

SUPPLEMENTARY INFORMATION

A CD25-biased interleukin-2 for autoimmune therapy engineered via a semi-synthetic organism

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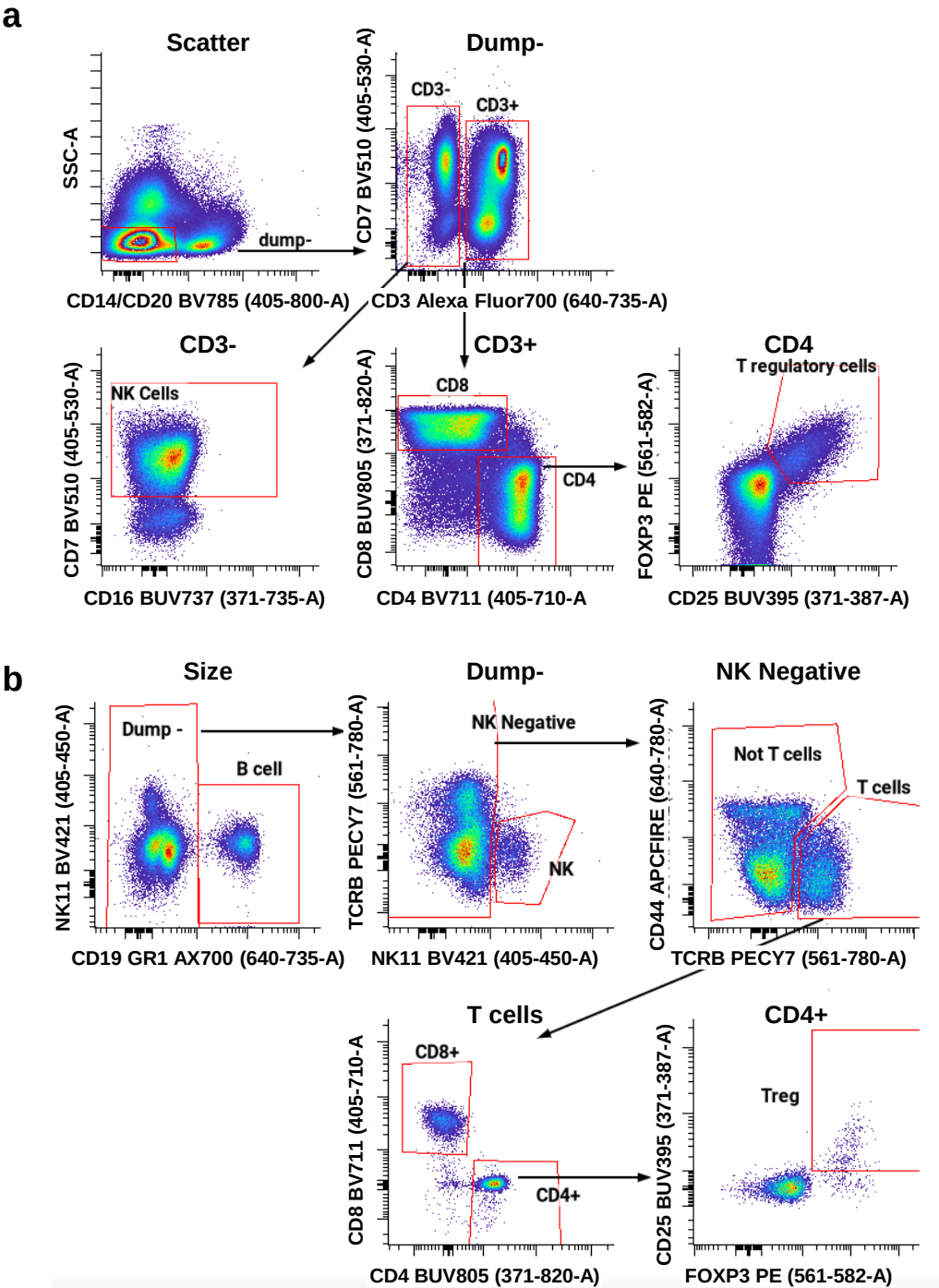
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Supplementary Figure 1. Representative gating strategy in (a) NHP and (b) mouse whole blood is shown.

