Supplemental Figure Legends

Figure S1. Biacore data show that 5 kDa is the optimal mPEG size for abolishing IL-2Rα interactions yet maintaining IL-2Rβ/γ interactions. Kd values from Biacore experiments testing IL-2 muteins with varying IL-2Rα blocking mPEG sizes for binding to human IL-2Rα or IL-2Rβ.

Figure S2. Human PBMC data show that a 5 kDa mPEG is sufficient for abolishing IL-2Rα interactions and that larger mPEG sizes reduce IL-2 β/γ potency. Human PBMCs were stimulated with IL-2 or IL-2 variants with 5, 10, or 30 kDa mPEG moieties attached to the IL-2Rα binding interface for 30 minutes at indicated concentrations then analyzed by flow cytometry for pSTAT5 in the indicated cellular populations. Shown are **(A)** pSTAT5 median fluorescence intensity (MFI) titration plots and **(B)** EC_{50} values.

Figure S3. IL-2 β/γ demonstrates higher cytotoxic potency compared to IL-2 variants with larger IL-2Rα blocking mPEGs. **(A)** NK cell cytotoxicity responses were measured against K562 targets at a fixed E:T ration of 1.25:1 and varying concentrations of IL-2 variants attached to 5, 10 or 30 kDa IL-2Rα blocking mPEGs. Data are plotted as mean ± SEM. **(B)** $CD8^+$ T cell cytotoxicity responses were measured against Raji targets in the presence of 1 nM CD3-CD20 bispecific antibody at a fixed E:T ration of 5:1 and varying concentrations of IL-2 muteins attached to 5, 10 or 30 kDa IL-2Rα blocking mPEGs. Data are plotted as mean ± SEM. **(C)** Pooled data from both donors for $CD8^+$ T cell and NK cell cytotoxicity assays plotted as mean ± SEM. Statistical analyses were performed using paired t-test. *, $p<0.05$.

Figure S4. Female C57BL/6 mice with established MC38 tumors were treated with buffer control or 60 μg TransCon IL-2 β/γ on Days 0, 7 and 14 (n=20 mice/group). Individual animal tumor volumes are shown.

Figure S5A. TransCon IL-2 β/γ increases $CD8^+/CD4^+$ and $CD8^+$/Treg ratios in mice. CT26 bearing BALB/C mice were administered two weekly doses of TransCon IL-2 β/γ intravenously. Peripheral blood was drawn 96 h after the first and second dose and analyzed by flow cytometry. Shown are $CD8^+/CD4^+$ and $CD8^+$/Treg ratios from blood and spleen samples as mean ± SEM (n=5 mice/group). Statistical analyses were performed using unpaired t-test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Figure S5B. TransCon IL-2 β/γ robustly activates effector function potential in $CD8^+$ T cell and NK cells in mice after a single dose. CT26 bearing BALB/C mice were administered two weekly 60 μg doses of TransCon IL-2 β/γ intravenously. Peripheral blood was drawn 96 h after the first dose and stimulated with BD Leukocyte Activation Cocktail for 4 h to induce cytokine production before analysis by flow cytometry. Shown are example example flow cytometry plots to illustrate gating (top) and aggregate data (bottom) for the proportion of Granzyme B^+ or IFN-γ^+ NK cells (left) or $CD8^+$ T cells (right) as mean ± SEM (n=3-4 mice/group). Statistical analyses were performed using unpaired t-test. *, $p<0.05$; ***, $p<0.001$.

Figure S6. IL-2 β/γ is IL-2Rβ/γ selective in human and cynomolgus monkey cells. Human or monkey blood was stimulated with control IL-2 or IL-2 β/γ for 30 minutes at the indicated concentrations then analyzed by flow cytometry for pSTAT5 in the indicated cellular populations. Data are shown as median fluorescence intensity (MFI) of pSTAT5 for two donors as mean ± SEM (top) and summarized as EC_{50} values (bottom).

Figure S7. TransCon IL-2 β/γ provides prolonged IL-2 β/γ systemic levels compared to pulsatile IL-2 levels seen with aldesleukin treatment. Cynomolgus monkeys were administered either 0.4 mg (average 0.044 mg/kg) per day of aldesleukin for five days or a single 1 mg dose (average 0.122 mg/kg) of TransCon IL-2 β/γ intravenously. Peripheral blood was drawn post-dosing and was analyzed for serum IL-2 levels or plasma IL-2 β/γ levels respectively. Shown are systemic IL-2 levels after aldesleukin dosing (left) or IL-2 β/γ levels as IL-2 equivalents after TransCon IL-2 β/γ dosing (right) as mean ± SEM (n=4/group).

Figure S8. TransCon IL-2 β/γ induces dose-dependent and robust increases in lymphocytes with minimal increases in eosinophils and induction of systemic inflammation markers in cynomolgus monkeys. Cynomolgus monkeys were bled before (predose) and at various timepoints after intravenous administration of TransCon IL-2 β/γ at 0.1, 0.3 and 0.9 mg/kg (n=1 animal/sex/group) **(A)** Total lymphocytes and eosinophils are presented as counts/μL and shown as mean ± SEM. **(B)** Serum concentrations of sCD25, IL-5, IL-6, MCP-1, IFN-γ and TNF-α are presented as mean ± SEM.

Figure S9. TransCon IL-2 β/γ increases the ratio of $CD8^+$ T cells, NK cells and γδ T cells to Tregs or $CD4^+$ T cells in cynomolgus monkeys. Cynomolgus monkeys were bled before (predose) and at various timepoints after intravenous administration of TransCon IL-2 β/γ at 0.1, 0.3 and 0.9 mg/kg (n=1 animal/sex/group). **(A)** Ratios of $CD8^+$ T cells, NK cells and γδ T cells to Tregs expressed as the fold change from predose ratios and shown as mean ± SEM. **(B)** Ratios of $CD8^+$ T cells, NK cells and γδ T cells to $CD4^+$ T cells expressed as the fold change from predose ratios and shown as mean ± SEM.

Figure S10. TransCon IL-2 β/γ induces more potent expansion of effector memory $CD8^+$ T cells compared to total $CD8^+$ T cells in cynomolgus monkeys. Animals were bled before (predose) and at various timepoints after intravenous administration of TransCon IL-2 β/γ at 0.1, 0.3 and 0.9 mg/kg (n=1 animal/sex/group). **(A)** Fold change compared to predose counts of Total (left) and Effector Memory (right) $CD8^+$ T cells shown as mean ± SEM. **(B)** Percentages of proliferating ($Ki67^+$) cells within Total (left) and effector memory (right) $CD8^+$ T cells shown as mean ± SEM.

Figure S11. TransCon IL-2 β/γ induces expansion of Granzyme B expressing $CD8^+$ T cells, NK cells and, γδ T cells in cynomolgus monkeys. Animals were bled before (predose) and at various timepoints after intravenous administration of TransCon IL-2 β/γ at 0.1, 0.3 and 0.9 mg/kg (n=1 animal/sex/group). **(A)** Raw cell counts and **(B)** Fold change of cell counts compared to predose levels for Granzyme B expressing $CD8^+$ T cells, NK cells and γδ T cells shown as mean ± SEM.

Figure S12A. IL-2 β/γ induces cytokine and cytotoxic effector molecules in human $CD8^+$ T cells. Supernatants were taken from $CD8^+$ T cell cocultures with Raji lymphoma cells from cytotoxicity experiments described in Figure 7 and analyzed for TNF-α, IFN-γ, FasL, Granzyme A, Granzyme B and Perforin. Data are shown over the full E:T range of the assay and displayed as mean ± SEM (n=3 donors).

Figure S12B. IL-2 β/γ induces cytokine and cytotoxic effector molecules in human NK cells. Supernatants were taken from NK cell cocultures with K562 erythroleukemia cells from cytotoxicity experiments described in Figure 7 and analyzed for TNF-α, IFN-γ, FasL, Granzyme A, Granzyme B and Perforin. Data are shown over the full E:T range of the assay and displayed as mean ± SEM (n=3 donors).

Figure S12C. IL-2 β/γ induces cytokine and cytotoxic effector molecules in human γδ T cells. Supernatants were taken from γδ T cell cocultures with Raji lymphoma cells from cytotoxicity experiments described in Figure 7 and analyzed for TNF-α, FasL, IL-17A, IFN-γ, Granzyme A, Granzyme B, Perforin and Granulysin. Data are shown over the full E:T range of the assay and displayed as mean ± SEM (n=2 donors).

Figure S13. IL-2 β/γ induces cytokine and cytotoxic effector molecules in endogenous TCR ligand activated human γδ T cells. γδ T cells were cocultured overnight with Daudi lymphoma cells. **(A)** γδ T cell cytotoxicity results against Daudi target cells after subtracting for background seen in conditions with targets alone. Pooled data are expressed as mean ± SEM (n=3 donors). **(B, C)** Supernatants were taken from γδ T cell and Daudi cocultures described in **(A)** and analyzed for TNF-α, FasL, IL-17A, IFN-γ, Granzyme A, Granzyme B, Perforin, and Granulysin. **(B)** Fold change values at an E:T ratio of 2.0. **(C)** Data over the full E:T range of the assay displayed as mean ± SEM (n=2 donors for all E:Ts except E:T = 8 where n=1 due to insufficient sample).

Figure S1: Biacore data suggests 5 kDa is the optimal size for abolishing IL-2Rα interactions yet maintaining IL-2Rβ/γ interactions

Size of biasing PEG residue	Biacore analysis (Kd values)	
	Binding towards IL-2Rα	Binding towards IL-2Rβ
NA (IL-2 control)	9.3 nM	280 nM
2 kDa	700 nM	670 nM
5 kDa	> 2,000 nM	1,000 nM
10 kDa	> 2,000 nM	1,900 nM
20 kDa	> 3,000 nM	4,500 nM

