

Supplementary Information

Article title:

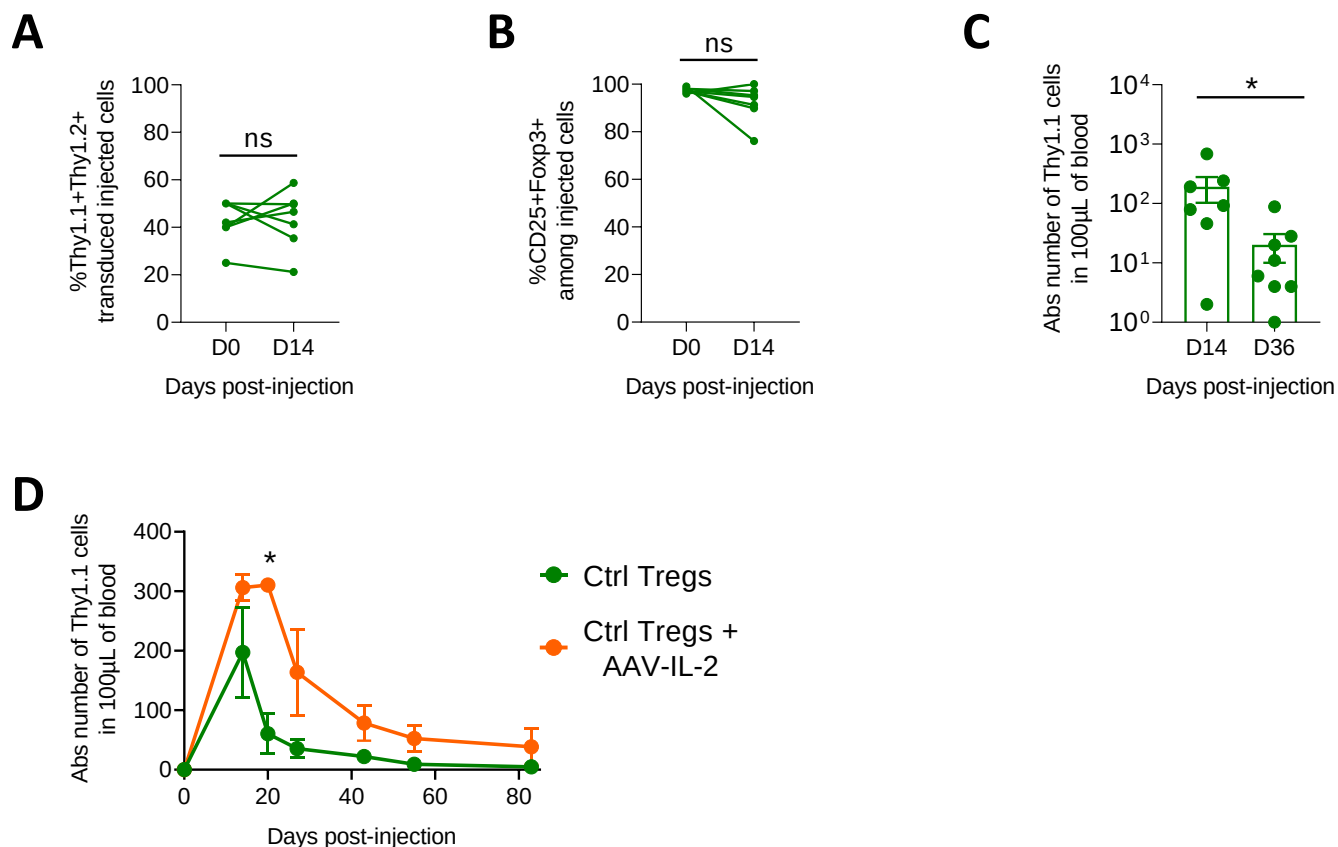
Enhancing regulatory T cell efficacy
through self-sufficiency for an
interleukin-2 partial agonist

