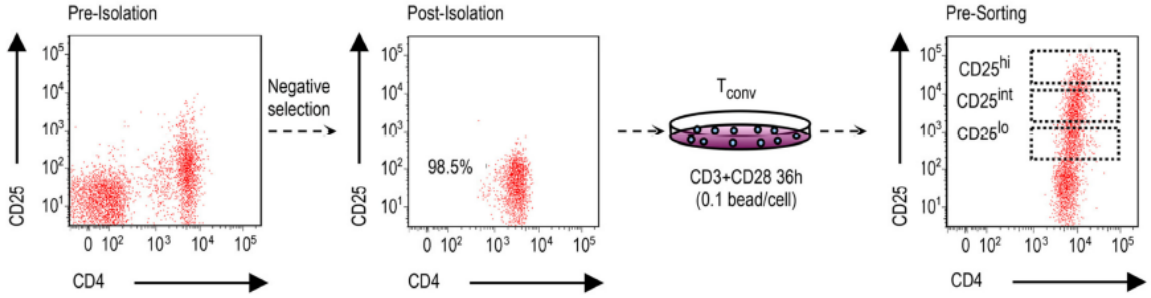
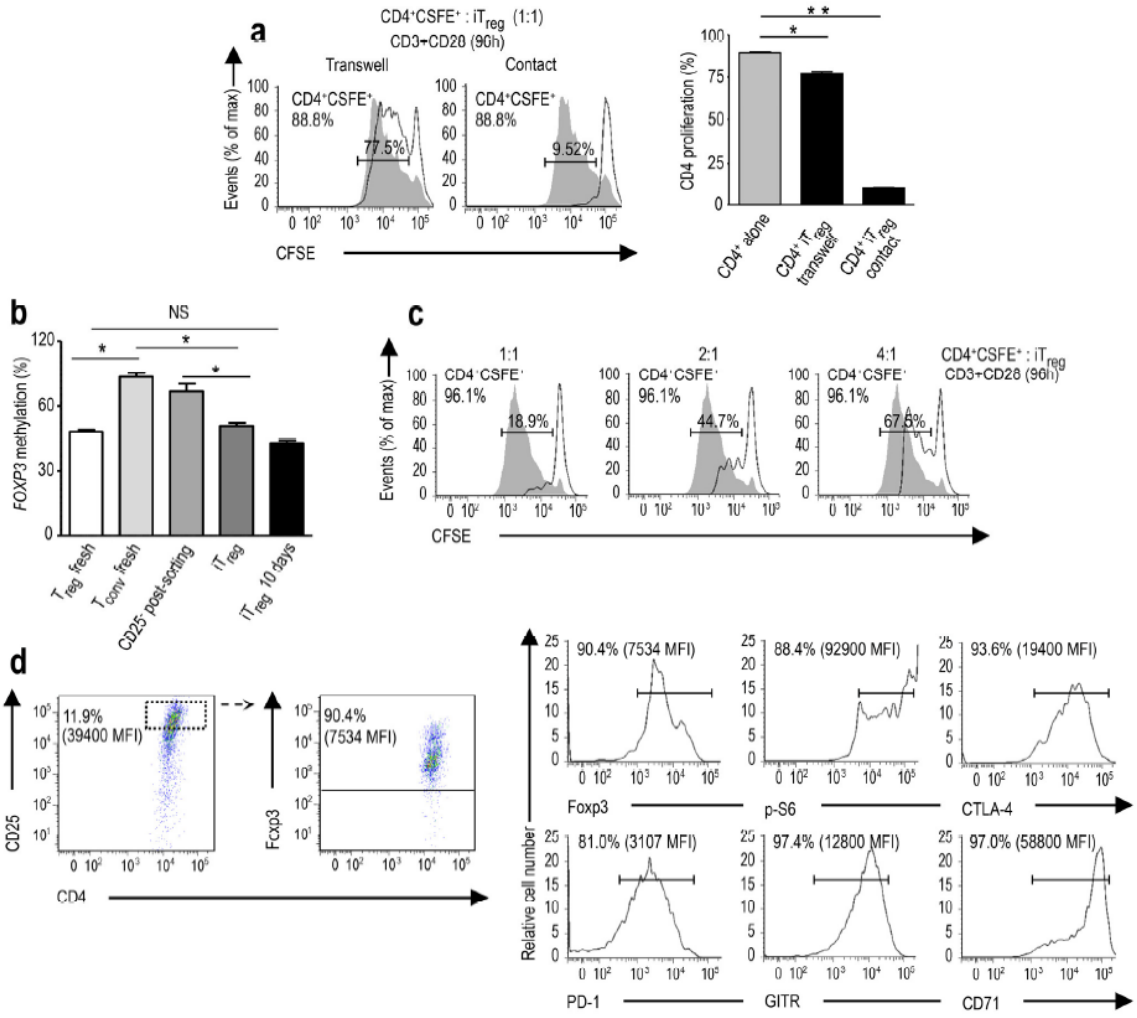
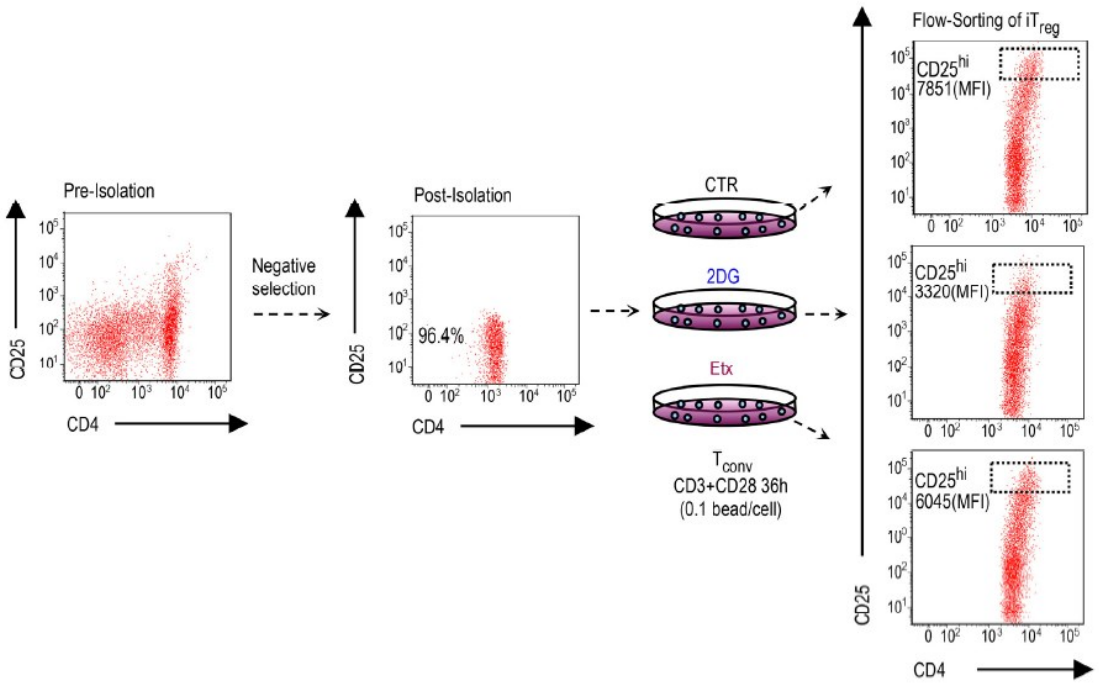
Supplementary Figures



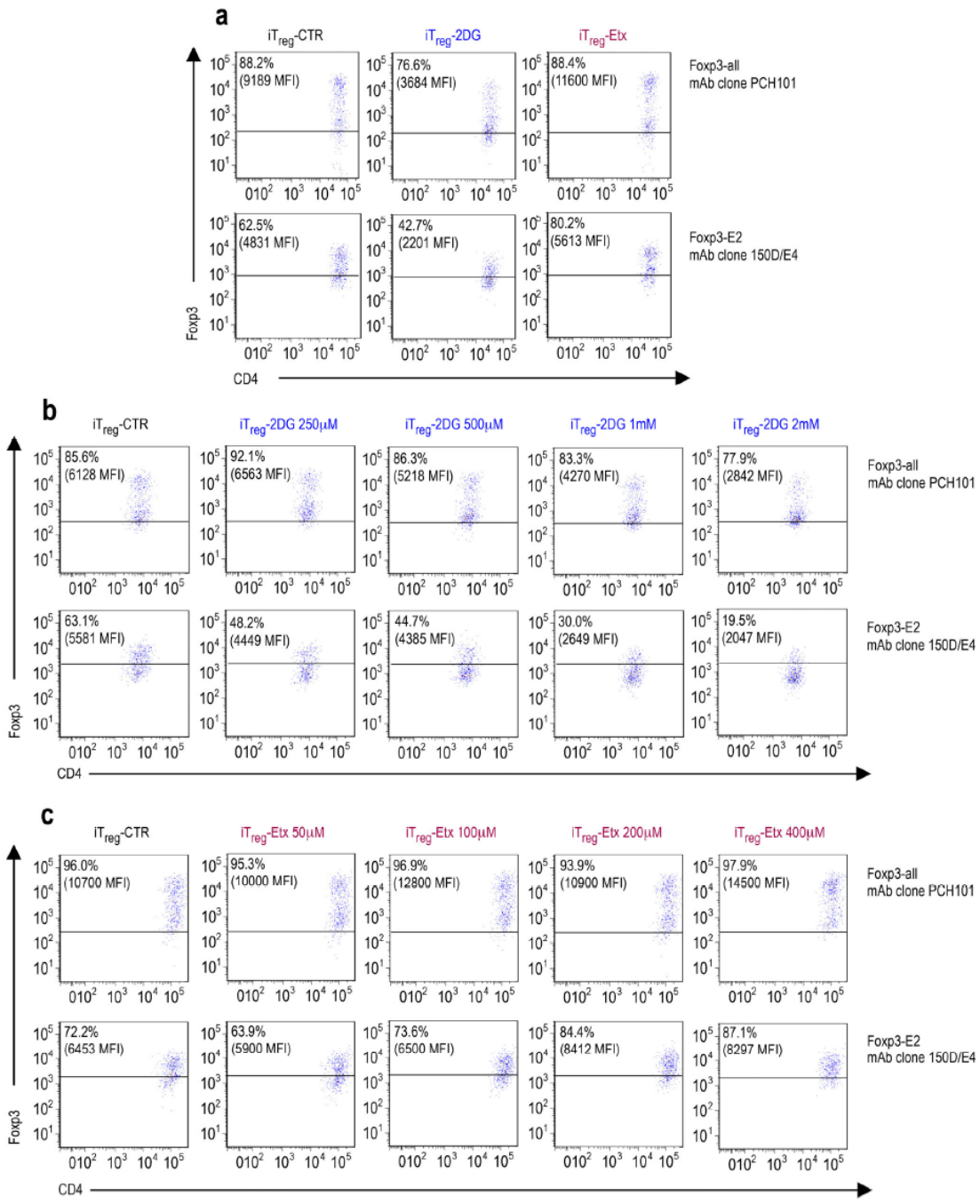
Supplementary Figure 1: Experimental procedure used for the generation of human iT_{reg}cells from activated T_{conv} cells. Human $CD4^+$ T cells were isolated from PBMCs by negative selection with magnetic beads, and activated with anti-CD3+CD28 beads (0.1 bead/cells) for 36h. Analyses and flow-sorting were performed on the different three regions according to the expression of the CD25 molecule ($CD25^{hi}$, $CD25^{int}$ and $CD25^{lo}$).



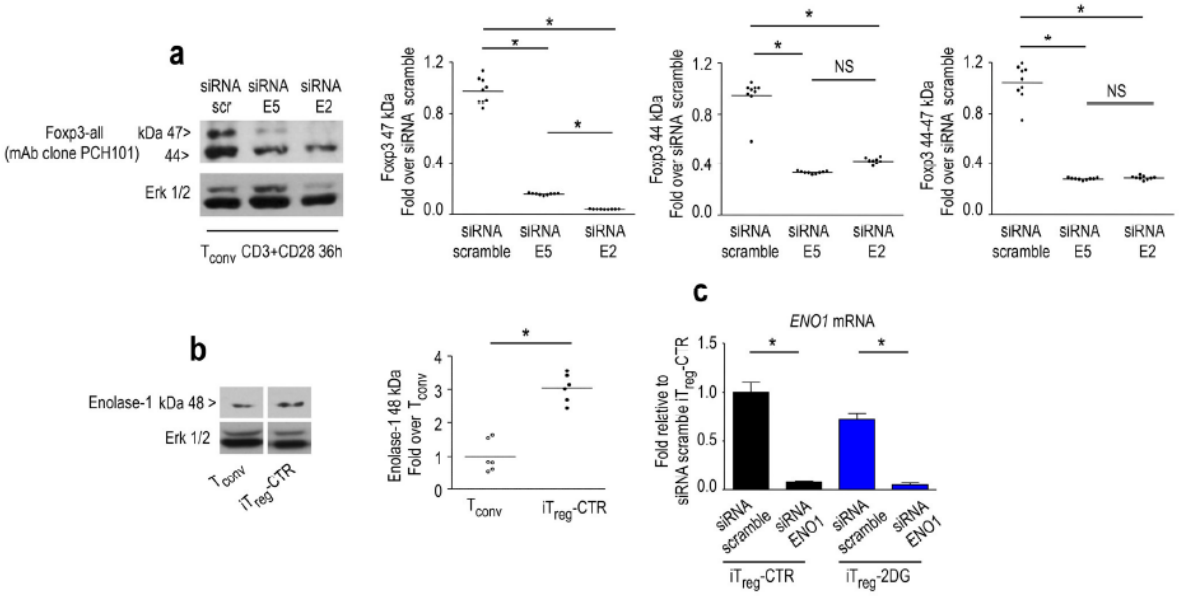
Supplementary Figure 2: Transwell experiments and stability of iT_{reg} cells' suppressive properties. (**a**) Suppression of T cell proliferation (96 hours) in transwell