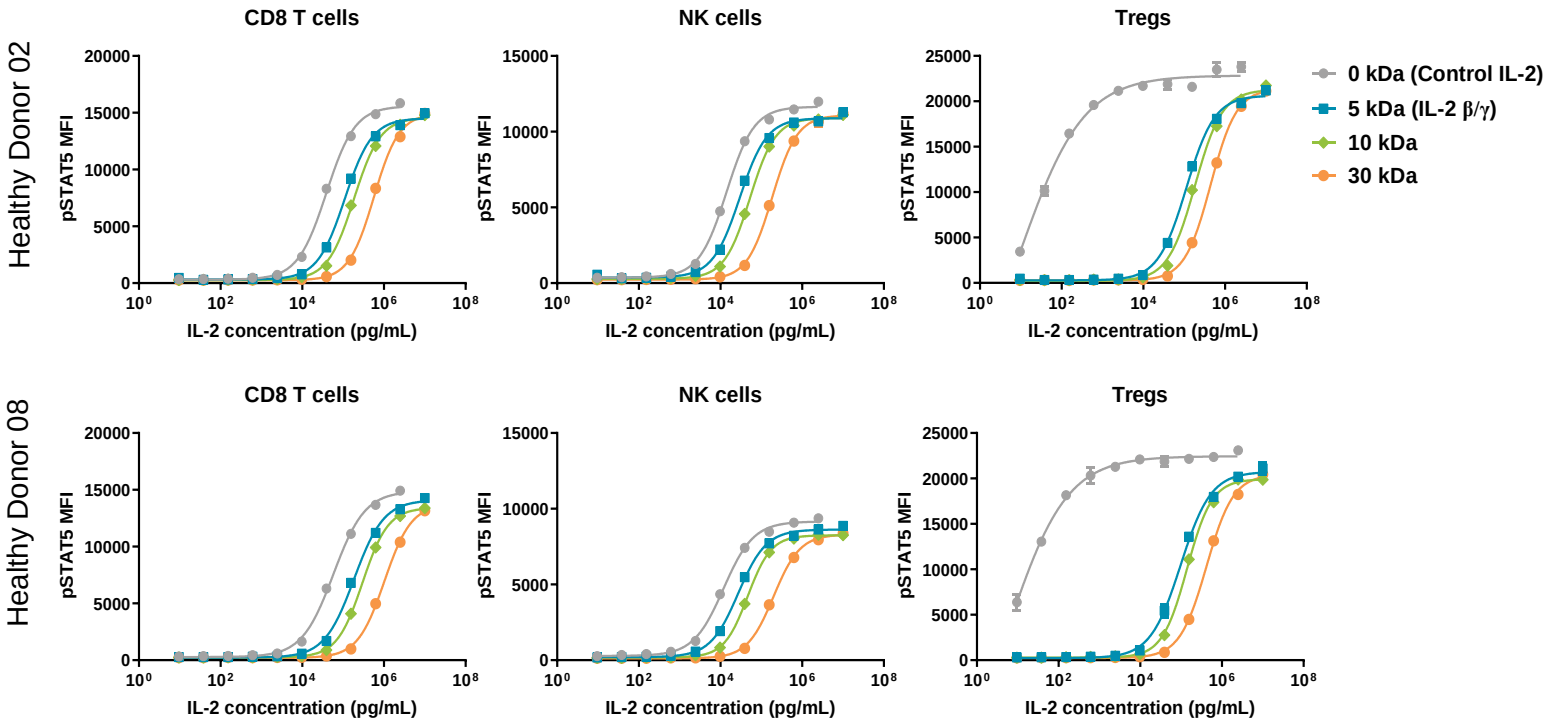
No detectable binding towards IL-2Rα

PEG Size

IL-2Rβ affinity

Figure S2: PBMC data show that a 5 kDa PEG is sufficient for abolishing IL-2Rα interactions and increasing PEG length reduces IL-2 β/γ potency

A)



B)

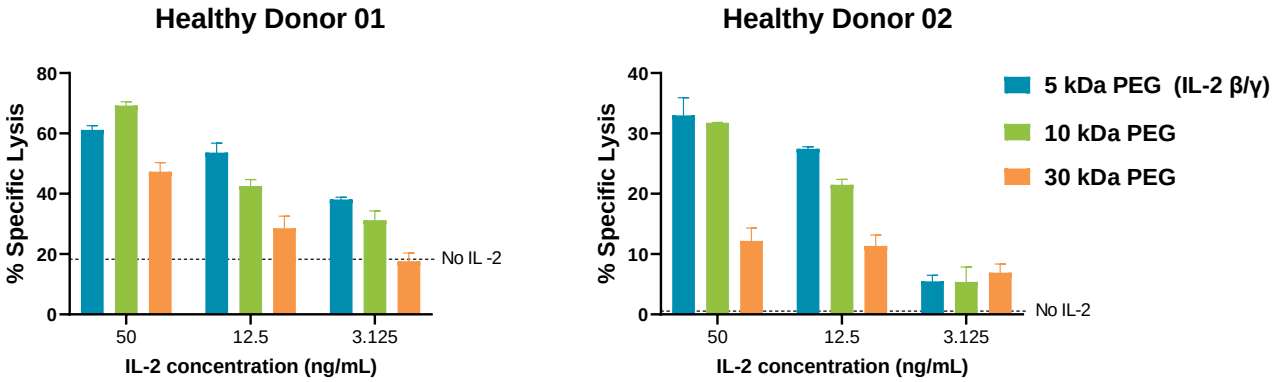
Size of biasing PEG residue	pSTAT5 EC_{50} potency analysis (Human PBMCs)		
	$CD8^+$ T cells (IL-2Rβ/γ potency)	NK cells (IL-2Rβ/γ potency)	Tregs (IL-2Rα/β/γ potency)
NA (IL-2 control)	48.75 ng/ml	13.06 ng/ml	< 0.1 ng/ml
5 kDa	148.31 ng/ml	28.41 ng/ml	105.47 ng/ml
10 kDa	242.50 ng/ml	49.41 ng/ml	162.87 ng/ml
30 kDa	802.17 ng/ml	192.25 ng/ml	432.88 ng/ml

Figure S3: IL-2 β/γ demonstrates higher cytotoxic potency compared to IL-2 variants with larger PEG lengths

A)

NK cells

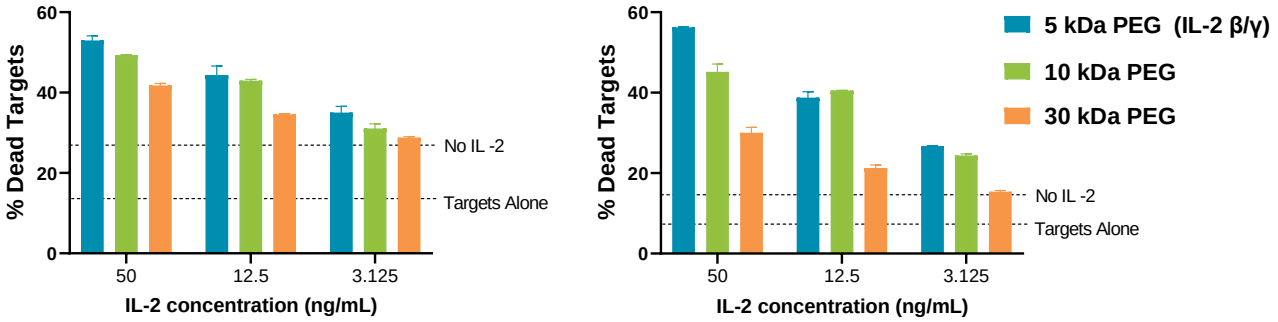
Fixed E:T = 1.25:1
with K562
Erythroleukemia
Targets
4h assay



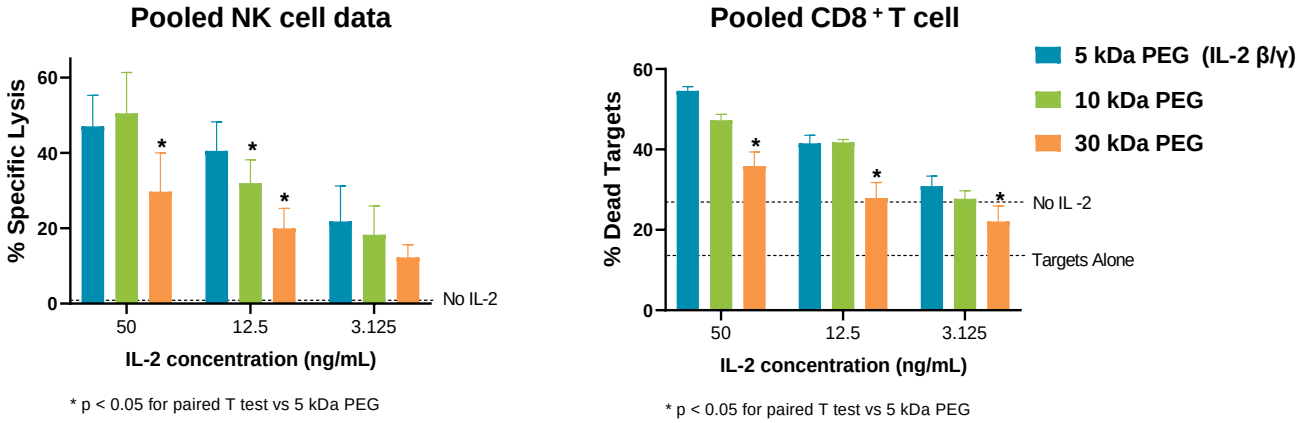
B)

CD8⁺ T cells

Fixed E:T = 5:1
with Raji
B cell Lymphoma
Targets
+ CD3-CD20 BsAb
20h assay



C)



* p < 0.05 for paired T test vs 5 kDa PEG

* p < 0.05 for paired T test vs 5 kDa PEG

IL-2 β/γ (5 kDa PEG) enhances NK and CD8⁺ T cell cytotoxicity more potently than variants with larger blocking PEGs

Figure S4: TransCon IL-2 β/γ shows anti-tumor effects in MC38 tumor bearing mice

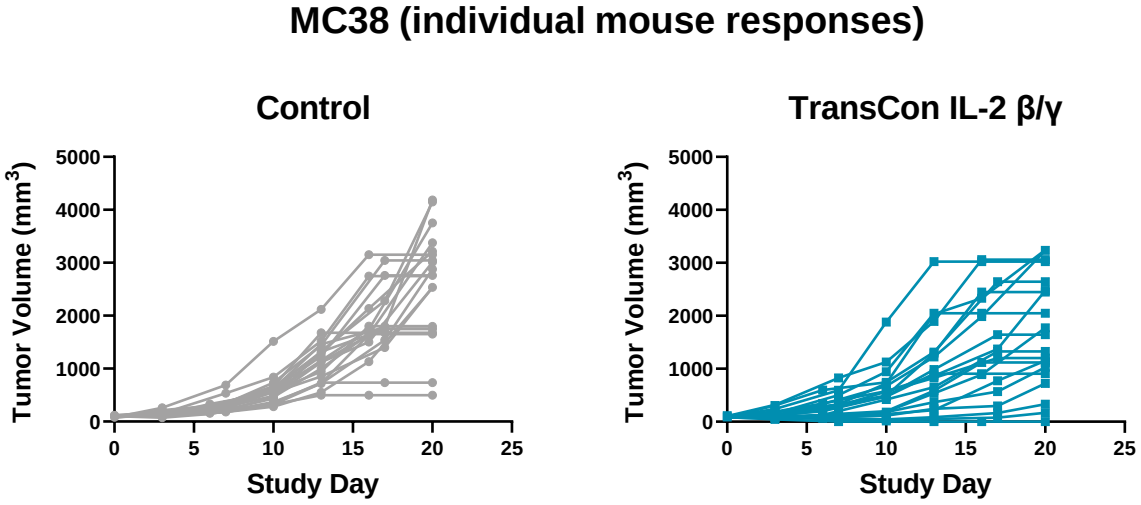


Figure S5A: TransCon IL-2 β/γ increases CD8+ / CD4 + and CD8+ / Treg ratios in mice