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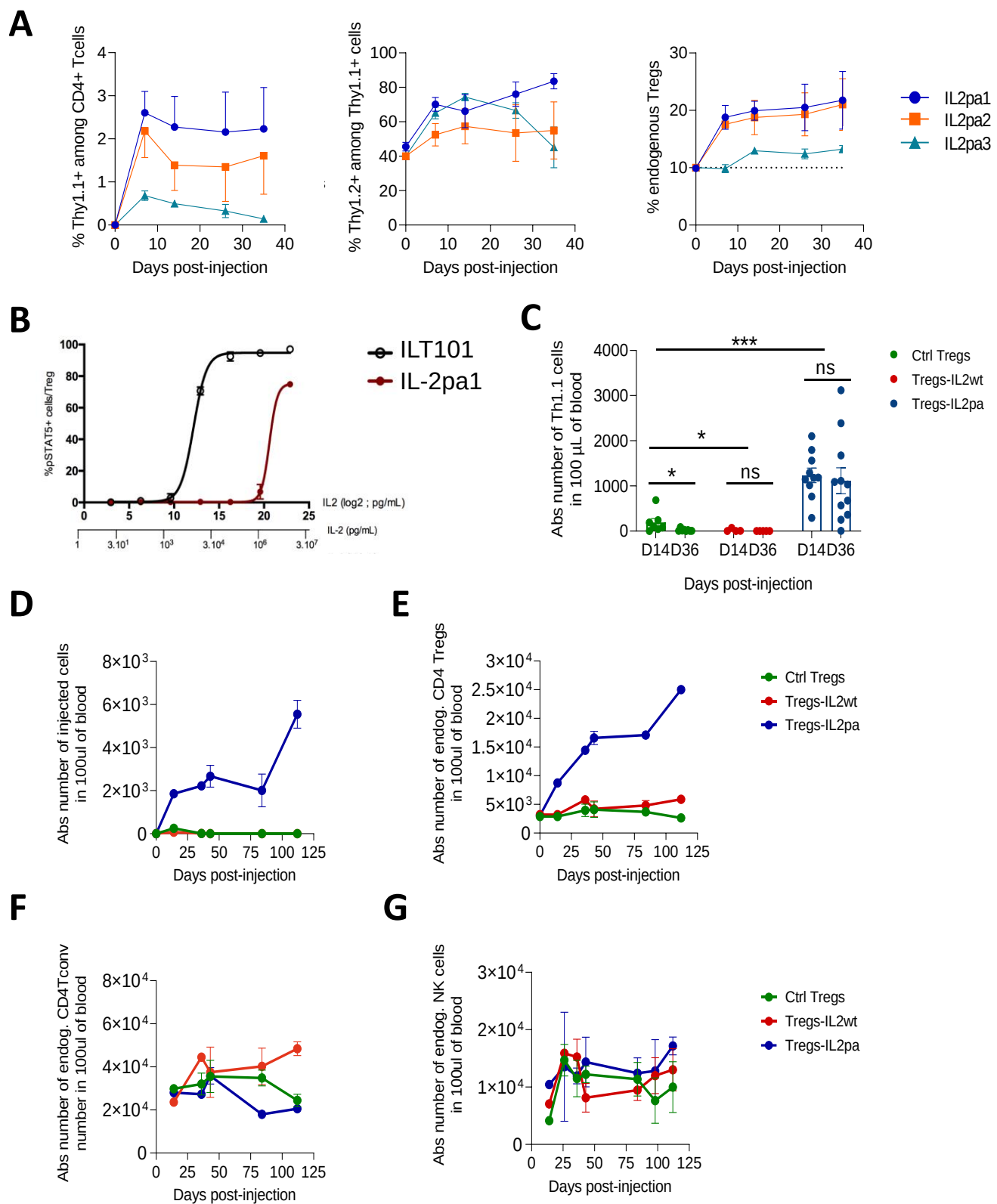
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