by flow-sorted iT_{reg} cells. Filled grey histograms correspond to proliferation *in vitro* of $CD4^+$CFSE-labelled T cells stimulated with anti-CD3+CD28 mAbs; black lines in histograms correspond to proliferation of $CD4^+$CFSE-labelled T cells co-cultured with flow-sorted iT_{reg} cells either in transwell plate or in cell-to-cell contact. Representative experiment of two (left panels) and percent of proliferation of two independent experiments (right panel). Statistical analysis derives from two independent experiments in technical triplicates (n = 6; data are shown as mean ± s.e.m.) *P < 0.05; **P < 0.001 by paired two-tailed Student's *t*-test. (**b**) Methylation of CpG island of *FOXP3* CNS2 region evaluated in: freshly isolated T_{reg} cells and T_{conv} cells, post-sorting $CD25^-$ and iT_{reg} cells, iT_{reg} cells upon 10 days of culture with anti-CD3+CD28 mAbs and IL-2 (50 IU/ml). Statistical analysis derives from two independent experiments in technical triplicates (n = 6; data are shown as mean ± s.e.m.)*P < 0.001; NS not significant by two-tailed Student's *t*-test. (**c**) Suppression of T cell proliferation (96 hours) by flow-sorted iT_{reg} cells after 10 days of culture with anti-CD3+CD28 and IL-2 (50 IU/ml). Filled grey histograms correspond to proliferation of $CD4^+$CFSE-labelled T cells stimulated *in vitro* with anti-CD3+CD28 mAbs; black lines in histograms correspond to proliferation of $CD4^+$CFSE-labelled T cells co-cultured with flow-sorted iT_{reg} cells at different ratios (from 1:1 to 4:1). Representative experiment of three. (**d**) Representative dot plots showing expression of CD25, CD4 and Foxp3 and other T_{reg} cell markers