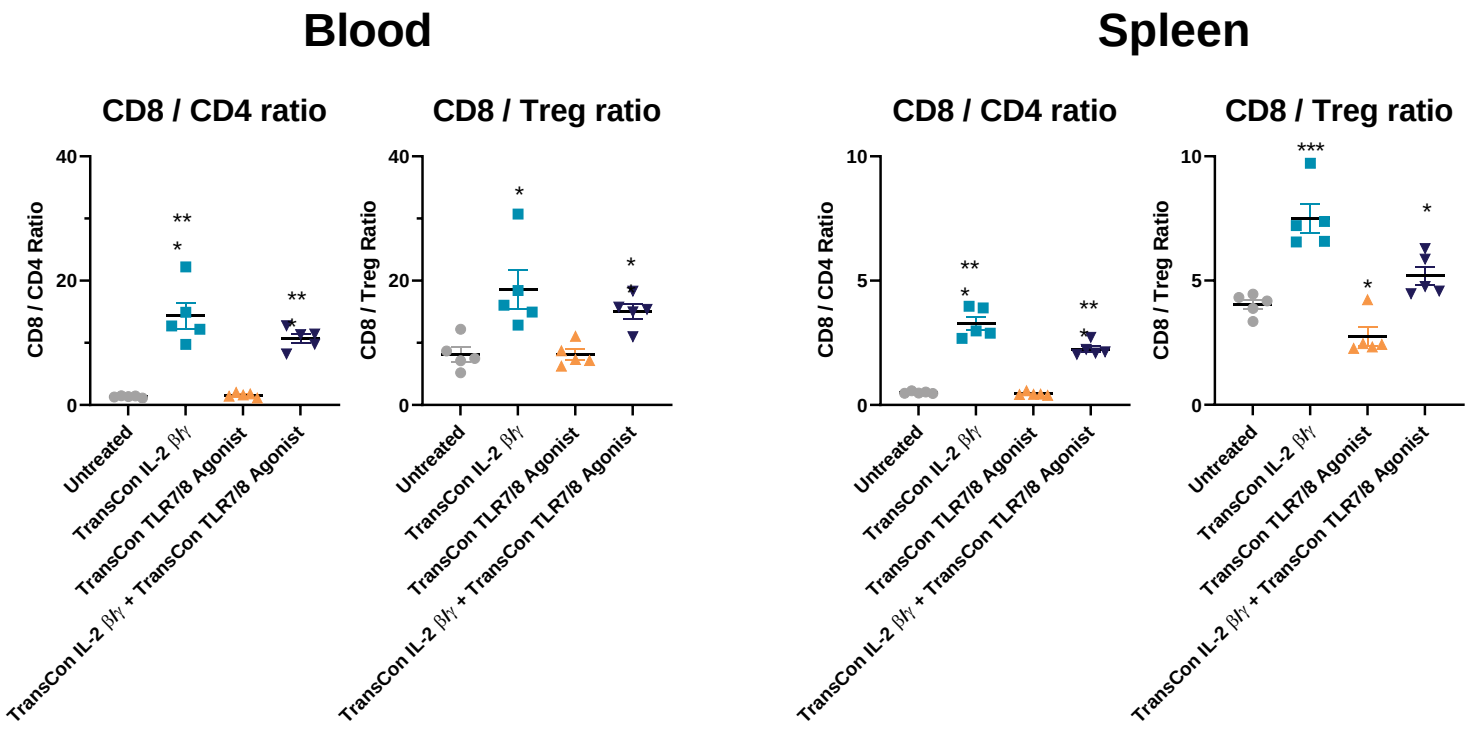


Figure S5B: TransCon IL-2 β/γ robustly activates effector function potential in CD8+ T and NK cells in mice

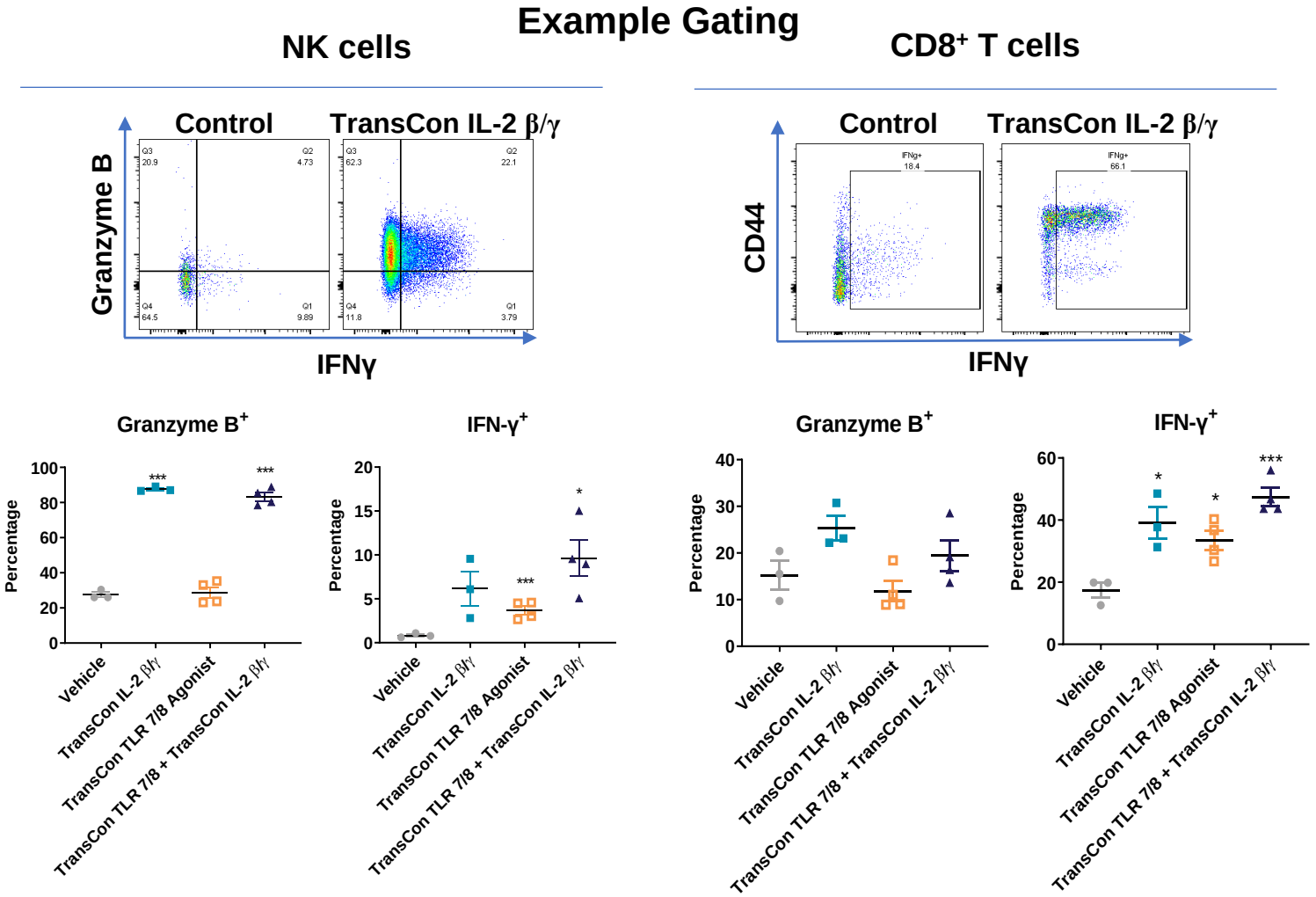
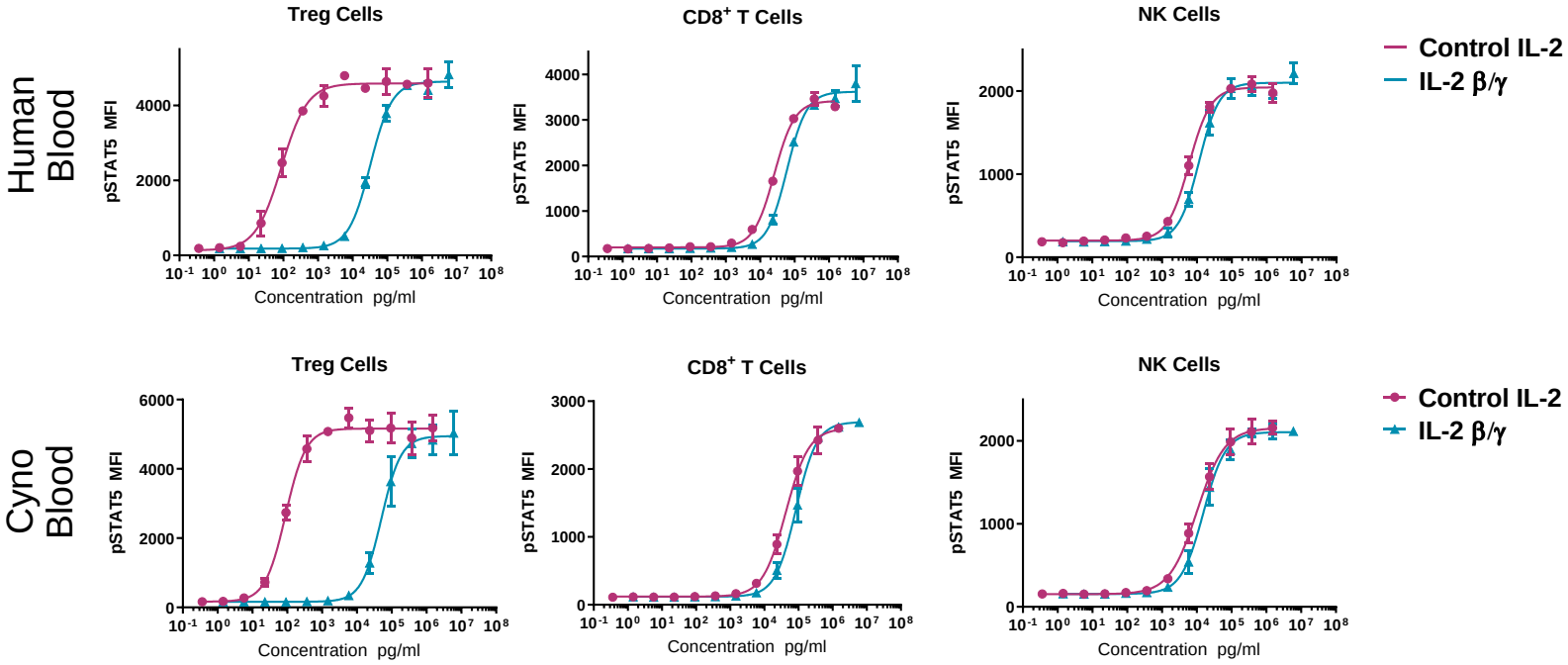