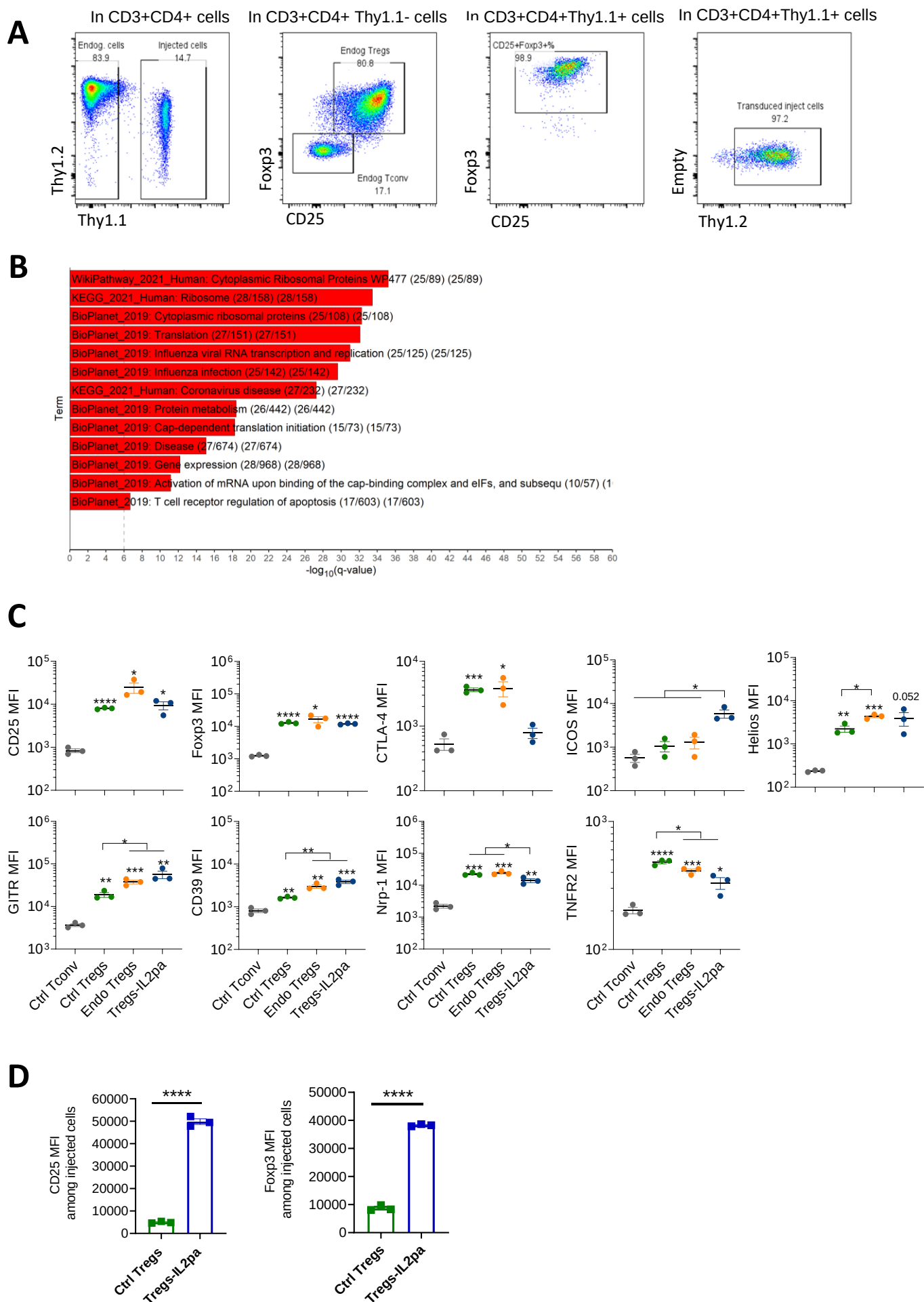
Supplementary Figure 1: Adoptively transferred ex vivo cultured Tregs have a limited lifespan in mice. (A)