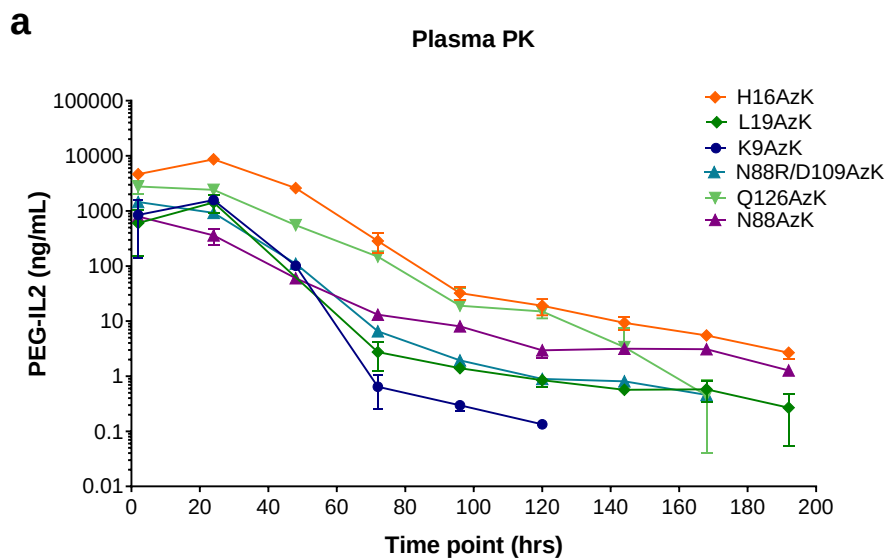
(a) For NHP, NK cells are defined as CD14-CD20-CD3-CD7+, CD8 T cells as CD14-CD20-CD3+CD8+, non-Treg CD4 as CD14-CD20-CD3+CD4+CD25-FoxP3-, and Tregs as CD14-CD20-CD3+CD4+CD25+FoxP3+. (b) For mouse, NK cells are defined as CD19-GR1-NK1.1+, CD8 T cells as CD19-GR1-NK1.1-TCRb+CD8+, non-Treg CD4 as CD19-GR1-NK1.1-TCRb+CD4+CD25-FoxP3-, and Tregs as CD19-GR1-NK1.1-TCRb+CD4+CD25+FoxP3+



Supplementary Figure 2. PK profile of IL-2 variants in C57BL/6 mice.

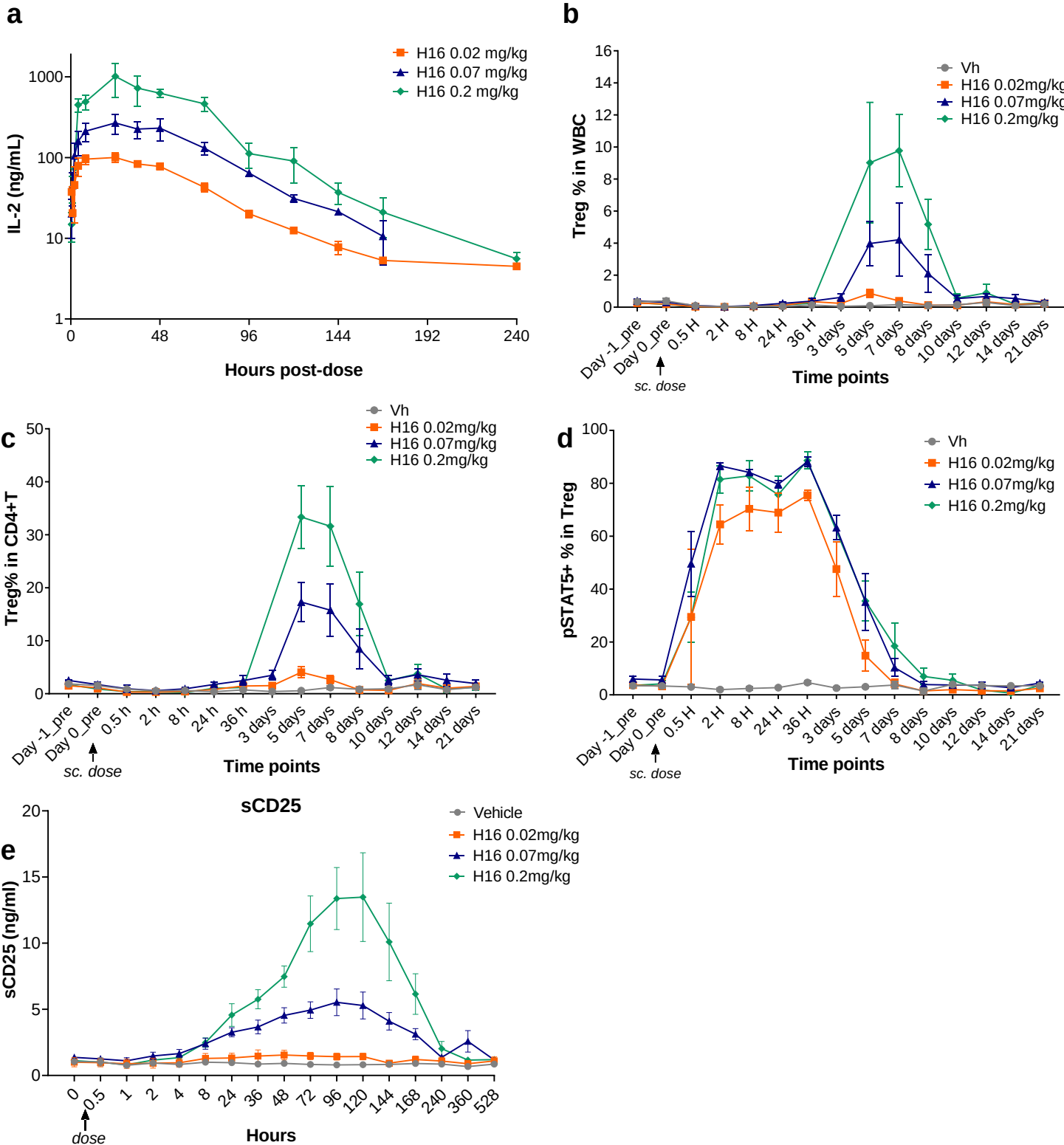
Compound screening in mice identified H16 as a pegylated IL-2 variant which significantly expanded Treg populations compared to other compounds. A single subcutaneous dose of the indicated variant was administered to C57BL/6 mice.