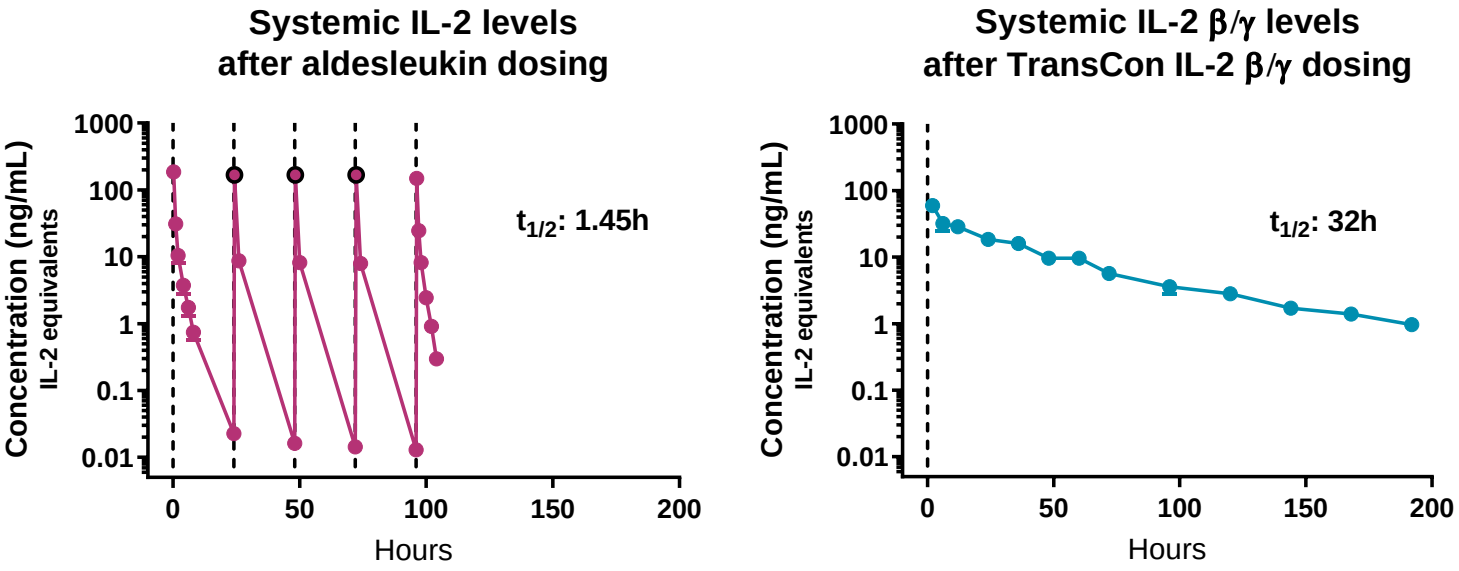
Figure S6: IL-2 β/γ is IL-2Rβ/γ selective in human and cynomolgus monkey cells



Summary of pSTAT5 Potency Values in Human and Cynomolgus Monkey Blood

EC_{50} (ng/mL)	Treg	$CD8^+$ T	NK
Human			
IL-2	0.09	25.69	6.01
IL-2 β/γ	34.50	62.15	11.84
Cynomolgus Monkey			
IL-2	0.09	45.10	10.52
IL-2 β/γ	52.16	86.4	16.26

Figure S7: TransCon IL-2 β/γ provides stable IL-2 β/γ systemic levels compared to pulsatile IL-2 levels seen with aldesleukin treatment.



Open circles on D2, D3, D4 represent data inputed based on the average 15' post dose data from D1 and D5

Dashed lines indicate dosing occasions

Figure S8: TransCon IL-2 β/γ, at ascending doses, induces robust lymphocyte responses with minimal eosinophilia and induction of systemic inflammation markers in cynomolgus monkeys

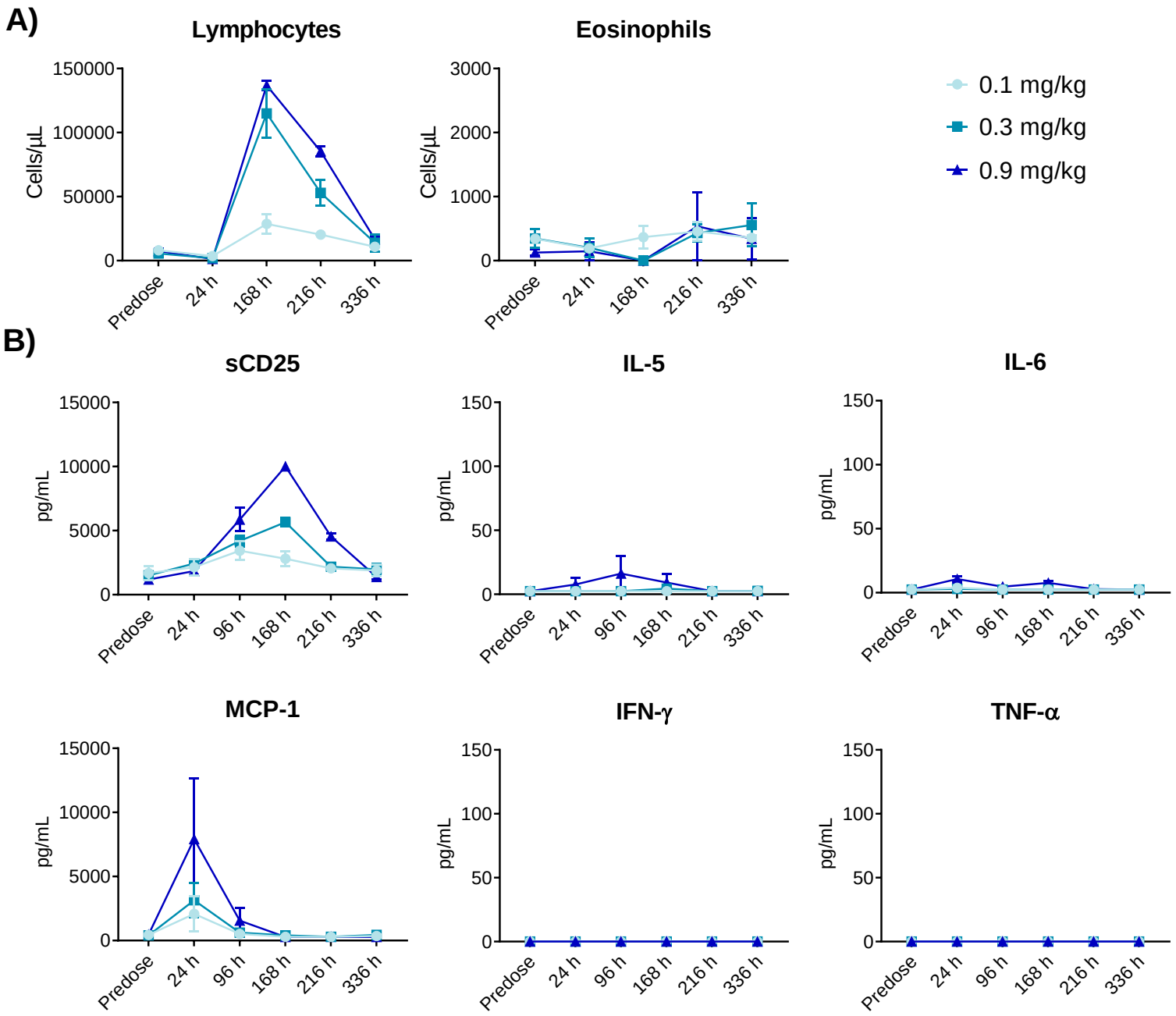
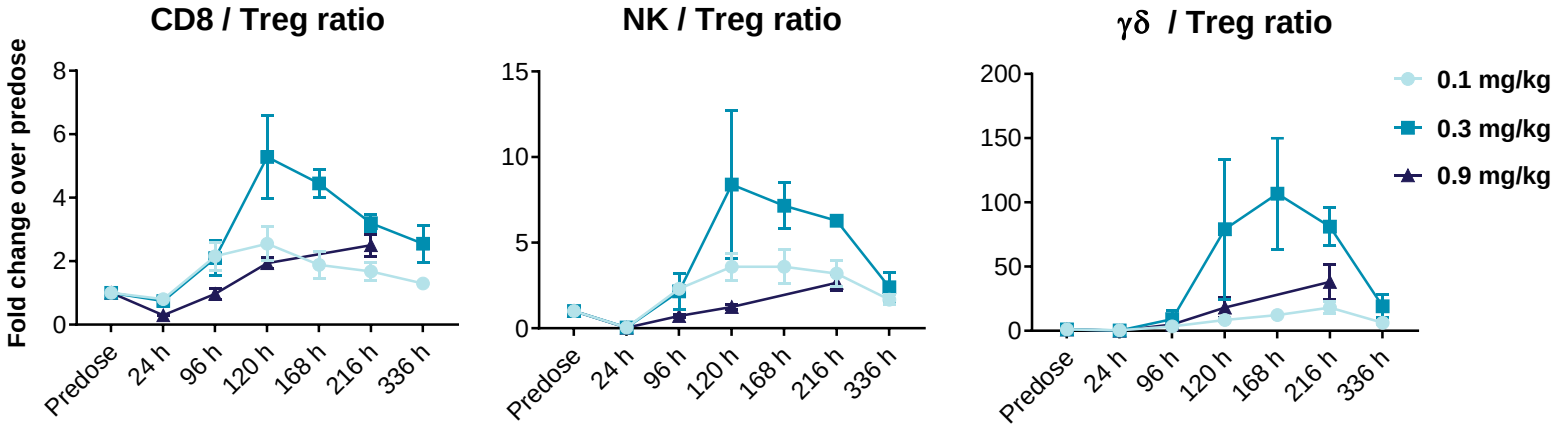


Figure S9: TransCon IL-2 β/γ increases the ratio of CD8⁺ T cells, NK cells and γδ T cells to Tregs or CD4⁺ T cells in cynomolgus monkeys

A)



B)