Proportion of transduced donor cells monitored as Thy1.2+ among Thy1.1+ injected cells, on the day of the cell injection and 14 days post-injection in the blood (n=7). **(B)** CD25+Foxp3+ phenotype of injected cells (n=7). **(C)** Absolute numbers of injected cells in 100 μL of blood, 14 and 36 days post-injection (n=8). A significant difference was observed between day 14 and day 36 ($p = 0.0272$). **(D)** Absolute numbers of Ctrl Tregs in 100 μL of blood from mice treated with an AAV-IL2 (orange, n=3) or not (green, n=8), from four independent experiments. A significant difference was found between AAV-IL2-treated and non-treated mice at day 20 ($p = 0.0357$). Data are depicted as mean $\pm$ SEM in panels (A), (B), (C), (D) and compared using a two-tailed Mann–Whitney test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$).



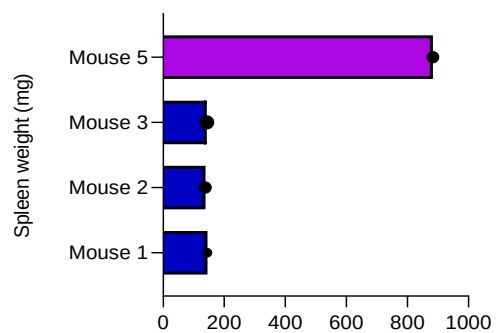
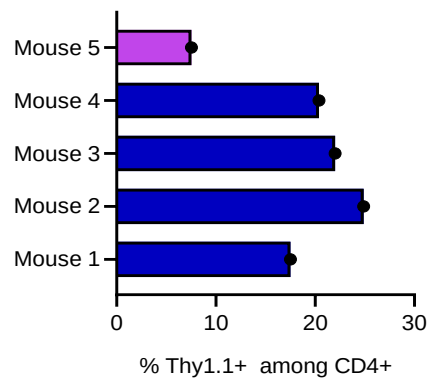
Supplementary Figure 2: IL-2-self-sufficient Treg survival in mice. (A) Comparison of three IL-2 partial agonists in immunocompetent mice. Sorted Thy1.1+ Tregs were transduced to express one of the tested IL-2 partial agonists along with the Thy1.2 reporter gene. The non-sorted transduced cells were injected into B6 Thy1.2 mice at a dose of 9×10^5 cells per mouse, and their survival was tracked in the blood ($n=4$ for the IL2pa1 group, $n=3$ for IL2pa2 and IL2pa3 groups). This figure shows the proportion in the blood of injected cells among CD4 cells, the proportion of transduced cells (Thy1.1+Thy1.2+) among the injected cells, and the percentage of endogenous CD4 Tregs. **(B)** Comparison of pSTAT5 profiles between recombinant human wild-type IL-2 (ILT101) and the selected IL-2 partial agonist. PBMC from healthy blood donors were incubated 10 min with increasing concentrations of IL-2 and stained to assess pSTAT5 expression in CD4 Tregs ($n=2$ per group). **(C)** Absolute numbers of injected cells among CD3+CD4+ cells on days 14 and 36 post-injection across four independent experiments with Ctrl Tregs ($n=8$), Tregs-IL2wt ($n=5$), and Tregs-IL2pa ($n=11$).

Significant differences were observed in the Ctrl Treg group between day 14 and day 36 ($p= 0.0272$), at day 14 between Ctrl Tregs and Tregs-IL2wt ($p= 0.0455$), and day 14 between Ctrl Tregs and Tregs-IL2pa ($p= 0.002$). Dynamics of absolute numbers of injected cells (**D**), endogenous CD4 Tregs (**E**), CD4 Tconv (**F**), and NK cells (**G**) in 100 μ L of blood, $n=2$ per group. Data are presented as mean $\pm$ SEM in the panel (A) to (G) and compared using a two-tailed unpaired t-test in panel (A) and a two-tailed Mann–Whitney test in the panel (C) (* $p<0.05$; ** $p<0.01$; *** $p<0.001$, **** $p<0.0001$).