in flow-sorted iT_{reg} cells after 10 days of culture with anti-CD3+CD28 mAbs and IL-2 (50 IU/ml). Percentages and MFI of positive cells are indicated. Representative experiment of three.



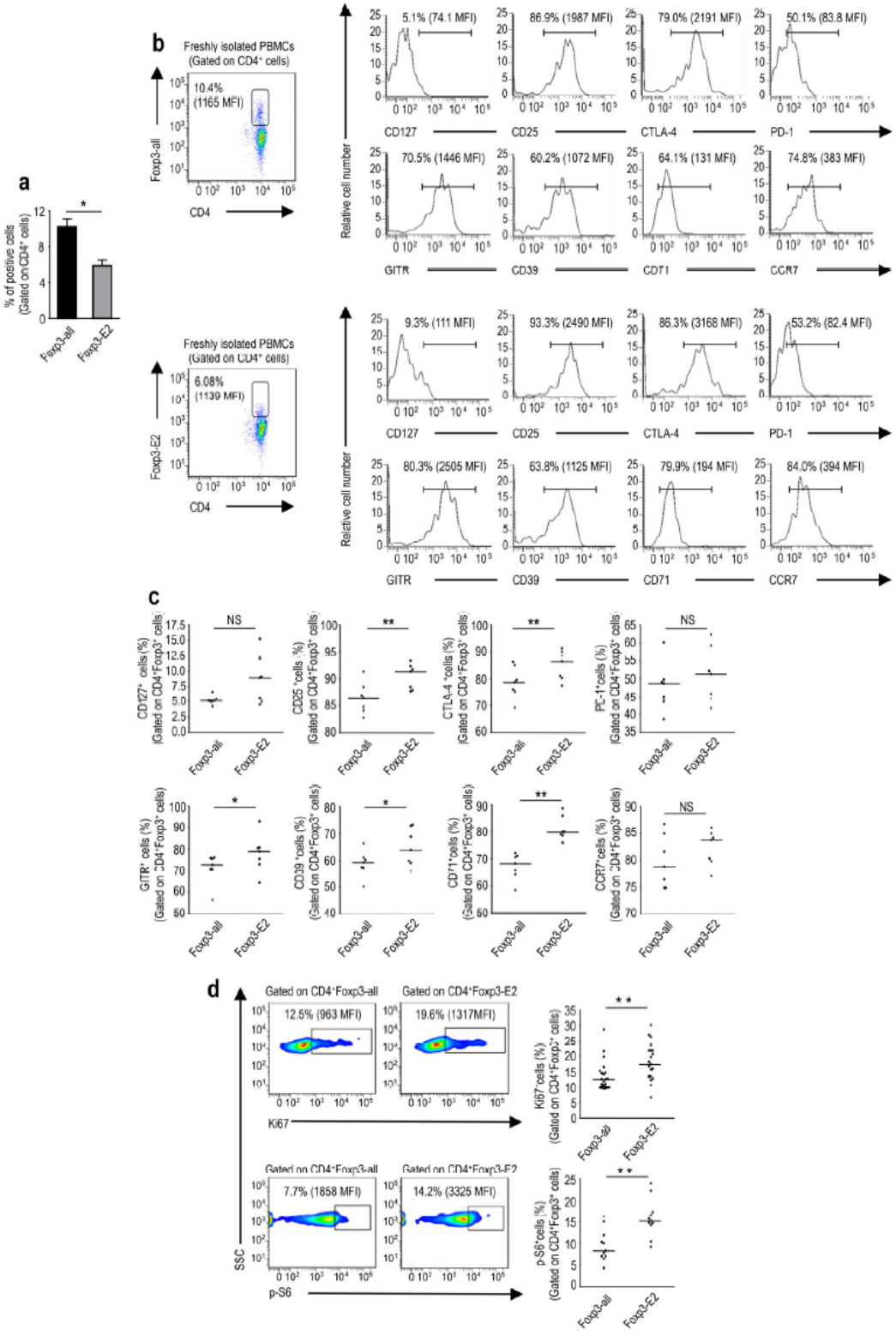
Supplementary Figure 3: Experimental procedure used for the generation of human iT_{reg}cells from T_{conv} cells in the presence of metabolic inhibitors. Human $CD4^+$ T cells were isolated from PBMCs by negative selection with magnetic beads, and activated with anti-CD3+CD28 beads (0.1 bead/cells) for 36h in the presence or absence of 2DG or Etx. Flow-sorting was performed on each $CD25^{hi}$ cell population, defined as iT_{reg}-CTR, iT_{reg}-2DG and iT_{reg}-Etx.



Supplementary Figure 4: Splicing variants of Foxp3 in iT_{reg}-CTR, iT_{reg}-2DG and iT_{reg}-Etx cells at various doses of inhibitors. (**a**) Representative flow cytometry plots showing levels of Foxp3 in iT_{reg}-CTR, iT_{reg}-2DG and iT_{reg}-Etx cells stained with either Foxp3 Ab PCH101 (against all Foxp3 splicing variants) (upper panels) or Foxp3 mAb 150D/E4 (specific for the Foxp3 exon 2 form) (lower panels). Percentages and MFI of positive cells are indicated. Representative of 3. (**b**) Representative flow cytometry plots showing levels of Foxp3-all (upper panels) and Foxp3-E2 (lower panels) in iT_{reg} cells generated in the presence of different doses of 2DG (250 µM, 500 µM, 1 mM and 2 mM). Representative of 3. (**c**) Representative flow cytometry plots showing levels of Foxp3-all (upper panels) and Foxp3-E2 (lower panels) in iT_{reg} cells generated in the presence of different doses of Etx (50 µM, 100 µM, 200 µM and 400 µM). Representative experiment of 3.