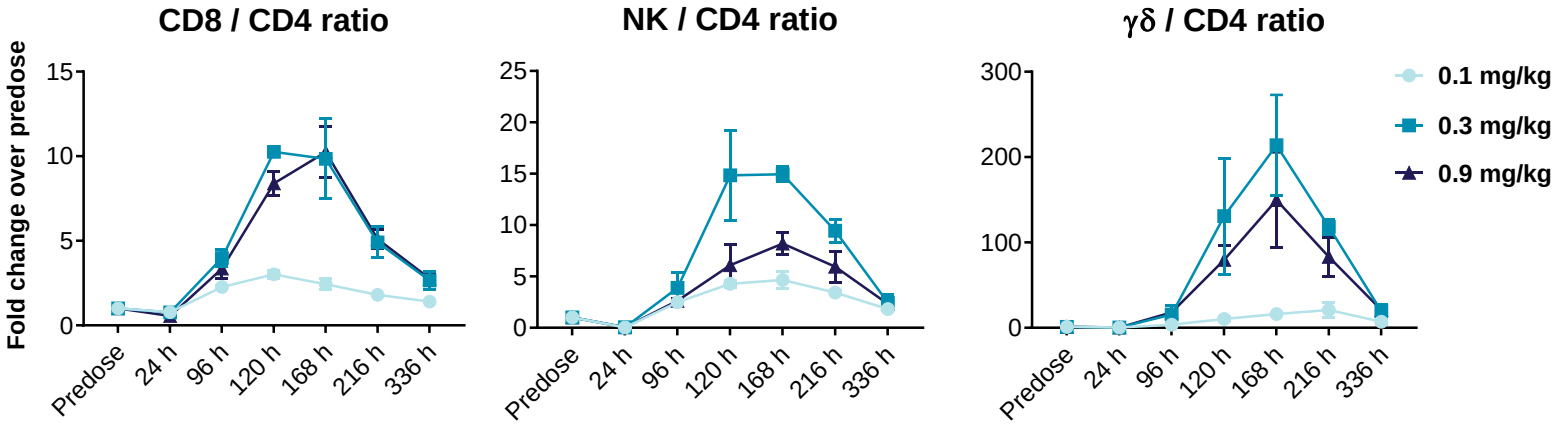


Figure S10: TransCon IL-2 β/γ induces more potent expansion of effector memory CD8+ T cells compared to total CD8 + T cells in cynomolgus monkeys

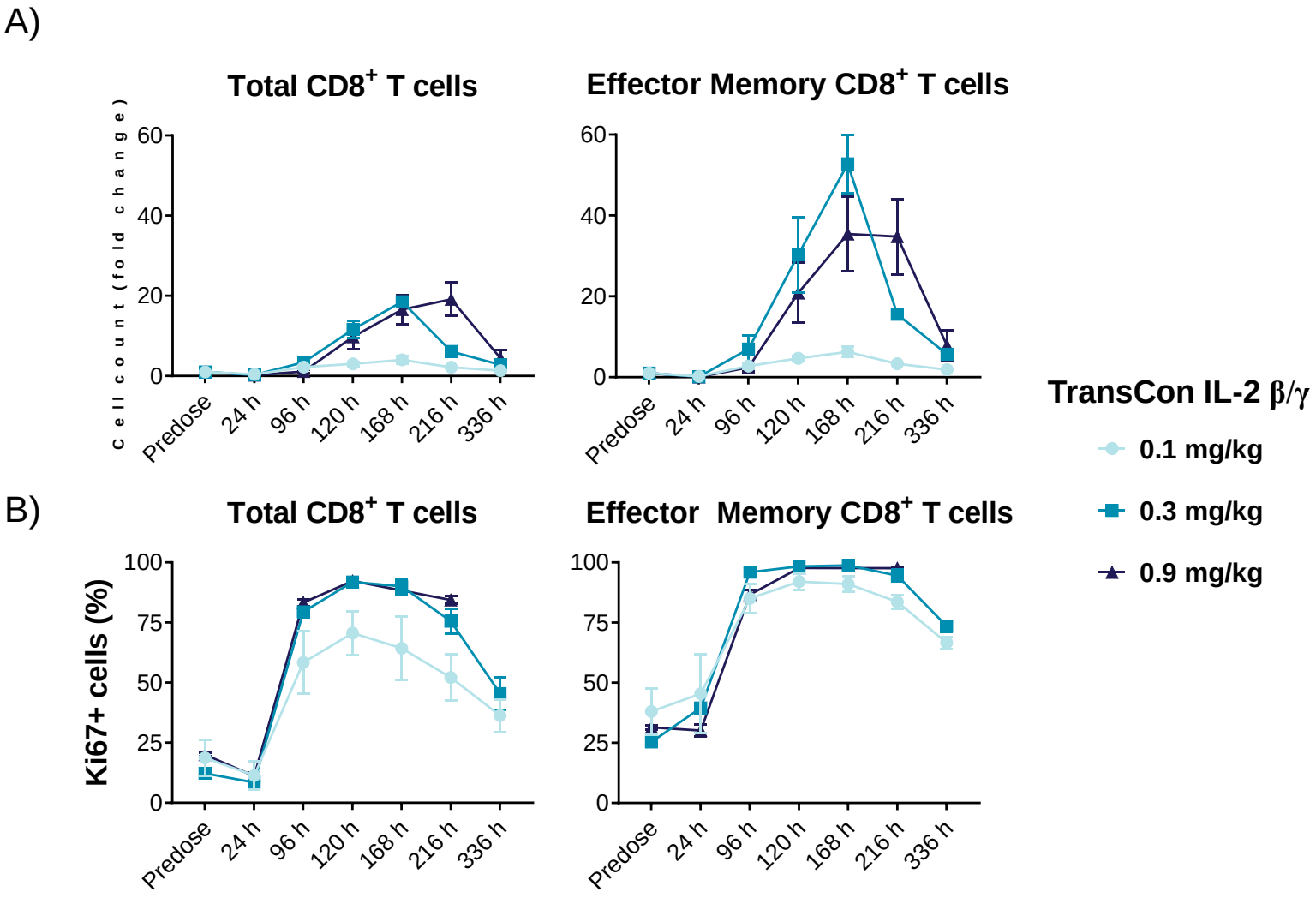


Figure S11: TransCon IL-2 β/γ induces expansion of Granzyme B expressing CD8⁺ T cells, NK cells and, γδ T cells.

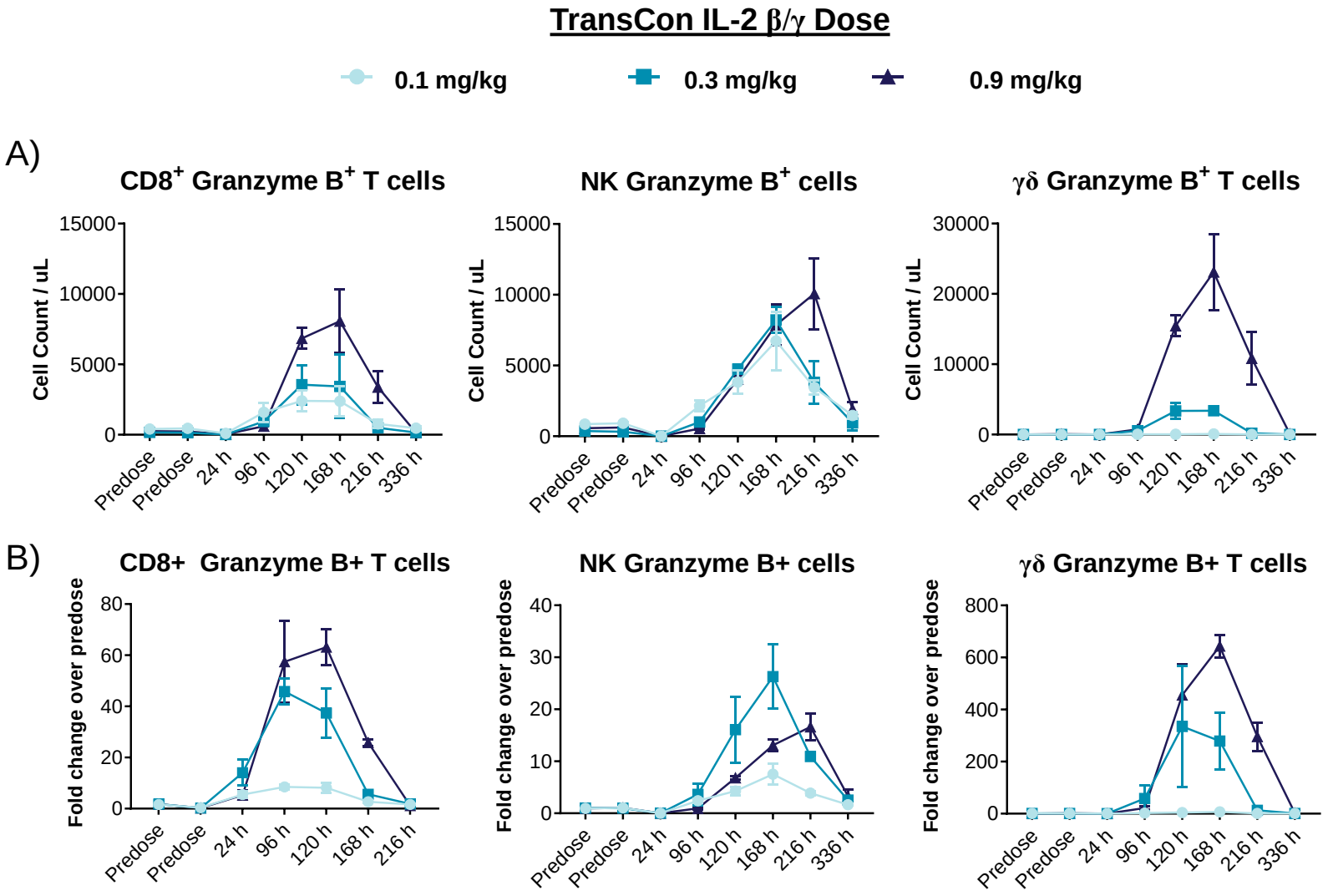


Figure S12A: IL-2 β/γ induces cytokine and cytotoxic effector molecules in human CD8+ T cells