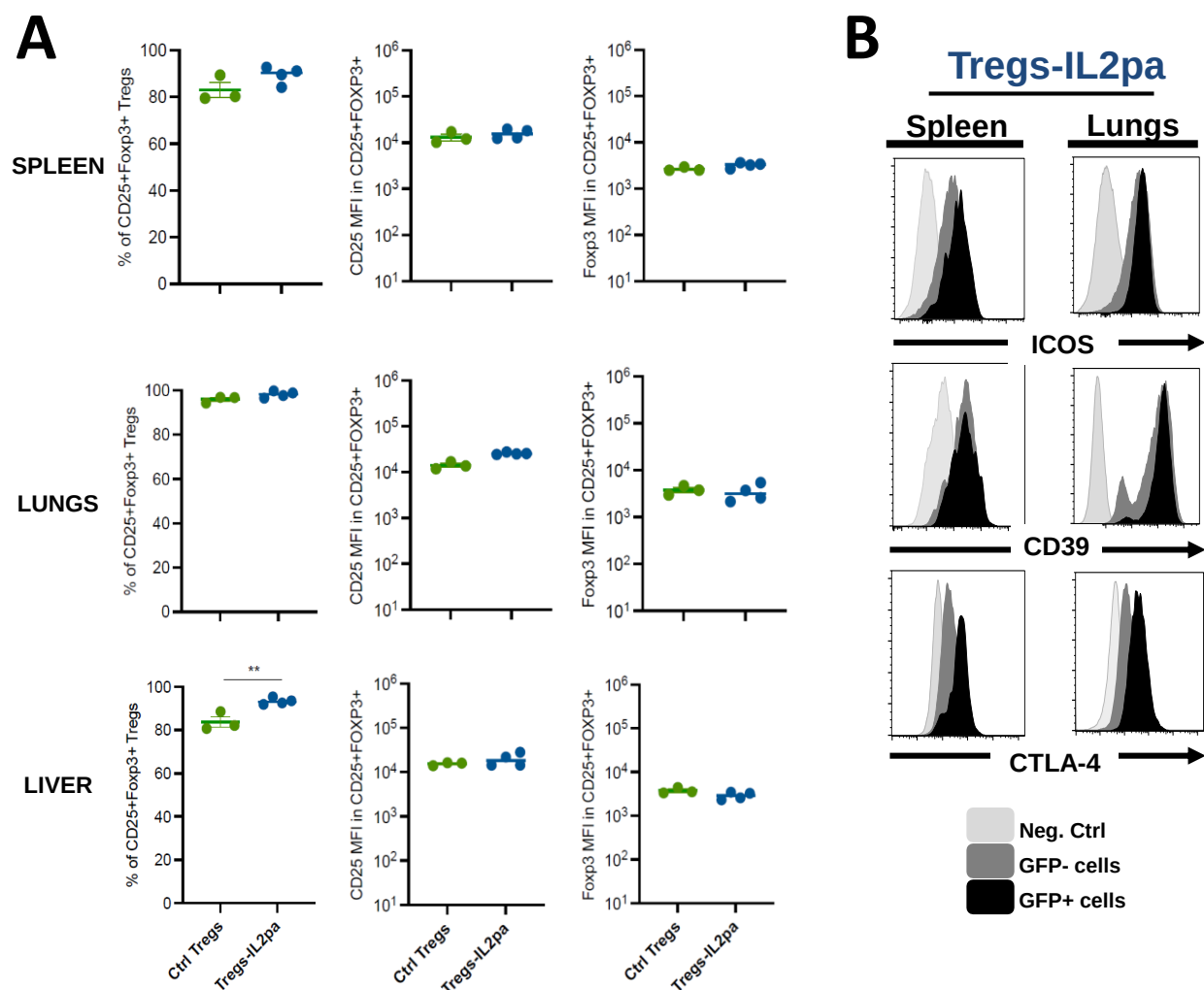


Supplementary Figure 3: Deep immunoprofiling of murine IL-2pa-self-sufficient Tregs. (A) Dot plots of splenocyte staining in a mouse six months post Tregs-IL2pa injection. The figure illustrates the proportion of injected cells, endogenous CD4 Tregs, and the expression of CD25 and Fcpx3 in the injected cells, as well as the proportion of transduced cells among the injected cells. (B) Bar plot showing the results of functional

enrichment analysis comparing the transcriptomes of Tregs-IL2pa and IL-2pa-stimulated Tregs. Each horizontal bar represents a biological pathway from the BioPlanet database, with the length proportional to its statistical significance, as indicated by the $-\log_{10}$ of the p-value. **(C)** Mean Fluorescence Intensity (MFI) of Treg activation/functional markers from blood flow cytometry stainings. Ctrl Tregs and Ctrl Tconv were obtained from unmanipulated mice (n=3) and IL-2pa-stimulated endogenous Tregs and Tregs-IL2pa, from mice that received Tregs-IL2pa (n=3). Stainings were performed 5 to 7 months post-injection. The calculated p-values have been reported in the Source Data file. **(D)** CD25 and Foxp3 MFI among the injected cells in RAG mice 36 days after cell injection, n=3 per group. Statistical differences were found in the MFI of CD25 and Foxp3 between Ctrl Tregs and Tregs-IL2pa ($p < 0.0001$). For panel (B), statistical analysis was performed using a two-sided Fisher's exact test, and p-values were adjusted for multiple comparisons. Data are presented as mean $\pm$ SEM in panels (C), and (D), and compared using a two-tailed unpaired t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$).