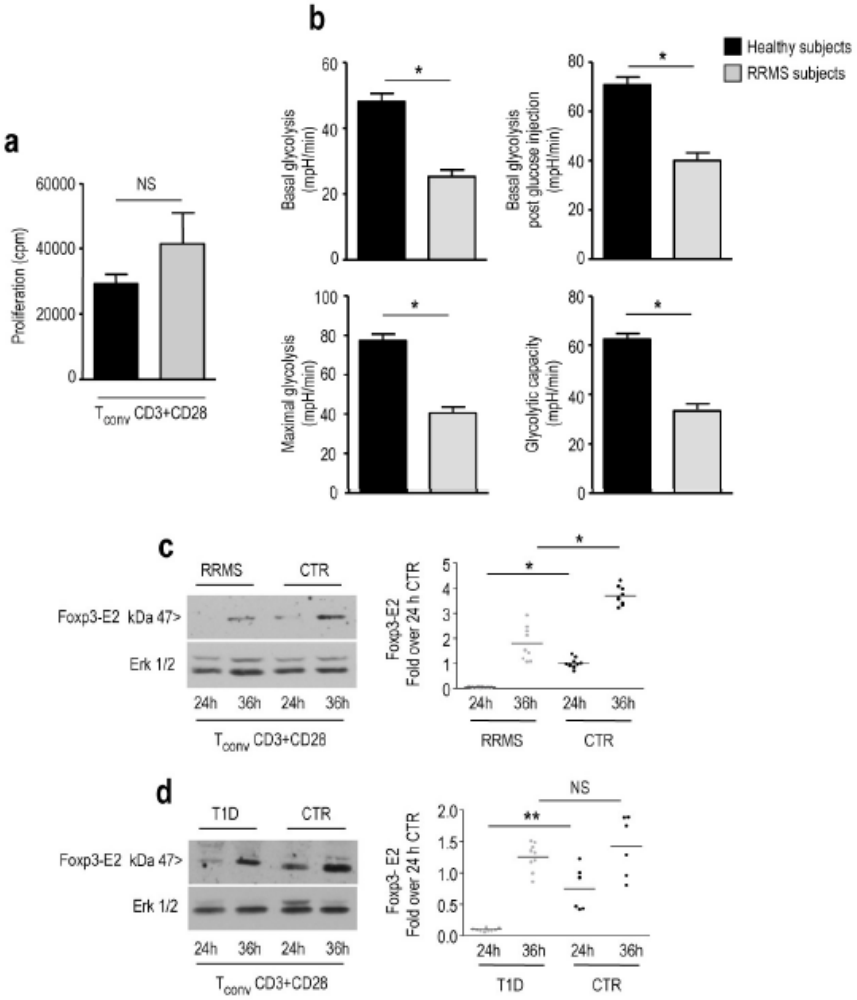


Supplementary Figure 5: Silencing of *FOXP3* splicing variants and *ENO1* mRNA in iT_{reg}cells. (a) Immunoblotting for Foxp3 (clone PCH101) showing specific silencing on T_{conv} cells stimulated for 36 hours with anti-CD3+CD28 mAbs in the presence of reported siRNA. One representative of three independent experiments. The graphs show relative densitometric quantitation of each Foxp3 band normalized on total Erk1/2 and shown as fold change over siRNA scrambled cells. Statistical analysis derives from three independent experiments in technical triplicates (n = 9; data are shown as individual data points and means) *P < 0.001; NS not significant by two-tailed Student's *t*-test. (**b**) Western blotting analysis of cytoplasmic enolase-1 (48 kDa) levels in freshly isolated T_{conv} cells and iT_{reg}-CTR cells. One representative experiment of two. Graph shows the absorbance values, shown as fold over T_{conv} cells, normalized on total Erk1/2. Statistical analysis derives from two independent experiments in technical triplicates (n = 6; data are shown as individual data points and means) *P < 0.01 by Wilcoxon. (**c**) RT-PCR for*ENO1* showing specific silencing on iT_{reg}-CTR and iT_{reg}-2DG in the presence of reported siRNA. The graphs show *ENO1* mRNA levels 18S-normalized and shown as fold over iT_{reg}-CTR siRNA scrambled cells. Statistical analysis derives from two independent experiments in technical triplicates (n = 6; data are shown as mean ± s.e.m.) *P < 0.0001 by paired two-tailed Student's *t*-test.