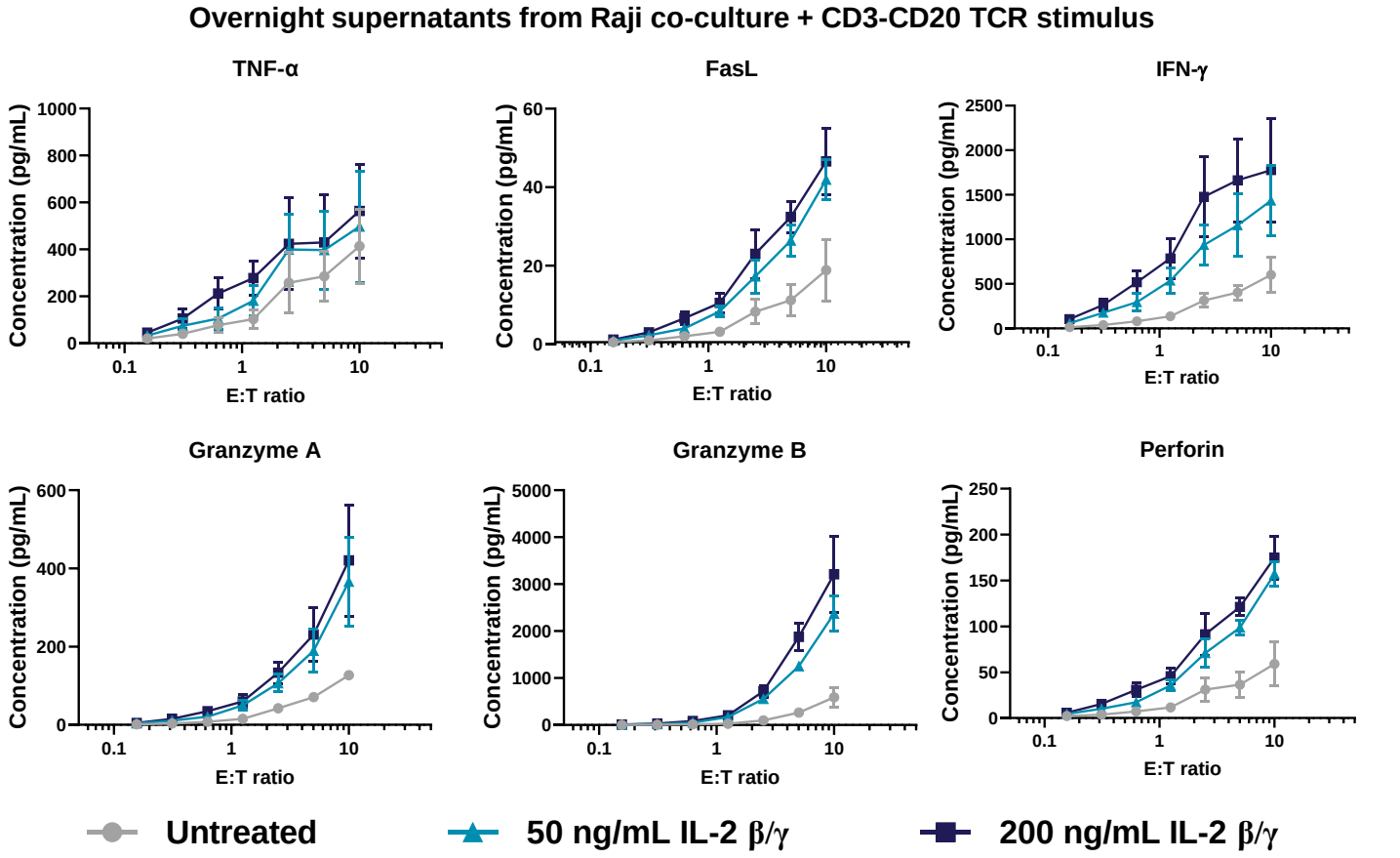


Figure S12B: IL-2 β/γ induces cytokine and cytotoxic effector molecules in human NK cells

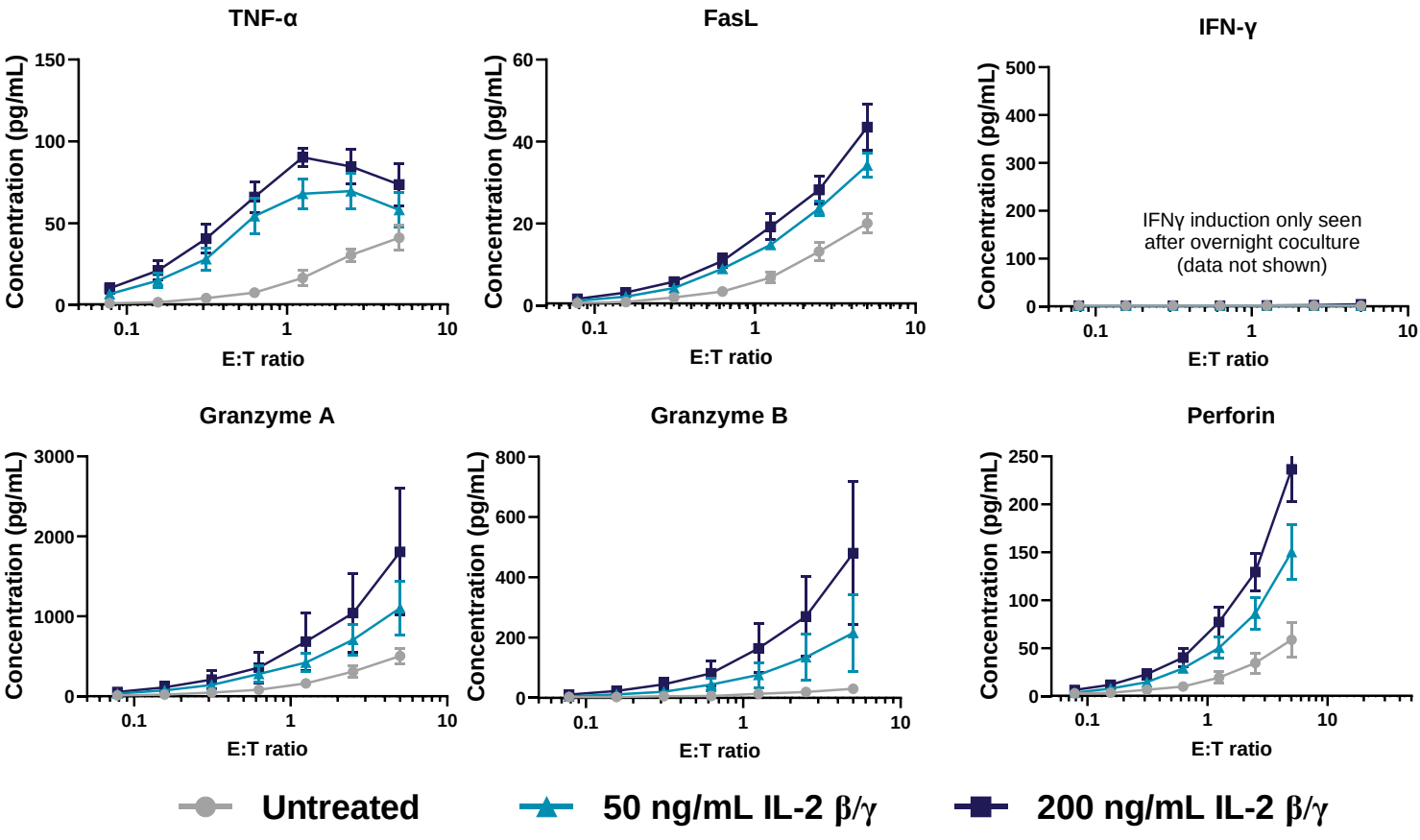


Figure S12C: IL-2 β/γ induces cytokine and cytotoxic effector molecules in human γδ T cells

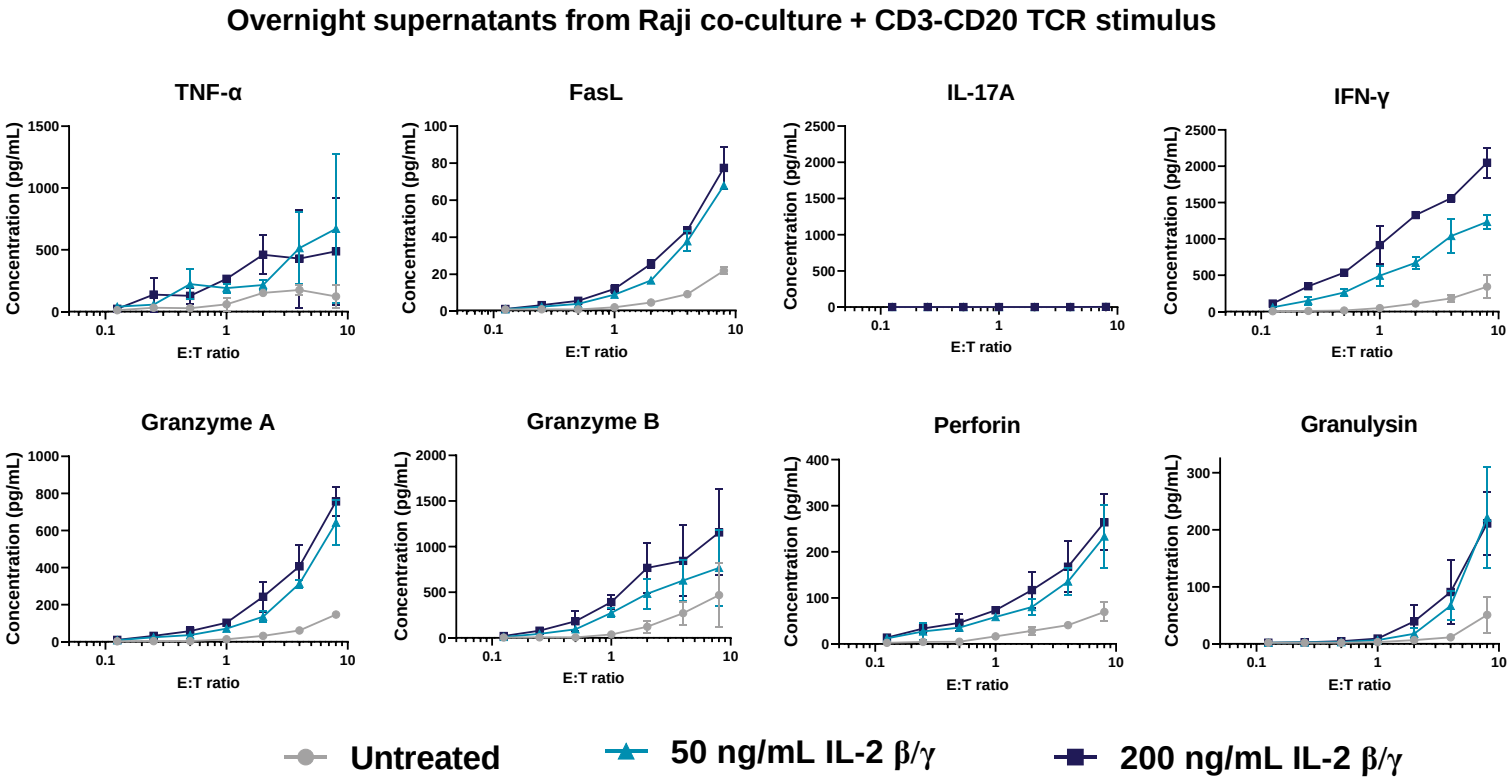
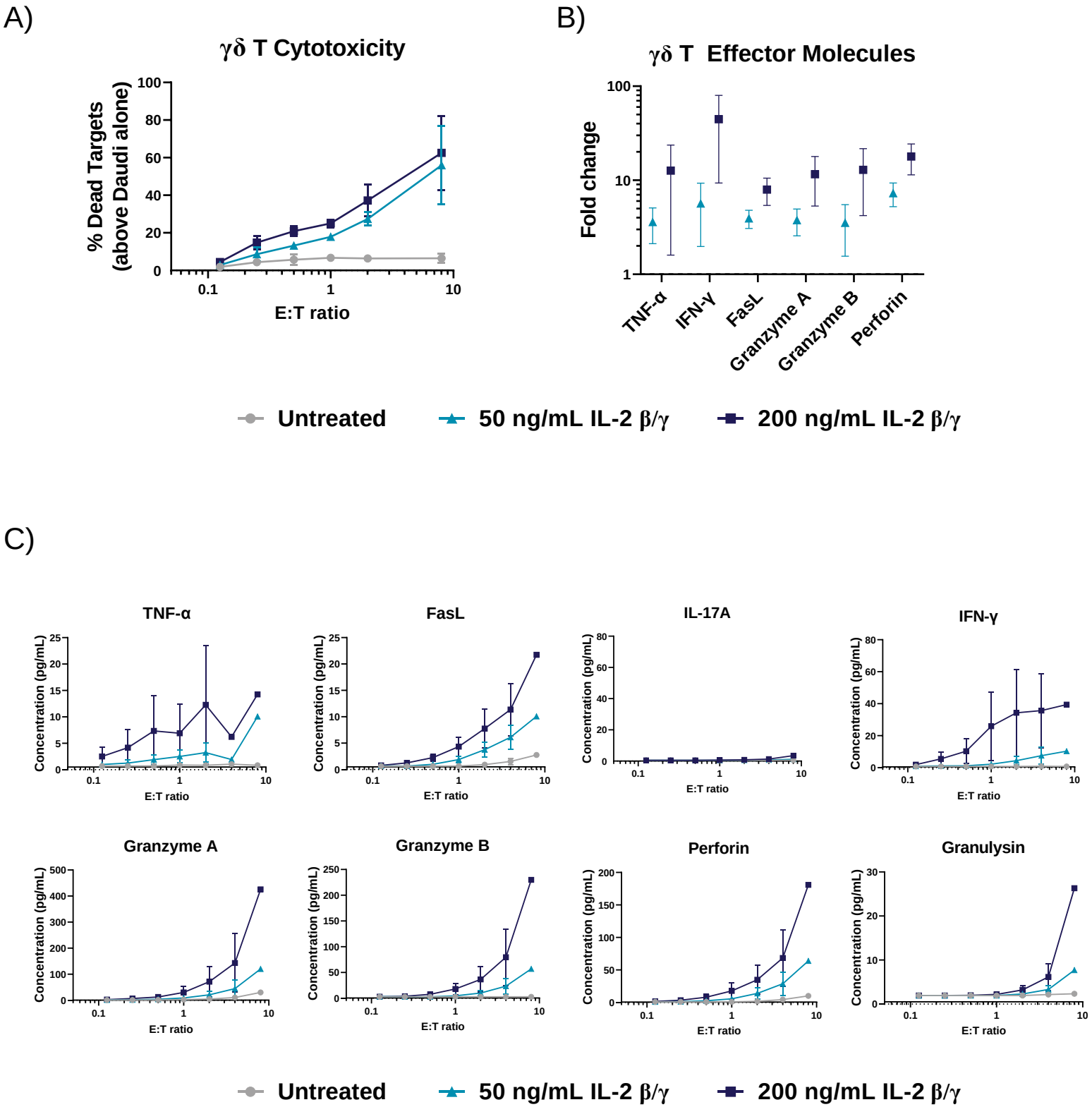