(a) Concentration is plotted as a function of time post dose. (N=3 ± SEM)



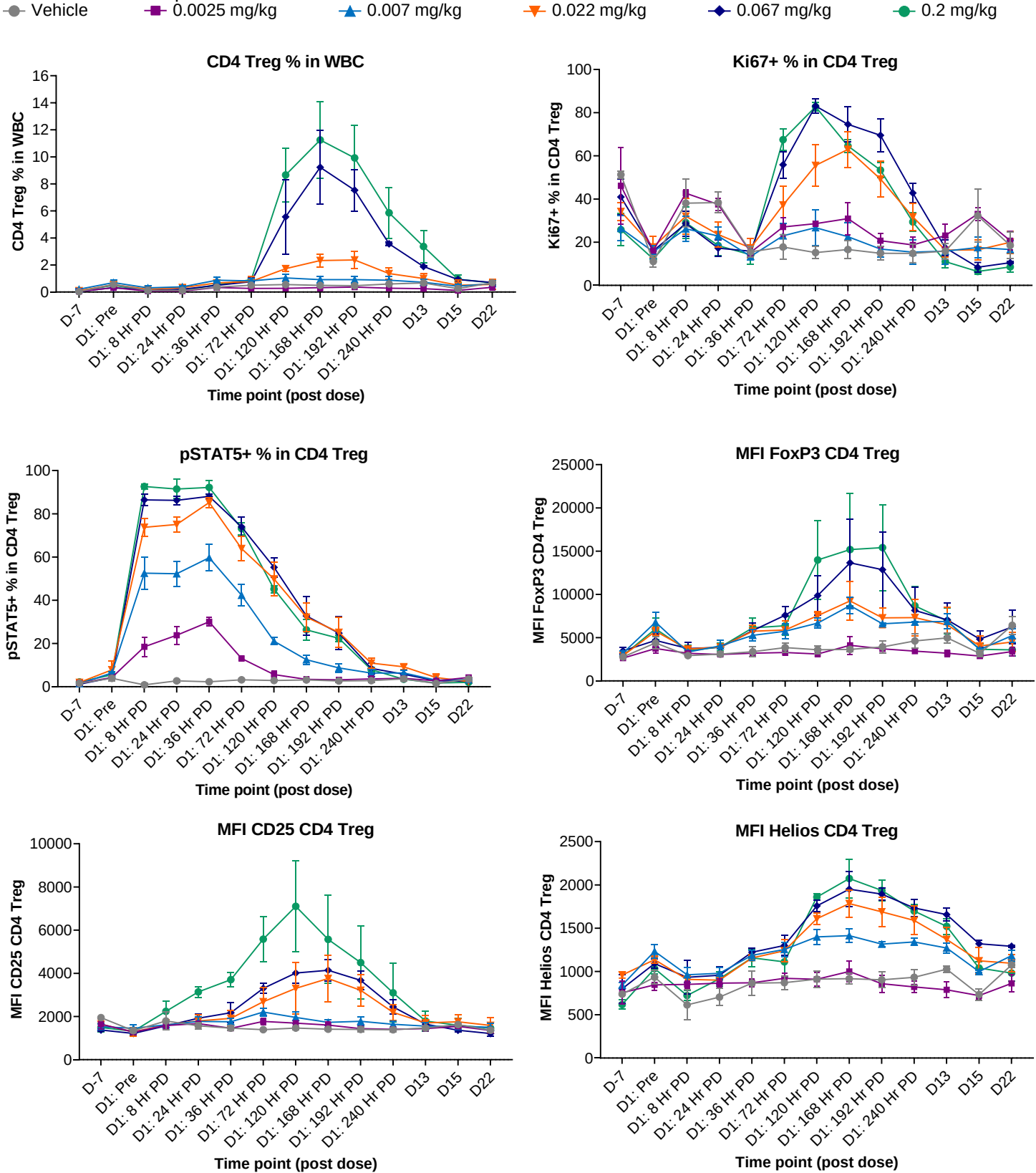
Supplementary Figure 3. H16 is a half-life extended IL-2 that promotes activation of Tregs in cynomolgus monkey.

H16 induces dose-dependent and large-scale Treg expansion and expression of biomarkers of differentiation and suppressive function in NHPs. (a) H16 was administered at 0.02, 0.07, or 0.2 mg/kg to cynomolgus monkeys. Pharmacokinetics of variants up to 240 hours post treatment. (b-e) H16 was administered at 0 (vehicle only), 0.07, or 0.2, mg/kg to cynomolgus monkeys, and peripheral blood samples were collected at the indicated times. Multi-color flow cytometry was used to quantitate the CD4+Treg as a (b) percentage of WBC (c) percentage of CD4 T cells, and (d) expression of pSTAT5. (At 0.2 mg/kg dose, day 3 time-point is excluded for poor sample collection). (e) Soluble CD25 (sCD25) was quantified from serum. Data represent mean $\pm$ SEM from three independent animals.



Supplementary Figure 4. SAR’336 promotes dose dependent Treg expansion and activation in NHP.

SAR’336 induces dose-dependent and large-scale Treg expansion and expression of biomarkers of differentiation and suppressive function in NHPs. THOR-809 was administered at 0.0025 – 0.2 mg/kg to cynomolgus monkeys and N88R/D107 at 0.3 mg/kg, and peripheral blood samples were collected at the indicated times. Multi-color flow cytometry was used to quantitate the CD4+Treg frequency and expression of Ki67, pSTAT5, FoxP3, CD25, and Helios. Data represent mean ± SEM from three independent animals.



Supplementary Table 1. Antibodies used for flow cytometry.

Species	Antigen	Fluorophore	Clone	Vendor	Catalog #	Dilution
Human	CD3	A488	UCHT1	Biolegend	300415	1:10
	CD4	PE/Cy7	RPA-T4	Biolegend	300512	1:10
	CD8	PerCP/Cy5.5	RPA-T8	BD	560662	1:10
	CD8	SB780	RPA-T8	eBioscience	78-0088-42	1:50
	CD127	eF506	eBioRDR5	eBioscience	69-1278-42	1:10
	CD25	APC	M-A251	Biolegend	356110	1:10
	CD56	BV421	HCD56	Biolegend	318328	1:10
Mouse	Fc block	-	93	Biolegend	101320	1:10
	FoxP3	APC	FJK-16S	eBioscience	17-5773-82	1:10
	CD25	PE/Cy7	3C7	Biolegend	101916	1:10
	CD8	APC	53-6.7	Biolegend	100712	1:10
	CD4	PerCP/Cy5.5	GK1.5	Biolegend	100434	1:10
	CD127	PE	A7R34	Biolegend	135010	1:10
	CD4	APC/Cy7	L3T4	eBioscience	A15384	1:100
	CD45RB	APC	C363.16A	eBioscience	17-0455-81	1:100
	CD25	PE	PC61.5	eBioscience	12-0251-82	1:100
	CD3	PerCP/Cy5.5	145-2C11	BD	553067	1:10
	CD4	BUV737	RM4-5	BD	612844	1:10
	CD8	PE/CF594	53-6.7	BD	562283	1:10
	CD19	BV510	SJ25C1	BD	562947	1:10
	CD25	APC	PC61	BD	557192	1:10
	NK1.1	PE Cy7	PK136	BD	552878	1:10
	FoxP3	BV421	MF-23	BD	562996	1:10
	CD45	BV510	30-F11	BD	563891	1:20
	TCRb	BV711	H57-597	BD	563135	1:20
	CD45	FITC	RM4-5	BD	553047	1:20
	CD8a	PE	53-6.7	BD	553033	1:20
	CD25	BV786	3C7	BD	564368	1:10
	CD44	PE/Cy7	IM7	BD	560569	1:40
	CD62L	BV650	MEL-14	BD	564108	1:5
	FoxP3	V450	MF23	BD	561293	1:10
NHP	CD3	A700	SP34-2	BD	8162839	1:50
	CD4	BV711	OKT4	BioLegend	317440	1:20
	CD7	BV510	M-T701	BD	563650	1:20
	CD8	BUV805	SK1	BD	564912	1:20
	CD14	BV785	M5E2	BioLegend	301840	1:200
	CD16	BUV737	3G8	BD	564434	1:200
	CD20	BV785	2H7	BioLegend	302356	1:200
	CD25	Biotin	M-A251	BioLegend	356124	1:100
	FoxP3	PE	259D	BioLegend	320208	1:20
	ICOS	BV421	C398.4A	BioLegend	313524	1:100
	Helios	A488	D8W4X	CST	56424BC	1:25
	Streptavidin	BUV395	n/a	BD	564176	1:250
	Ki-67	PE/Cy7	SolA15	eBioscience	25-5698-82	1:500

Supplementary Table 2. Potency of IL-2 variants in CD8 T cells and Tregs.

Compound	NK cells (pg/mL)	CD8+ T cells (pg/mL)	Treg cells (pg/mL)	CD8+/Treg ratio
rhIL-2	4586	31024	75	414
V91K ¹	20537	102255	142	720
S127AzK	20210	92190	150	615
T131AzK	24207	132922	258	515
T123AzK	33396	152928	269	569
E100AzK	35088	195823	483	405
N119AzK	34010	143380	535	268
M23AzK	143620	539824	1030	524
K9AzK	169578	1100679	2217	496
N26AzK	258531	1188859	2459	483
N88R ²	2312847	15025734	11082	1356
Q126AzK	3676807	19722480	29454	670
H16AzK	2545257	12070108	34976	345
L19AzK	6756768	22436430	93205	241
N88R/D109AzK ³	2780819	12503386	175805	71
N88AzK	3298113	11111537	323201	34
D20AzK	2643930	9505217	1129455	8

¹Compound based on Gavin MA. US Patent US20140286898A1. Interleukin-2 muteins for the expansion of t-regulatory cells. 2014.

²Shanafelt, A. B. et al. A T-cell-selective interleukin 2 mutein exhibits potent antitumor activity and is well tolerated in vivo. Nat. Biotechnol. 18, 1197–1202 (2000).

³Compound based on Greve J. US Patent US10035836B1. Modified IL-2 variants that selectively activate regulatory T cells. 2015. AzK was incorporated at D109 position and compound was pegylated using the pegylation strategy employed for other variants.

Supplementary Table 3. IL-2 and SAR'336 binding constants to human IL-2 receptor.

Sample	Surface	k_a (M ⁻¹ s ⁻¹)	k_d (s ⁻¹)	K_D (nM)
IL-2	IL-2Ra	1.3e8	1.2	9.29
	IL-2Rb	9.00e5	0.2634	293
SAR'336	IL-2Ra	3.88e6	0.1257	32.4
	IL-2Rb	NO BINDING DETECTED		

Supplementary Table 4. EC50 (pg/mL) of IL-2 and SAR'336 by pSTAT5 detection in human cells.

Variant	Donor	<i>CD8 T cell</i>	<i>NK cell</i>	<i>Treg</i>
IL-2	1	12,669	6,047	24
	2	11,120	4,020	21
	3	14,590	3,010	19
	4	16,537	4,307	34
SAR'336	1	-	-	1,499,594
	2	-	-	2,223,949
	3	-	-	928,362
	4	-	-	1,824,464