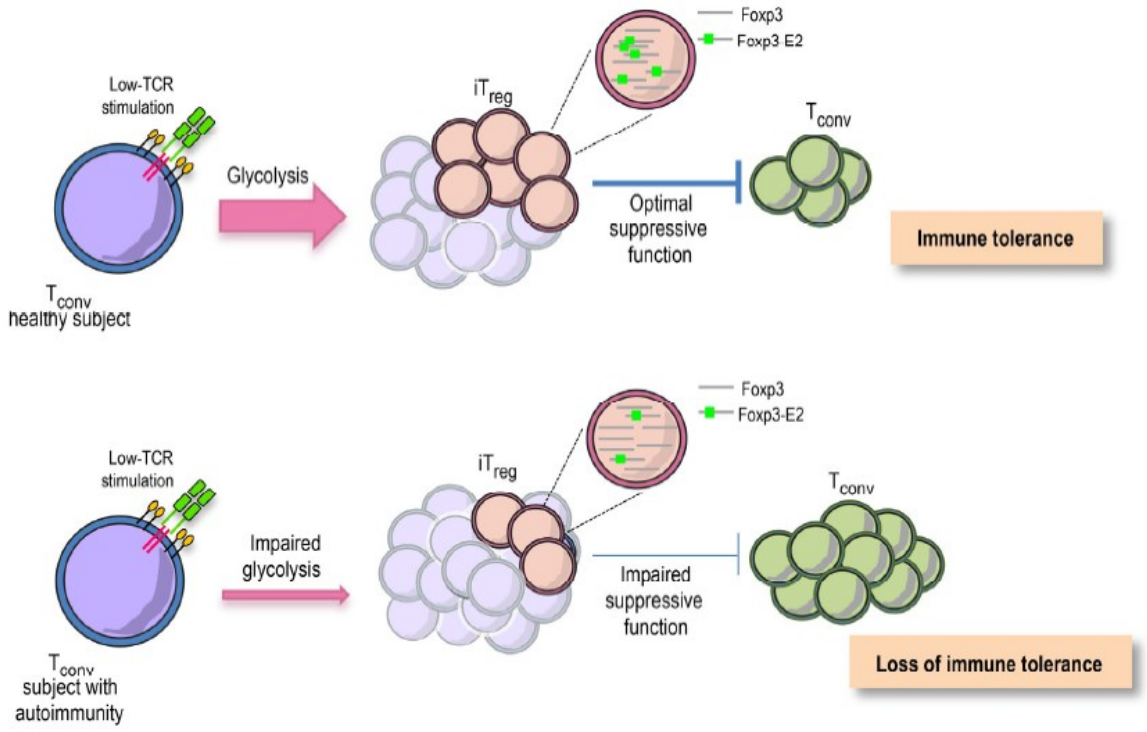


Supplementary Figure 6: Characterization of $CD4^{+}Foxp3\text{-}E2^{+}$ T cells from PBMCs of human healthy donors. (**a**) Percentage of $Foxp3\text{-}all^{+}$ cells (black column) and $Foxp3\text{-}E2^{+}$ cells (grey column) in freshly isolated PBMCs of human healthy donors (n = 19), gated on $CD4^{+}$ cells, (cumulative data are shown as

mean ± s.e.m.) *P < 0.0001 by paired two-tailed Student's*t*-test. (**b**) Representative dot plots showing expression of T_{reg} cell-specific markers in freshly isolated PBMCs of human healthy donors, gated on $CD4^+$Foxp3-all cells (upper panel) and $CD4^+$Foxp3-E2 cells (lower panel). MFI and percent of expression of reported markers are indicated. Representative experiment of three. (**c**) Percentage of expression of T_{reg} cell-specific markers in freshly isolated PBMCs of human healthy donors, gated on $CD4^+$Foxp3-all cells and $CD4^+$Foxp3-E2 cells. Statistical analysis derives from three independent experiments, data are shown as individual data points and means (n = 7).**P < 0.005; *P < 0.05; NS not significant by paired two-tailed Student's *t*-test. (**d**) Percentage of $ki67^+$ and $p\text{-}S6^+$ cells gated on $CD4^+$Foxp3-all cells and $CD4^+$Foxp3-E2 cells in freshly isolated PBMCs of human healthy donors. One representative plot out of four (left panel). MFI and percentage of expression of reported markers are indicated. Statistical analysis derives from cumulative data of four independent experiments, data are shown as individual points and means (n = 21 for ki67, n = 11 for p-S6) (right panel)**P < 0.005 by paired two-tailed Student's *t*-test.



Supplementary Figure 7: Selective impairment of glycolysis and Foxp3-E2 expression in iT_{reg} cells from autoimmune subjects. (**a**) Cumulative data of T_{conv} cells proliferation after anti-CD3+CD28 mAbs stimulation, in RRMS (n = 25) and healthy (n = 42) subjects, measured as (^{3}H) thymidine incorporation. Data are shown as mean ± s.e.m.; NS not significant by Wilcoxon. (**b**) Basal glycolysis (after glucose addition), maximal glycolysis (after oligomycin addition) and glycolytic capacity (calculated as the difference of oligomycin-induced ECAR and 2DG-induced ECAR) of T_{conv} cells from naive-to-treatment RRMS (n = 6) and healthy subjects (n = 14). Cumulative data are expressed as mean ± s.e.m. *P < 0.001 by Wilcoxon. (**c**) Western blot analysis of Foxp3-E2 protein after 24 and 36 hours of *in vitro* stimulation with anti-CD3+CD28 mAbs of T_{conv} cells from RRMS and healthy subjects. Graphs show the relative densitometric quantitation of Foxp3-E2 bands normalized to total Erk1/2, shown as fold change over T_{conv} cells from healthy donor, 24 hour after *in vitro*stimulation with anti-CD3+CD28 mAbs. One representative of three independent experiments. Statistical analysis derives from three independent experiments performed on three healthy and three RRMS subjects, in technical triplicates (n = 9; data are shown as individual data points and means) *P < 0.001 by Wilcoxon. (**d**) Western blot analysis of Foxp3-E2 protein after 24 and 36 h of *in vitro* stimulation with anti-CD3+CD28 mAbs of T_{conv} cells from T1D and healthy subjects. Graphs show relative densitometric quantitation of Foxp3-E2 bands normalized to total Erk1/2, shown as fold change over T_{conv} cells from healthy donor, 24 h after *in vitro* stimulation with anti-CD3+CD28 mAbs. One representative of three experiments. Statistical analysis from three independent experiments performed on two healthy and three T1D subjects in triplicates (n = 6 for healthy and n = 9 for T1D; data shown as individual data points and means) **P < 0.005; NS not significant by Wilcoxon.



Supplementary Figure 8: Model of the glycolytic control of Foxp3-E2 splicing variants in human iT_{reg} cells in health and autoimmune diseases: T_{conv} cells from healthy subjects engage glycolysis during *in vitro* weak-TCR stimulation, which leads to generation of fully suppressive iT_{reg} cells representing the highly proliferative and glycolytic fraction of T_{conv} cells ($CD25^{hi}$, $ki67^{+}$, $p\text{-}S6^{+}$, $Foxp3\text{-}E2^{+}$) (upper panel). On the contrary, in subjects with autoimmunity, T_{conv} cells show an impaired glycolysis during TCR stimulation, which accounts for an altered Foxp3-E2 induction, reduced generation and function of iT_{reg} cells and loss of immune tolerance (lower panel).

Supplementary Table 1. Table reports specific primers used for PCR experiments.

	PRIMERS
FOXP3-Δ2 Fw	5'- GCCCAACCCCAGGCCTGGCAAGC -3'
FOXP3-Δ2 Rev	5'- ATTTGGGAAGGTGCAGAGCAGT -3'
FOXP3-E2 Fw	5'- CACACTGCCCCTAGTCATGG -3'
FOXP3-E2 Rev	5'- GCATGAAATGTGGCCTGTCCT -3'
FOXP3-all Transcripts Fw	5'- AACATGCGACCCCCTTTCACCTAC -3'
FOXP3-all Transcripts Rev	5'- GCCCCCTTCTCGCTCTCCAC -3'
18S Fw	5'-TCCCCATGAACGAGGAATTC-3'
18S Rev	5'-GGCCTCACTAAACCATCCAA-3'
ChIP Promoter Fw	5'- GGTTGGCCCTGTGATTTATTTTAG -3'
ChIP Promoter Rv	5'- GTGTGGAAGCCGCAGACCTC -3'
ChIP CNS2 Fw	5'- TGAGAAACCCAGTCAGAAAGG -3'
ChIP CNS2 Rev	5'- GGCCAGAGCTAAGAATTCTCC -3'
ChIP CNS3 Fw	5'- GGACAAGGACCCGATGCCCAACC -3'
ChIP CNS3 Rev	5'- CTCCCGCCCAGTGCCACAGTAAAG -3'
CNS2 A Fw	5'- GACATCACCTACCACATCC -3'
CNS2 A Rev	5'- TATCGGGGTCTGCATCTGG -3'
CNS2 B Fw	5'- TGAGAAACCCAGTCAGAAAGG -3'
CNS2 B Rev	5'- GGCCAGAGCTAAGAATTCTCC -3'
H19 Fw	5'-GAGCCGCACCAGATCTTCAG-3'
H19 Rev	5'-TTGGTGGAACACACTGTGATCA-3'
UEB2B Fw	5'-CTCAGGGGTGGATTGTTGAC-3'
UEB2B Rev	5'-TGTGGATTCAAAGACCACGA-3'
ENO1 Fw	5' TGGCAACTCTGAAGTCATCCTG 3'
ENO1 Rev	5' TGTGGTAAACCTCTGCTCCAATG 3'