Figure S13 IL-2 β/γ induces cytotoxicity and cytokine production in endogenous TCR ligand activated human γδ T cells (Daudi coculture)



Supplemental Methods (SM)

Construction of IL-2 β/γ and TransCon IL-2 β/γ

IL-2 containing an engineered cysteine within the IL-2Rα binding region and a C125S stabilizing mutation was produced recombinantly in *E. coli* or CHO cells. For *E. coli*, the IL-2 mutein was harvested from inclusion bodies and refolded, then purified by size exclusion and ion-exchange chromatography. For CHO, cell culture clarification was performed by depth filtration and the IL-2 mutein was purified by chromatographic and filtration techniques. After purification of the IL-2 mutein and mild reduction with tris (2-carboxyethyl) phosphine, a 5 kDa mPEG-maleimide was selectively and permanently attached to the engineered cysteine within the IL-2Rα binding region followed by a basic incubation step to give IL-2 β/γ, which was either purified (for *in vitro* studies) or conjugated to a branched 40 kDa mPEG-Linker molecule to create the TransCon IL-2 β/γ prodrug. Subsequently, TransCon IL-2 β/γ was purified by chromatography. Similar methods were applied in earlier studies to generate bioactive IL-2 variants conjugated to mPEGs of varying lengths to determine the optimal mPEG length at the engineered cysteine to abolish IL-2Rα interactions yet maintain IL-2Rβ/γ binding and activity.

pSTAT5 analysis from whole blood

The following antibodies and gating definitions were used for pSTAT5 blood analyses. All subsets were analyzed for median fluorescence intensities (MFI) of pSTAT5 and half maximal effective concentration (EC_{50}) values were calculated for each compound across all cell types.

Table SM 1A: Human blood pSTAT5 analysis flow cytometry reagents

Antibody	Color	Cat. #	Clone	Vendor
CD3	BV786	300472	UCHT1	Biolegend
CD25	Biotin	356124	M-A251	Biolegend
CD8	BUV805	612889	SK1	BD
CD56	BV711	318336	HCD56	Biolegend
CD16	BV711	302044	3G8	Biolegend
CD4	BUV737	612748	SK3	BD
CD15	PECY7	323030	W6D3	Biolegend
Streptavidin	BV421	405225	N/A	Biolegend
STAT5	AX647	562076	47/Stat5 (pY694)	BD
Foxp3	PE	320208	259D	Biolegend

Table SM 1B: Human blood gating definitions for pSTAT5 analysis:

Population	Lymphocyte Gating Definition
NK cells	CD15- CD3- CD56/CD16+
Tregs	CD15- CD3+ CD4+ CD25+ Foxp3+
CD8 T cells	CD15- CD3+ CD8+

Table SM 1C: Cynomolgus monkey blood pSTAT5 flow cytometry reagents

Antibody	Color	Cat. #	Clone	Vendor
CD3	AX700	557917	SP34-2	BD
CD4	BV711	317440	OKT4	Biolegend
CD7	BV510	563650	M-T701	BD
CD8	BUV805	612889	SK1	BD
CD14	BV785	301840	M5E2	Biolegend
CD11b	BV785	301346	ICRF44	Biolegend
CD16	BUV737	612786	3G8	BD
CD20	BV785	302356	2H7	Biolegend
CD25	Biotin	356124	M-A251	Biolegend
Streptavidin	BUV395	564176	N/A	BD
STAT5	AX647	612599	47/Stat5 (pY694)	BD
Foxp3	PE	320208	259D	Biolegend

Table SM 1D: Cynomolgus monkey gating definitions for pSTAT5 analysis:

Population	Lymphocyte Gating Definition
NK cells	CD11b- CD14- CD20- CD3- CD16+ CD7+
Tregs	CD11b- CD14- CD20- CD3+ CD4+ CD25+ Foxp3+
CD8 T cells	CD11b- CD14- CD20- CD3+ CD8+

pSTAT5 analysis from PBMCs

The following antibodies and gating definitions were used for pSTAT5 PBMC analyses. All subsets were analyzed for MFI of pSTAT5 and EC_{50} values were calculated for each compound across all cell types.

Table SM 1E: Human PBMC pSTAT5 flow cytometry reagents

Antibody	Color	Cat. #	Clone	Vendor
Foxp3	AF488	320112	206D	Biolegend
CD45RA	PE	304108	HI100	Biolegend
STAT5	AX647	562076	47/Stat5 (pY694)	BD
CD8	AX700	301028	RPA-T8	Biolegend
CD4	BV421	300532	RPA-T4	Biolegend
CD16	BV570	302036	3G8	Biolegend
CD3	BV605	300460	UCHT1	Biolegend
CD56	BV711	362542	5.1H11	Biolegend
CD25	BV785	356140	M-A251	Biolegend

Table SM 1F: Human PBMC gating definitions for pSTAT5 analysis:

Population	Lymphocyte Gating Definition
NK cells	CD3- CD56+
Tregs	CD3+ CD4+ CD25+ Foxp3+
CD8 T cells	CD3+ CD8+

All subsets were analyzed for MFI of pSTAT5 and EC_{50} values were calculated for each compound across all cell types.

Additional ARRIVE (Animal Research: Reporting of In Vivo Experiments) details for mouse experiments

Mouse tumor experiments were performed inoculating approximately double the number of mice to be enrolled in the study (as indicated in figure legends) in order to meet pre-dose target tumor volume average and range. Mice were randomized and enrolled into the study using the Matched Distribution method for randomization in the StudyLog™ software. 8-10 animals per treatment condition were used to assess efficacy in order to overcome the expected variability in syngeneic mouse tumor studies while still minimizing the number of animals used. To minimize potential confounding effects, treatments with similar agents were performed together, measurements for tumor volumes and body weights were performed in a consistent order (by treatment group), and animals were all housed in the same room. The studies were not run in a blinded fashion. CT26 experiments used naïve healthy female BALB/c mice sourced by Crown Bioscience, China or purchased from Taconic Biosciences (USA). MC38 experiments used naïve healthy female C57BL/6 mice purchased from The Jackson Laboratory (Jax, USA). Mouse work was performed at Ascendis Inc, Redwood City CA (California Department of Public Health Certificate 081) or at Crown Bioscience, Taicang City, China (Animal use permit number: AN-1903-05-1067). Mouse work at Ascendis Pharma was reviewed and approved by the Ascendis Inc. Redwood City Institutional Animal Care and Use Committee (IACUC) (Protocol 20200507-ASN-02 and Study Reference VIV-M-032) and performed in accordance with the US Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals and the USDA Animal Welfare Act. Mouse work at Crown Bioscience was approved by the Crown Bioscience IACUC (Study Reference E4649-U1920, approval reference AN-1903-05-1067) and performed in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Flow cytometry analysis in CT26 mouse experiments

In the first experiment, blood was stimulated in vitro with Leukocyte Activation Cocktail, with BD GolgiPlug™ (BD Biosciences) for 5 hours at 37°C before FACS staining and acquisition on a BD Fortessa flow cytometer. Cells were preincubated with Fc-Block prior to surface staining then underwent RBC lysis (Bio-gems). Cells were then fixed and permeabilized (eBiosciences, Foxp3 kit) and stained with intracellular antibodies. In some experiments, cells were acquired in the presence of 123count Ebeads (eBiosciences), and in others, cells were acquired in a fixed volume to enable cell count calculations. In the 96 h post-dose data from this experiment there was one data point from the control group and one data point from the TransCon IL-2 β/γ group which were deemed to be technical outliers and excluded based on the questionable quality of the flow cytometry data observed.

In the second experiment, blood and spleen were harvested. Blood was lysed with 1X RBC lysis buffer (Bio-gems) and single cell suspensions for spleen were prepared by established methods. Cells were stained for surface antigens together with Fc-Block (purified anti-mouse CD16/CD32, eBioscience), then fixed and permeabilized (Foxp3 / Transcription Factor Staining Buffer Set, eBioscience) and stained with intracellular antibodies. Cells were acquired on an Agilent NovoCyte Quanteon flow cytometer, using a fixed volume to enable cell count calculations. The following antibodies and gating definitions were applied:

Table SM 2A: Flow cytometry reagents for analysis of CT26 bearing mice (first experiment)

Markers	Fluorochrome	Cat #	Clone	Vendor
CD45	BV711	103147	30-F11	Biolegend
CD3	BUV395	740268	17A2	BD Bioscience
CD4	BUV737	564298	GK1.5	BD Bioscience
CD8	FITC	100706	53-6.7	eBioscience
CD25	BV510	102041	PC61	Biolegend
CD335	BV605	137619	29A1.4	Biolegend
CD44	BV421	103040	IM7	Biolegend
Ly6C	BV785	128041	HK1.4	Biolegend
CTLA4	PE	106306	UC10-4B9	Biolegend
Foxp3	PerCP-Cy 5.5	45-5773-82	FJK-16S	eBioscience
TNF-α	APC	506308	MP6-XT22	Biolegend
IFN-γ	PE-Cy7	25-7311-41	XMG1.2	eBioscience
GranzymeB	PE-eFluor 610	61-8898-82	NGZB	eBioscience
Live / Dead	eFluor 780	65-0865-18	NA	eBioscience

Table SM 2B: Populations analyzed used in CT26 tumor bearing mice (first experiment)

Population	Lymphocyte Gating Definition
NK cells	CD45+ CD3- NKp46+
Tregs	CD45+ CD3+ CD4+ CD25+ Foxp3+
CD4 T cells	CD45+ CD3+ CD4+
CD8 T cells	CD45+ CD3+ CD8+

Table SM 2C: Flow cytometry reagents for analysis of CT26 bearing mice (second experiment)

Markers	Fluorochrome	Cat #	Clone	Vendor
Ki67	AF488	558616	B56	BD
CD3e	PerCP-eF710	46-0033-82	eBio500A2	eBioscience
CD25	APC	17-0251-82	PC61.5	eBioscience
CD8	AX700	56-0081-82	53-6.7	eBioscience
CD45	APC-eF780	47-0451-82	30-F11	eBioscience
Foxp3	PE	12-5773-82	FJK-16s	eBioscience
NKp46	PE-eF610	61-3351-82	29A1.4	eBioscience
CD19	PE-Cy5	115510	6D5	Biolegend
CD62L	PE-Cy7	25-0621-82	MEL-14	eBioscience
Granzyme B	Pacific Blue	515408	GB11	Biolegend
CD11b	BV570	101233	M1/70	Biolegend
ICOS	BV605	313538	C398.4A	Biolegend
CD4	BV650	100469	GK1.5	Biolegend
PD-1	BV711	748265	RMP1-30	BD
CD44	BV785	103059	IM7	Biolegend
Live/Dead	eFluor 506	65-0866-14	NA	eBioscience

Table SM 2D: Populations analyzed used in CT26 tumor bearing mice (second experiment)

Population	Lymphocyte Gating Definition
NK cells	CD45+ CD19- CD3- NKp46+
Tregs	CD45+ CD19- CD11b- CD3+ CD4+ CD25+ Foxp3+
CD4 T cells	CD45+ CD19- CD11b- CD3+ CD4+
CD8 T cells	CD45+ CD19- CD11b- CD3+ CD8+

ARRIVE (Animal Research: Reporting of In Vivo Experiments) details for cynomolgus monkeys

The studies were performed in Mauritian cynomolgus monkeys. Due to the small number of animals, no blinding or randomization was performed. Two to four animals per group was regarded

as sufficient to assess toxicity and/or PK/PD effects, while minimizing animal usage, see details for each study below. No animals were excluded from analysis.

The first studies testing aldesleukin (Study Reference 8713-1903) and TransCon IL-2 β/γ (Study Reference 8713-1904) were performed at the University of Louisiana at Lafayette New Iberia Research Center (NIRC), Lafayette, USA (USDA Animal Welfare Act Certificate Number: 72-R-0007). Four monkeys of ages 6.9-7.8 years old and body weights 7.7-10.6 kg and 4 monkeys of ages from 10.0-15.6 and body weights from 7.6–8.3 kg, respectively, at time of dosing were selected for the studies. Animals were tested for enteric parasites and bacterial pathogens during pre-study physical exams. Monkeys were acclimatized to their designated housing for at least 3 days prior to the dosing and were chair trained prior to study initiation. Animals were observed twice daily throughout the study for changes in general appearance and behavior and daily for signs of adverse effects. The animals were single housed for the duration of the study. In order to reduce confounding effects, animals were dosed in a consistent order (for aldesleukin daily dosing) and blood samples were collected at specified timepoints relative to the timing of dose administration for each animal. These studies were reviewed and approved by university of Louisiana Lafayette's Animal Care and Use Committee (IACUC approval number 2019-8713-016) prior to the study. During the study, the care and use of animals were conducted in accordance with the regulations of the USDA Animal Welfare Act (i.e., relevant sections of Section 9, Parts 1, 2 and 3 of the Code of Federal Regulations).

The second monkey study testing different dose levels of TransCon IL-2 β/γ was performed at Labcorp Early Development Laboratories Ltd Harrogate, UK (Project License Number: P98E2EBFD). Six monkeys were allocated from the stock colony in order to provide 3 healthy animals of each sex. At the start of dosing, animals were 111 to 130 weeks old and weighed

between 2.46 and 3.69 kg. All animals were tested for tuberculosis while at the premises of the breeder. Upon arrival, all animals were given a clinical inspection for ill health and tested for tuberculosis. Animals were acclimated to the facility for 13 to 20 weeks and to the study room for 2 to 4 weeks. A veterinary inspection was performed before the start of dosing to ensure suitability for the study. Animals were housed in pens that conform to the Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes (Home Office, 2014). Animals of the same sex were housed in the same pen. Animals were observed at least twice daily. Medical treatment necessary to minimize suffering and distress, including euthanasia, was the responsibility of the attending licensed animal technicians and/or Veterinary Surgeons. This study was approved by the Animal Welfare Ethical Review Body (AWERB) for Labcorp Harrogate (Study Reference: 8448706) and was performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

Flow cytometry and cytokine analysis in cynomolgus monkeys

Peripheral blood taken from study animals was lysed (PharmLyse) and stained for surface markers (CD3, CD4, CD8, CD159, CD14, CD25, CD28, and γδ TCR) before fixation and permeabilization (eBiosciences Foxp3 kit) and staining for intracellular markers (Ki67, Granzyme B and Foxp3). Cells were also stained with counting beads (Invitrogen) for assessment of absolute counts.

No prespecified quality metrics were defined for exclusion or flow cytometry data however post data acquisition analysis revealed some likely technical outliers due to failed intracellular staining processes which were excluded from analysis by the contract service laboratory performing the experiment. Specifically, the FoxP3 and Ki67 results at 168 and 336 hours postdose for animals receiving 0.9 mg/kg were excluded from analysis due to a possible error in the intracellular staining of these two markers.

Table SM 3A: Reagents used in Cynomolgus Monkey flow cytometry analysis

Marker / Reagent	Color	Cat.	Clone	Vendor
CD3	BV605	562994	SP34-2	BD
CD4	BV711	563913	L200	BD
CD8	AF700	344724	SK1	Biolegend
CD14-BUV737	BD	612763	M5E2	Bioscience
CD25	BUV395	564034	2A3	BD
CD28	PE-Cy5	555730	CD28.2	BD
CD45	BV786	563861	D058-1283	BD
CD95	BV421	562616	DX2	BD
CD159a (NKG2A)	PC7	B10246	Z199	Beckman Coulter
FoxpP3	PE	560046	259D/C7	BD
Granzyme B	AF647	560212	GB11	BD
KI67	FITC	556026	B56	BD
TCR γ/δ	APC/Fire™ 750	331228	B1	Biolegend
mouse-IgG1	PE	555749	MOPC-21	BD
TCR Vδ2	BV421	331428	B6	Biolegend
TCR Vγ9	PE-Cy7	331320	B3	Biolegend

Table SM 3B: Populations analyzed in Cynomolgus monkey flow cytometry data

Population	Gating Definition
Lymphocytes	CD14-CD45+
T cells	CD14-CD45+CD3+
CD4+ T cells	CD14-CD45+CD3+CD4+
Tregs	CD14-CD45+CD3+CD4+CD25+Foxp3+
CD8+ T cells	CD14-CD45+CD3+CD8+
γδ T cells	CD14-CD45+CD3+CD4-CD8-γδTCR+
NK cells	CD14-CD45+CD3-NKG2A+
Naïve CD4+ T cells	CD14-CD45+CD3+CD4+CD28+CD95-
Naïve CD8+ T cells	CD14-CD45+CD3+CD8+CD28+CD95-
Central memory CD4+ T cells	CD14-CD45+CD3+CD4+CD28+CD95+
Central memory CD8+ T cells	CD14-CD45+CD3+CD8+CD28+CD95+
Effector memory CD4+ T cells	CD14-CD45+CD3+CD4+CD28-CD95+
Effector memory CD8+ T cells	CD14-CD45+CD3+CD8+CD28-CD95+

Serum levels of IL-5, IL-6, IFN-γ and MCP-1 were measured using an ELISA/Multiplex methods using the Luminex 100 platform and BioPlex 200 software. Serum TNF-α was detected using the MSD U-Plex non-human primate TNF-α Kit and an MSD Sector Imager 600. Detection of serum

sCD25 was performed using the R&D Systems Quantikine Human CD25/IL-2Rα ELISA Kit and a SpectraMax i3x.

Anatomical Pathology

An anatomical pathology examination was performed by a board-certified pathologist at Labcorp. Approximately 50 organs and tissues were examined, including lung, kidney, liver, spleen, brain, heart, various lymph nodes and lymphoid tissues, nerves, reproductive organs and organs of the digestive system. Organ weights and macroscopic observations were recorded, and histopathology was performed to assess microscopic changes. In the absence of a control group, all findings were compared to historical control data from this laboratory.

In vitro human lymphocyte cytotoxicity and cytokine assays

Human PBMCs were purified from Leukoreduction chambers by Ficoll density centrifugation (GE Healthcare) and used to purify NK cell (average 93% purity), $CD8^+$ T cell (average 87% purity overall with 92% purity within T cells), or γδ T cell (average 78% purity overall with 98% purity within T cells) populations using negative selection kits (EasySep™ Human NK Cell Isolation Kit, EasySep™ Human $CD8^+$ T Cell Isolation Kit, and EasySep™ Human Gamma/Delta T Cell Isolation Kit from StemCell Technologies; and TCRγ/δ^+ T Cell Isolation Kit, human, from Miltenyi Biotec).

NK cell cytotoxicity assays used K562 erythroleukemia target cells. Briefly, 5,000-10,000 labeled (BATDA loaded) K562 cells were incubated with NK cells at Effector to Target (E:T) ratios from 5 to 0.78 for 4 h at 37°C, together with 1.25 mM probenecid to promote BATDA retention. Maximum release was determined by lysing target cells using 0.1% Triton X-100. Specific lysis

was calculated as (experimental release – spontaneous release) / (maximum release - spontaneous release).

$CD8^+$ T cytotoxicity assays used Raji target cells (ATCC) in the presence of 1 nM of a CD3-CD20 bispecific antibody (Creative BioLabs) in a flow cytometry assay. Briefly, 10,000 Raji cells, marked with 50 nM CellTrace Far Red (CTFR, