

# PNAS

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Supplementary Information for

Epigenetic conversion of conventional T cells into regulatory T cells by CD28 signal deprivation

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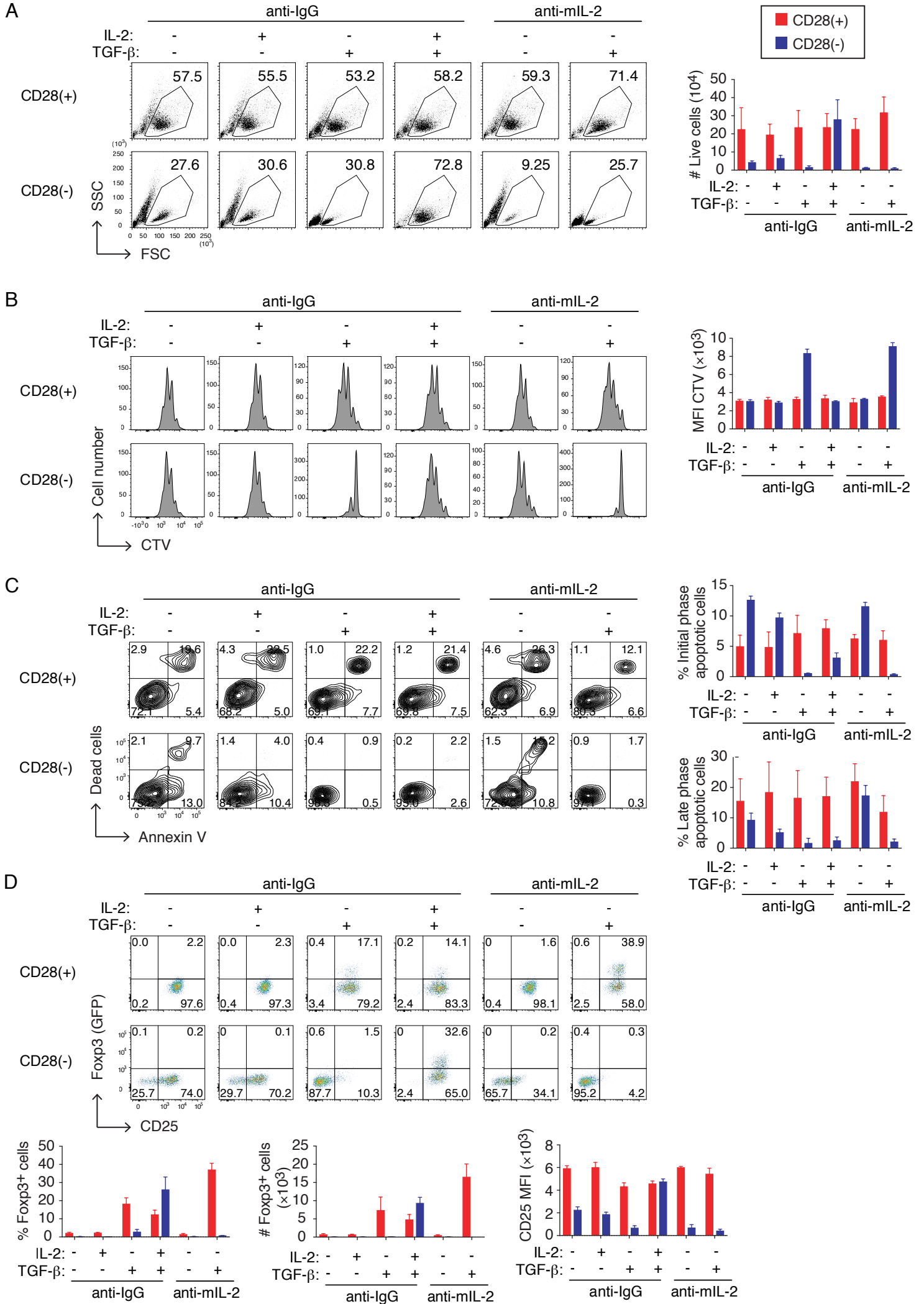
**This PDF file includes:**

Figures S1 to S8  
Tables S1

**Other supplementary materials for this manuscript include the following:**

None.