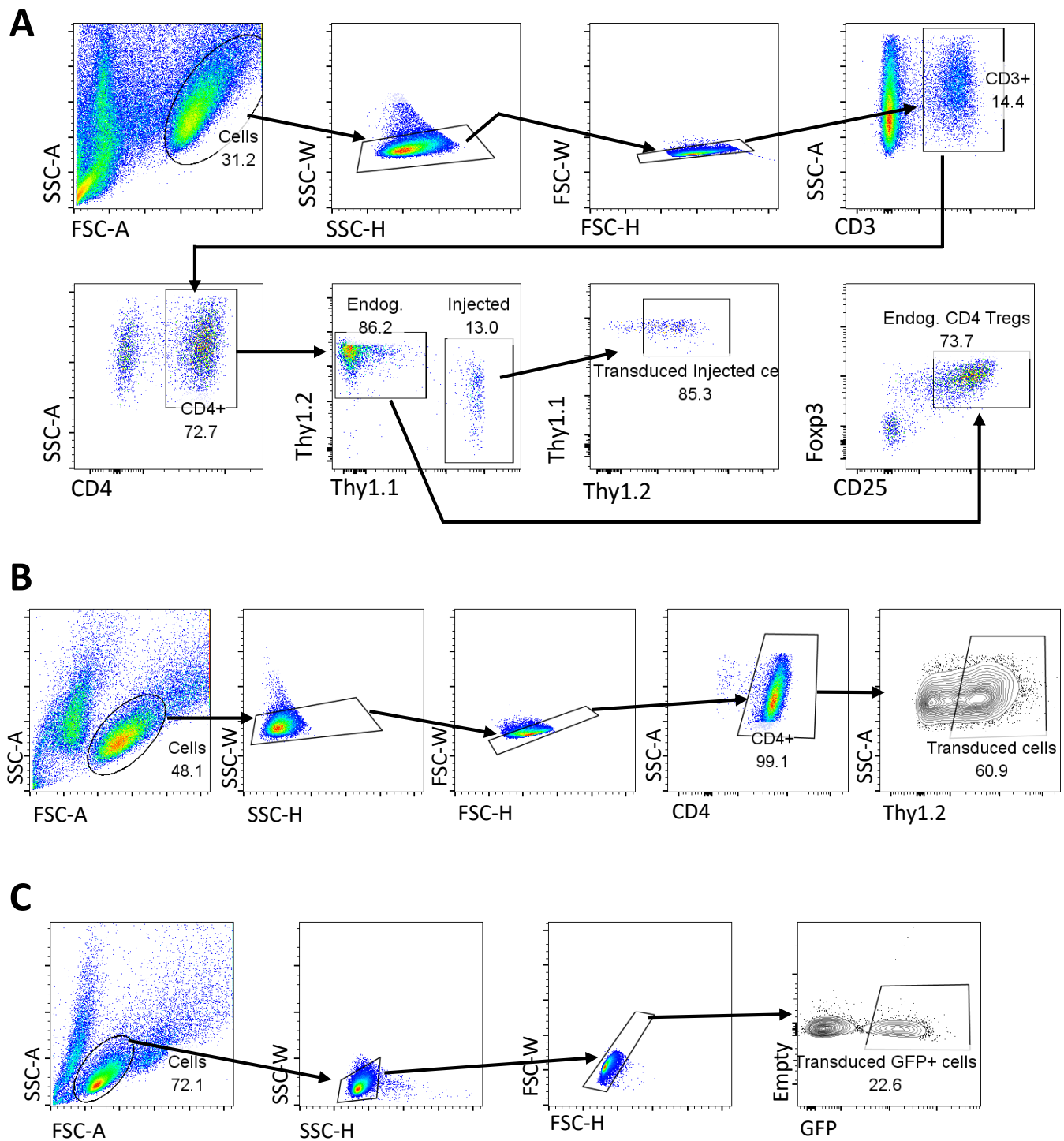
A**B**

Supplementary Figure 4: Diminished control of multiorgan autoimmunity in mouse 5 from the Treg-IL2pa group. (A) Spleen weight the day of euthanasia, and **(B)** proportion of injected cells in mice blood 10 days after the first DT injection, with data for mouse 5 shown in purple.



Supplementary Figure 5: Characterization of human Tregs-IL2pa injected into immunodeficient NSG mice.

(A) Percentage of CD25+Foxp3+ cells among injected cells in the organs (first column). CD25 Mean Fluorescence Intensity (MFI) among CD25+Foxp3+ injected cells (second column) and the Foxp3 MFI among the same population (third column) in NSG spleen, lungs, and liver. In Ctrl Tregs $n=3$ and $n=4$ in Tregs-IL2pa. Statistical difference was found in the percentage of CD25+Foxp3+ in the liver between Ctrl Tregs and Tregs-IL2pa ($p=0.0075$). **(B)** Expression profiles of ICOS, CD39, and CTLA-4 in Tregs-IL2pa isolated from spleen and lung ($n=1$). Data are presented as mean $\pm$ SEM in panels (A) and were analyzed using a two-tailed unpaired t-test ($*p<0.05$; $**p<0.01$; $***p<0.001$; $****p<0.0001$).



Supplementary Figure 6: Gating strategy applied for cell sorting. (A) Gating strategy to sort endogenous CD4 Tregs and injected transduced Tregs for experiments presented in Fig. 3a, b, c, e, f, g. **(B)** Gating strategy to sort transduced Thy1.2+ cells in Fig. 4a. **(C)** Gating strategy to sort transduced GFP+ cells in Fig. 5e, f, g, h, i.

	Target	Fluorochrome	Clone	Dilution	Brand	Catalog number
Anti-mouse antibodies	CD3	PE 610	145-2C11	1/200	Invitrogen	61-0031-82
	CD4	V500	RM4-5	1/400	BD Biosciences	560782
	CD8	SuperBright 600	53-6.7	1/200	Invitrogen	63-0081-82
	CD335	AlexaFluor 700	29A1.4	1/200	Invitrogen	56-3351-82
	CD90.1	PE	HIS51	1/200	Invitrogen	12-0900-83
	CD90.2	APC	53-2.1	1/400	Invitrogen	17-0902-82
	Foxp3	FITC	FJK-16s	1/100	Invitrogen	11-5773-82
	CD25	PC7	PC61.5	1/200	Invitrogen	25-0251-82
	CD4	BUV496	RM4-5	1/20	Invitrogen	364-0042-80
	CD8	BUV805	53-6.7	1/40	Invitrogen	368-0081-80
	CD90.1	APC	OX-7	1/800	BD	561409
	CD90.2	BV480	53-2.1	1/400	BD	566082
	CD25	BV786	PC61.5	1/40	Invitrogen	417-0251-80
	GITR	PE	DTA-1	1/40	Biolegend	126309
	ICOS	APC-Cy7	C398.4A	1/20	Biolegend	313529
	CD39	BUV395	Y23-1185	1/40	BD	567264
	CTLA4	PE	UC10-4B9	1/100	Invitrogen	12-1522-82
	HELIOS	eFluor 450	22F6	1/20	Invitrogen	48-9883-41
	TNFR2	BV421	TR75-89	1/200	BD	564088
	NRP1	PECy7	3E12	1/80	Biolegend	145211
Anti-human antibodies	CD3	ECD	UCHT1	1 µl /test	Beckman	A07748
	CD4	Pacific Blue	13B8.2	1 µl /test	Beckman	B49197
	CD8	Krome Orange	B9.11	3 µl /test	Beckman	B00067
	Foxp3	APC	259D	10 µl /test	Beckman	B30650
	CD25	PE	B1.49.9	5 µl /test	Beckman	A07774
	CD127	PC7	R34.34	5 µl /test	Beckman	A64618
	CD45	AF750	J33	10 µl /test	Beckman	A79392
	CD39	PE	MZ1823C8	10 µl /test	Miltenyi	130-093-503
	CTLA4	CyChrome	BNI3	20 µl /test	Invitrogen	555854
	LAP	PECF594	TW4-2F8	5 µl /test	BD	562490
	GFP	FITC	FM264G	5 µl /test	Biolegend	338008
	ICOS	PE	DX29	20 µl /test	BD	557802
	STAT5	PE	Tyr694	5 µl /test	Invitrogen	12-9010-42
	CellTrace™ Violet Cell		-	1/1000	Thermofisher	C34557

Supplementary Table 1: List of antibodies