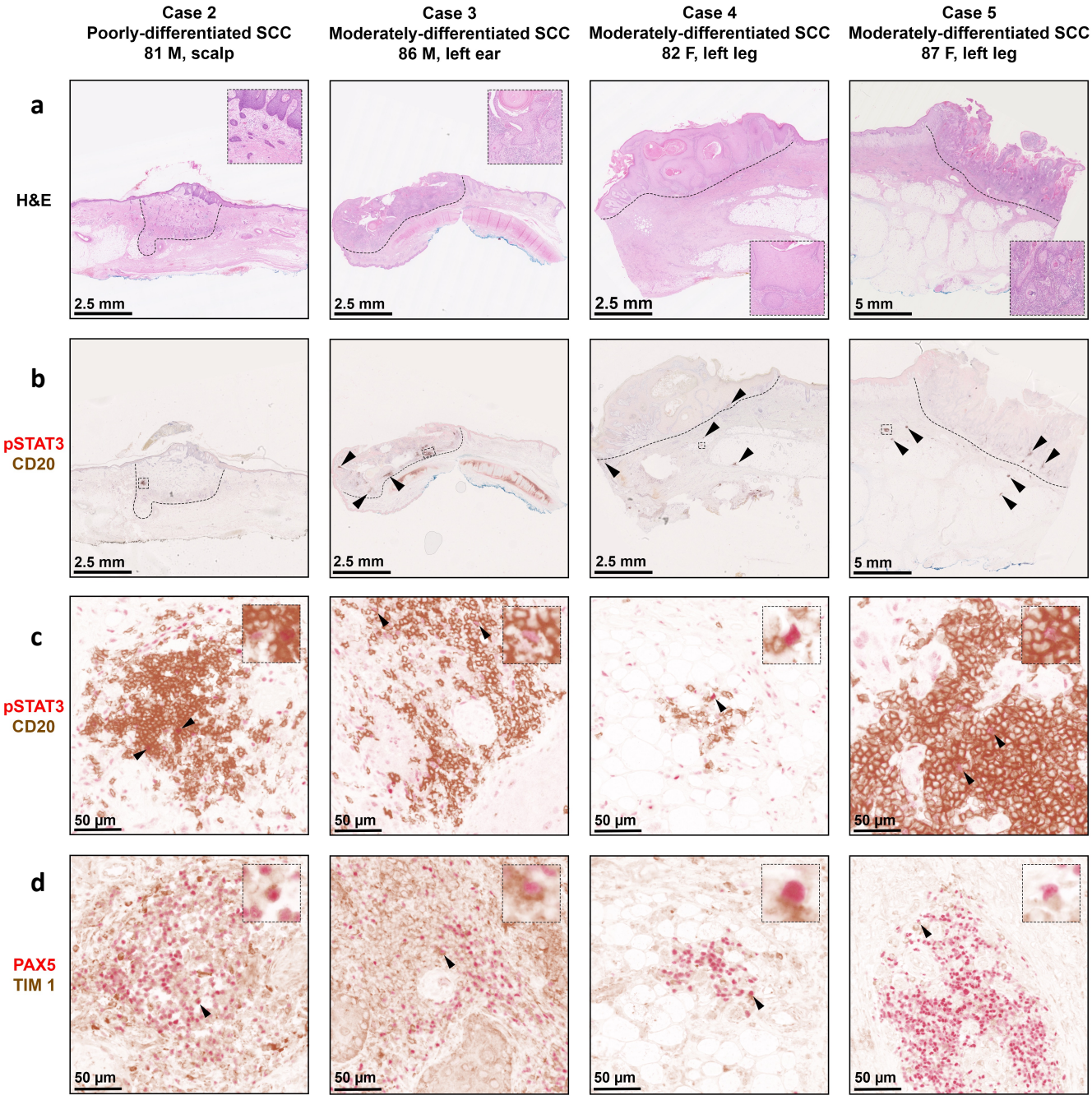
Supplementary Figure 9



Supplementary Figure 9

(a) Representative FACS plots of live $CD19^{+}CD73^{-}$ B cells to demonstrate gating strategy for $CD19^{+}CD73^{-}CD25^{+}CD71^{+}$ B Cells from human peripheral blood. Gating is on fluorescence minus one controls (FMO). **(b)** Representative FACS plots and summarised data of % IL-10 expression by $CD24^{hi}CD38^{hi}$, $CD24^{hi}CD27^{+}$ and $CD73^{-}CD25^{+}CD71^{+}$ Breg subsets from human peripheral blood. Gating is on FMO controls. Data from experiments performed with cells from different healthy donors ($n = 5$) are presented. Each dot is an individual response. **(c)** Representative FACS plots of % TIM-1^{+} B cells in whole $CD19^{+}$ B cells, $CD73^{-}CD25^{+}CD71^{+}$, $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cell subsets in peripheral blood of healthy human donors. Gating is on FMO controls. **(d)** Representative FACS plots of % $CD154^{+}$ B cells in whole $CD19^{+}$ B cells, $CD73^{-}CD25^{+}CD71^{+}$, $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cell subsets in peripheral blood of healthy human donors. Gating is on FMO controls. **(e)** Representative FACS plots of FACS-sorted $CD73^{-}CD25^{-}CD71^{-}$, $CD73^{-}CD25^{+}CD71^{+}$ enriched, TIM-1^{+} and TIM-1^{-} B cells from human peripheral blood. **(f)** Summarised data of % TIM-1^{+} and $CD154^{+}$ B cells within $CD19^{+}$ B cells in peripheral blood of healthy human donors (n=10) and age-matched patients with SCC (n=8). Each dot is an individual response. **(g)** Summarised data of % IL-10 expression within $CD73^{-}CD25^{+}CD71^{+}$ B cells in peripheral blood of healthy human donors (n=10) and age-matched patients with SCC (n=8). Each dot is an individual response**.** Error bars in **(b, f, g)** represent Median + interquartile range. One way ANOVA with Dunn's multiple comparisons test **(b)** and 2-tailed Mann Whitney test **(f, g)** were used. Source data are provided as a Source Data file.

Supplementary Figure 10



Supplementary Figure 10

(a) Hematoxylin and eosin (H&E) stained histological sections demonstrating invasive squamous cell carcinomas in an additional four patients (hashed line delineates tumour boundary; higher magnification image demonstrating representative histological features in top or bottom right-hand corners). **(b)** Double immunohistochemistry staining for pSTAT3 (red) and CD20 (brown) demonstrates the peritumoral accumulation of $CD20^+$ B cells (arrows), predominantly arranged as clusters, both within the tumours themselves and in proximity to their invasive margins (hashed line). **(c)** Higher magnification images of the annotated B cell clusters shown in (b) (hashed boxes), demonstrating double positive cells (arrows). **(d)** On the adjacent section, a single cell double stained for PAX5 (a master regulator of B cell development; red) and TIM 1 (brown) is identified in each cluster (arrows; higher magnification images of these cells in top right-hand corners). Each case in **(a, b, c, d)** represents 1 patient (n=4 patients).

Supplementary Table 1

Antibody (Human)	Fluorochrome	Concentration	Supplier	Catalogue Number
CD19	APC-Cy7	1:100	BD Pharmingen	557791
CD25	PE-Cy7	1:100	BD Pharmingen	557741
CD24	PE	1:100	BD Pharmingen	555428
CD38	FITC	1:100	BD Pharmingen	555459
CD127	PE	1:100	BD Pharmingen	557938
IgD	PE-Cy7	1:100	BD Pharmingen	561314
pSTAT3	Alexa Fluor 647	1:6	BD Pharmingen	557815
pSTAT5	Alexa Fluor 488	1:6	BD Pharmingen	562077
CD20	PE-Cy7	1:100	eBioscience	25-0209-42
CD4	PE-Texas Red	1:100	eBioscience	61-0049-42
CD8	FITC	1:100	eBioscience	11-0088-42
γCR	PE	1:100	eBioscience	12-1329-41
CD27	eFluor 450	1:100	eBioscience	48-0279-42
CD138	eFluor 450	1:100	eBioscience	17-1389-41
CD1d	APC	1:100	eBioscience	17-0016-42
CD5	FITC	1:100	eBioscience	11-0058-42
CD21	eFluor 450	1:100	eBioscience	9048-0219-025
CD71	APC	1:100	eBioscience	17-0719-41
CD73	eFluor 450	1:100	eBioscience	48-0739-41
LAP	PE	1:100	eBioscience	12-9829-41
IL-10	eFluor 660	1:4	eBioscience	50-7108-41
TNFα	FITC	1:4	eBioscience	11-7349-41
IFNγ	PE	1:4	eBioscience	12-7319-41
CD154	PE	1:100	eBioscience	12-1548-42
IgM	PE	1:100	eBioscience	12-9998-42
CD40	APC	1:100	eBioscience	17-0409-41
CD80	FITC	1:100	eBioscience	11-0809-42
CD86	PE	1:100	eBioscience	12-0869-42
FAS-L	PE	1:100	eBioscience	12-9919-41
PD-L1	PE-Cy7	1:100	eBioscience	25-5983-41
PD-L2	APC	1:100	eBioscience	17-5888-41
TIM-1	PE	1:100	Biolegend	353903
IL-10R	APC	1:100	Biolegend	308811
CD122	APC	1:100	Biolegend	339007
IL-6Rα	PE	1:100	Biolegend	352803
CD154	FITC	1:100	Biolegend	310804
CD10	PE-Texas Red	1:100	Beckman Coulter	41116015
CD45	APC	1:100	Invitrogen	MHCD45054
CD154	APC	1:100	R&D Systems	FAB617A
CD25	PE	1:100	R&D Systems	FAB1020P-025
CD154	Unconjugated	[10µg/ml]	R&D Systems	MAB617
CD40	Unconjugated	[10µg/ml]	R&D Systems	MAB6322
IL-10	Unconjugated	[10µg/ml]	R&D Systems	MAB217
IL-10Rα	Unconjugated	[10µg/ml]	R&D Systems	MAB274
CD122	Unconjugated	[10µg/ml]	R&D Systems	MAB224
CD25	Unconjugated	[10µg/ml]	R&D Systems	MAB223

Antibody (Human)	Fluorochrome	Concentration	Supplier	Catalogue Number
CD80	Unconjugated	[10µg/ml]	R&D Systems	MAB140
CD86	Unconjugated	[10µg/ml]	R&D Systems	MAB141
FASL	Unconjugated	[10µg/ml]	R&D Systems	MAB126
PD-1	Unconjugated	[10µg/ml]	R&D Systems	AF1086
IL-6	Unconjugated	[10µg/ml]	R&D Systems	MAB2061
IL-6R	Unconjugated	[10µg/ml]	R&D Systems	MAB227R
$IgG_{1κ}$	Unconjugated	[10µg/ml]	R&D Systems	MAB002
$IgG_{2aκ}$	Unconjugated	[10µg/ml]	R&D Systems	MAB004
CD25	Unconjugated	[10µg/ml]	R&D Systems	MAB1020
TIM-1	Unconjugated	[10µg/ml]	Biolegend	353902
CD20	Unconjugated	1:100	Leica Biosystems	PA0200
pSTAT3	Unconjugated	1:100	Abcam	ab76315
TIM-1	Unconjugated	1:100	Abcam	ab47635
PAX5	Unconjugated	1:500	Abcam	ab109443