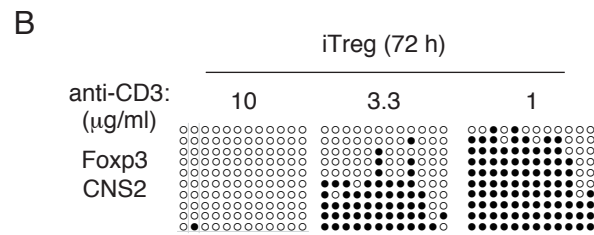
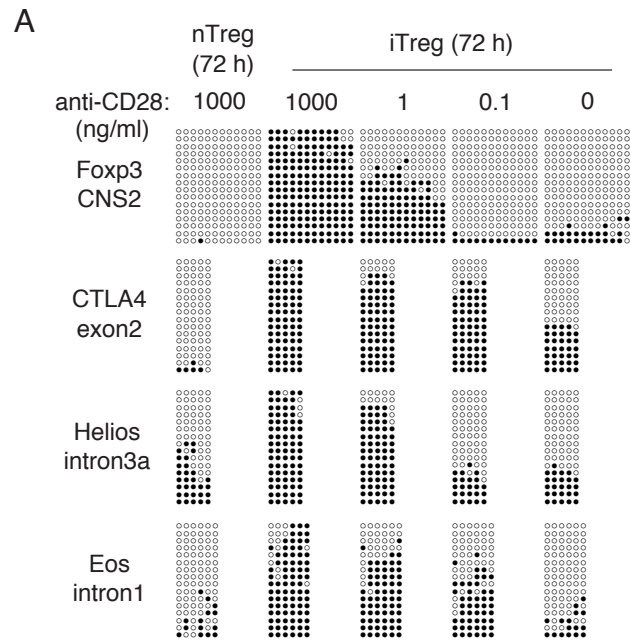
# Figure S1



**Figure S1. *In vitro* effects of IL-2 and TGF- $\beta$  on the induction of iTregs and their activation, proliferation, and apoptosis.**

**(A)** FSC/SSC profiles of CD4<sup>+</sup> T cells stimulated for 3 days under indicated conditions as shown in Fig. 1A. Anti-mouse IL-2 neutralizing mAb or control anti-IgG (1  $\mu$ g/ml) was added as indicated. A representative result of more than three independent experiments (left). Total numbers of live cells gated as shown in Fig. (A) (right). Bar graphs show mean  $\pm$  SD (n=3). **(B)** Proliferation of CTV-stained CD4<sup>+</sup> T cells stimulated for 3 days under indicated conditions as shown in Fig. 1A. A representative result of more than three independent experiments (left). MFI (mean  $\pm$  SD) of CTV staining (n=3) (right). **(C)** Apoptosis in CD4<sup>+</sup> T cells assessed by AnnexinV and LiveDead dye. A representative result of more than three independent experiments (left). "Early phase apoptotic cells" indicate the population of AnnexinV<sup>+</sup>LiveDead<sup>-</sup> cells (upper right figure); "Late phase apoptotic cells" indicate the population of AnnexinV<sup>+</sup>LiveDead<sup>+</sup> cells (n=3) (lower right figure). **(D)** CD25 and Foxp3 (eGFP) expression. A representative result of more than three independent experiments (left). Percentages of Foxp3<sup>+</sup> cells among CD4<sup>+</sup> T cells (lower left figure); the numbers of Foxp3<sup>+</sup> cells (lower middle), and the levels (as MFI) of CD25 expression (n=3) (lower right).

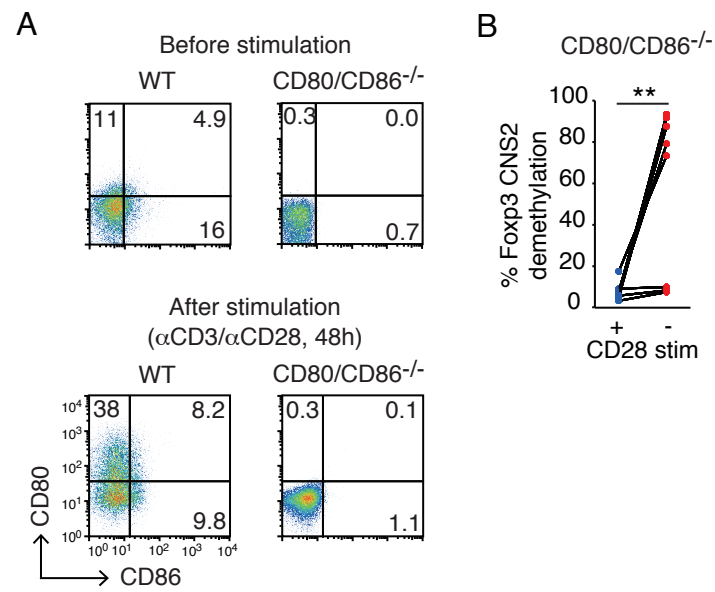
Figure S2



**Figure S2. Effects of the degree of CD28 blockade and TCR strength on Treg-DR demethylation in iTregs.**

**(A)** Dose-dependent effects of soluble anti-CD28 mAb on Treg-DR hypomethylation in iTreg generation. CD4<sup>+</sup> Tconvs were stimulated with anti-CD3 mAb, IL-2 and TGF- $\beta$  at graded concentrations of anti-CD28 mAb. A representative result of two independent experiments. **(B)** The effects of TCR strength on Foxp3 CNS2 hypomethylation in CD28(-) iTregs. Tconvs were stimulated, in the absence of anti-CD28 mAb, by anti-CD3 mAb plated-coated at graded concentrations, together with IL-2 and TGF- $\beta$ . A representative result of two independent experiments.

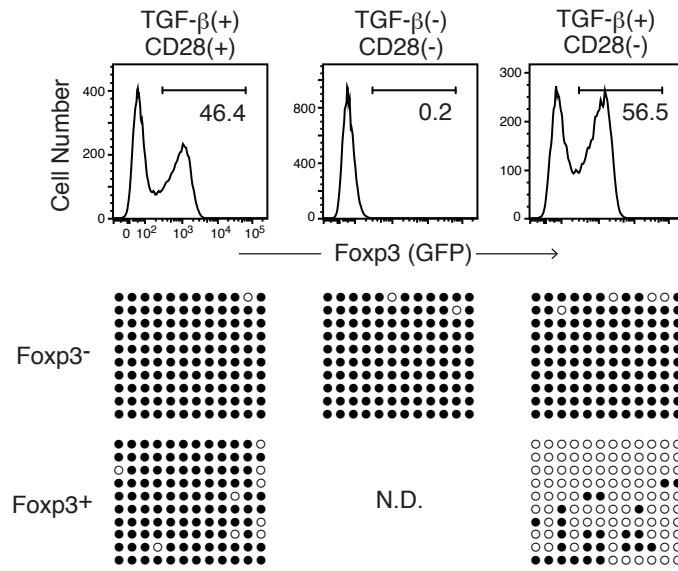
Figure S3



**Figure S3. Effects of T-cell expression of CD80/CD86 on Treg-DR demethylation in iTregs.**

**(A)** CD80 and CD86 expression by CD4<sup>+</sup> Foxp3<sup>-</sup> Tconv from peripheral lymph node of WT (left) or CD80/CD86-double KO mice (right) before (upper panel) or after (lower panel) stimulation with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb for 48 hours. A representative result of two independent experiments. **(B)** Foxp3 CNS2 hypomethylation in CD28(+) or CD28(-) iTregs prepared from CD80/86 KO mice (n=8), \*\*P<0.01 (Paired Student's t-test).

Figure S4

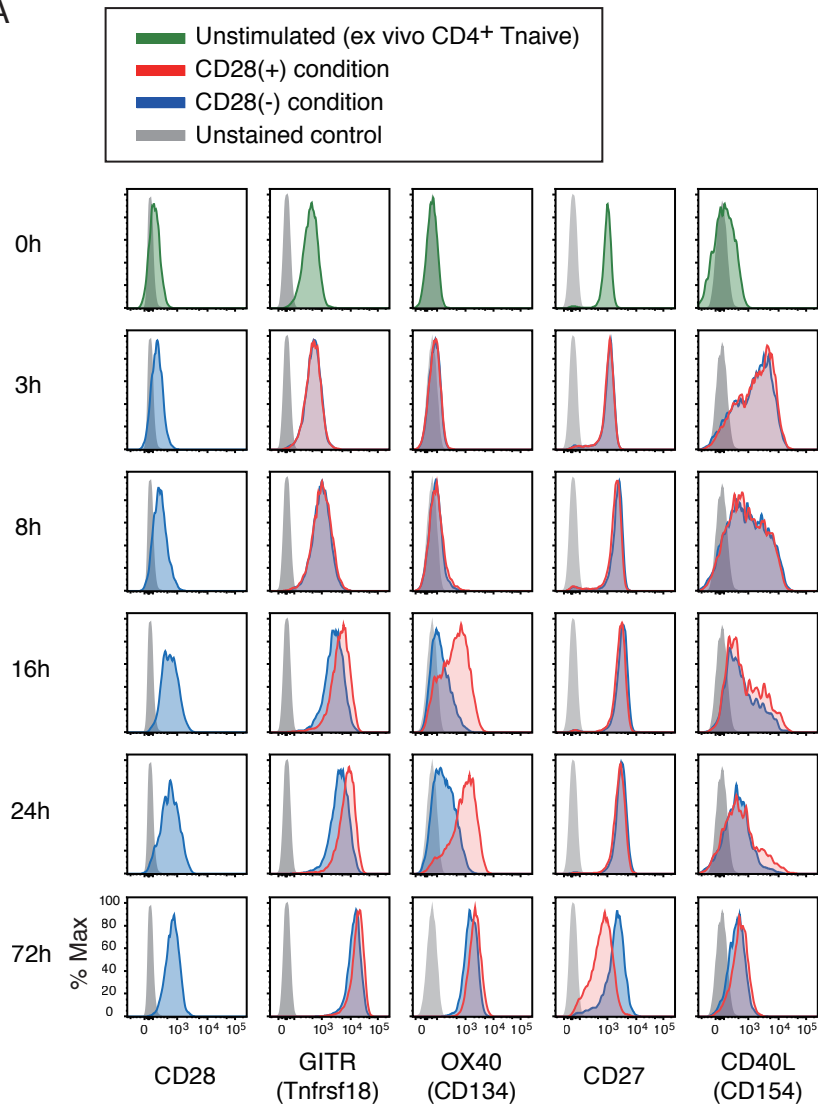




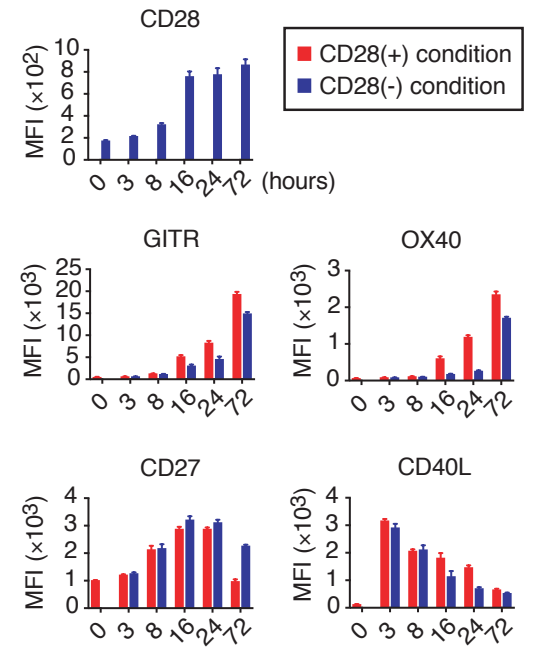
**Figure S4. Discrete Treg-specific methylation or demethylation in Foxp3<sup>+</sup> or Foxp3<sup>-</sup> cells.** Foxp3 CNS2 hypomethylation status in Foxp3<sup>+</sup> or Foxp3<sup>-</sup> cells in the indicated iTreg generating conditions. A representative result of two independent experiments.

Figure S5

A



B

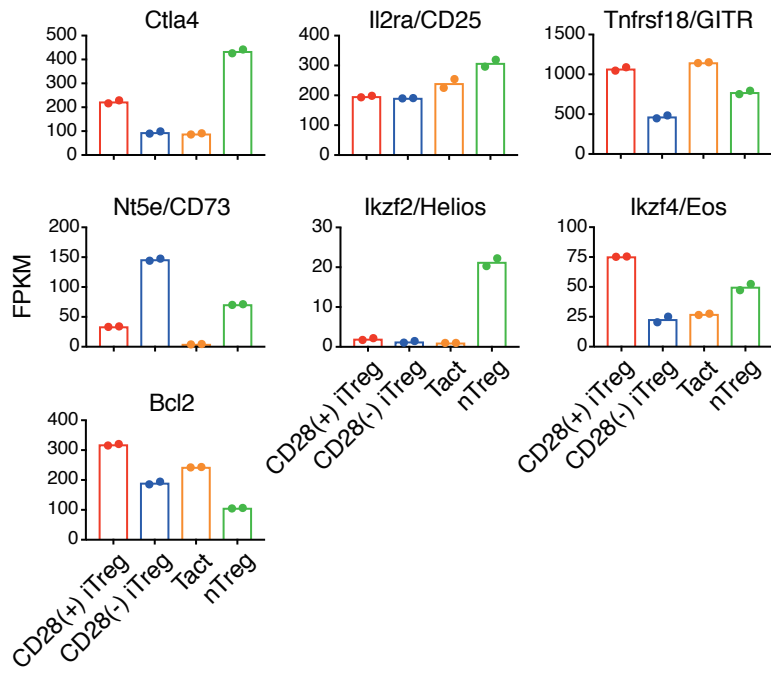


**Figure S5. Expression of T-cell accessory molecules by stimulated Tconv.**

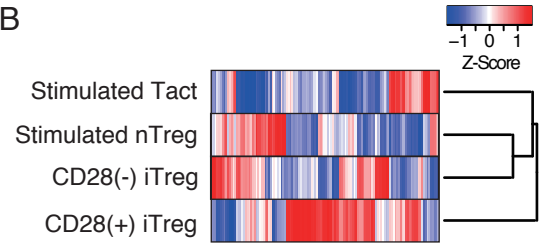
**(A)** Kinetics of the expression of T-cell accessory molecules (CD28, GITR, Ox40, CD27, CD40L) by CD28(+) or CD28(-) iTregs in the presence of IL-2 and TGF- $\beta$  at indicated time points. A representative FACS plot (n=3). **(B)** MFI of the staining in (A) (mean  $\pm$  SD, n=3).

Figure S6

A



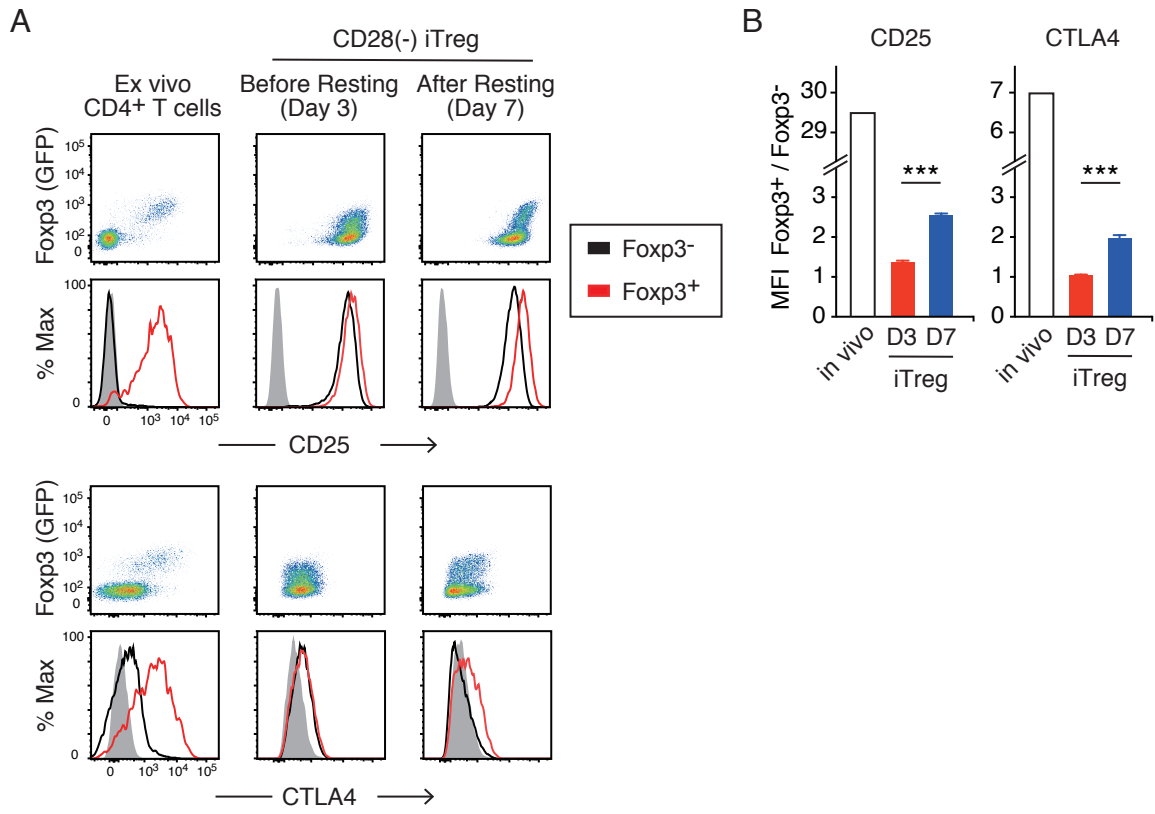
B



**Figure S6. Expression of Treg-signature gene RNA in CD28(-) iTregs.**

**(A)** RNA-seq results of CD28(+) and CD28(-) iTregs, activated nTregs and activated Tconvs (Tact) stimulated with anti-CD3/CD28 and IL-2 for 3 days. FPKM values of representative Treg-signature genes in two independent experiments are shown. **(B)** Hierarchical clustering of genes listed in GO0007249; “I-kappaB kinase NF-kappaB signaling”.

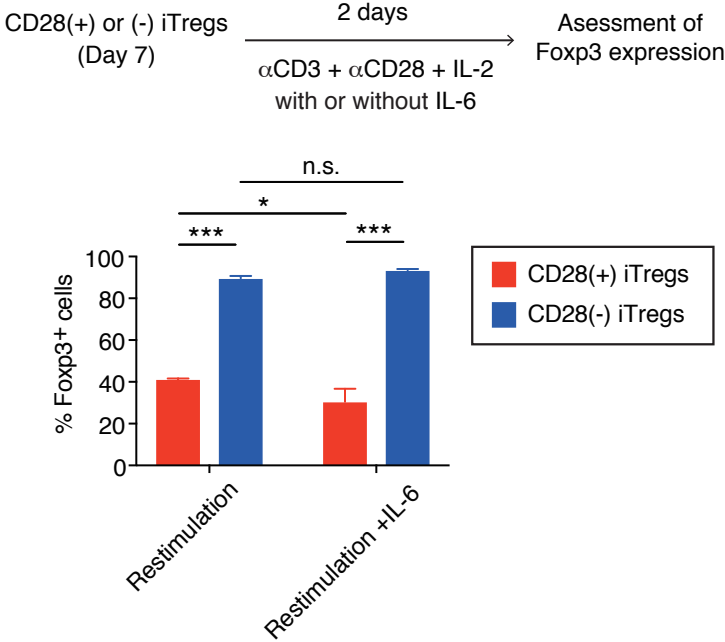
Figure S7



**Figure S7. Expression levels of CD25 and CTLA-4 in CD28(-) iTregs before and after “resting” with IL-2.**

**(A)** CD25 and CTLA-4 expression by Foxp3<sup>+</sup> or Foxp3<sup>-</sup> cells in CD28(-) iTreg generation before (Day 3) and after (Day 7) resting culture. **(B)** Ratio of CD25- or CTLA-4-staining MFI of Foxp3<sup>+</sup> versus Foxp3<sup>-</sup> cells (n=3). \*\*\*P<0.001 (Student's t-test).

Figure S8





**Figure S8. *In vitro* stability of Foxp3 expression in CD28(-) iTregs in the presence of IL-6.**

Percentages of Foxp3<sup>+</sup> cells among CD28(+) or CD28(-) iTregs cultured for 7 days as shown in Fig. 4D and restimulated with anti-CD3/CD28 and IL-2 for 2 days in the presence or absence of 10 ng/ml IL-6. Data show mean  $\pm$  SD of three independent experiments. \*P<0.05, \*\*\*P<0.001 (Student's t-test).

**Table S1. Antibodies used in this study.**

Antibody	Clone	Application	Vendor
anti-mouse CD62L	MEL-14	FCM	BD
anti-mouse CD11c	HL3	FCM	BD
anti-mouse CD152 (CTLA-4)	UC10-4B9	FCM	eBioscience
anti-mouse CD16/32	93	FCM	BD
anti-mouse CD25	PC61	FCM	BD
anti-mouse CD28	37.51	Stimulation/FCM	BD
anti-mouse CD3 $\epsilon$	145-2C11	Stimulation	BD
anti-mouse CD4	RM4-5	FCM	BD
anti-mouse CD80	16-10A1	FCM	eBioscience
anti-mouse CD86	PO3.1	FCM	eBioscience
anti-mouse GITR	DTA-1	FCM	BD
anti-mouse IFN $\gamma$	XMG1.2	FCM	BD
anti-mouse IL-17A	TC11-18H10.1	FCM	BD
anti-mouse IL-4	11B11	FCM	BD
anti-human/mouse CD44	IM7	FCM	BD
anti-DO11.10	KJ1-26	FCM	BD
anti-mouse/rat FoxP3	FJK-16s	FCM	eBioscience
anti-mouse c-Rel	1RELAH5	FCM	eBioscience
anti-mouse Ox40	OX-86	FCM	eBioscience
anti-mouse CD27	LG.3A10	FCM	BD
anti-mouse CD40L	MR1	FCM	eBioscience
anti-mouse IL-9	RM9A4	FCM	BioLegend
anti-IgG antibody	RTK2071	Neutralization	BioLegend
anti-mIL-2 antibody	JES6-1A12	Neutralization	BioLegend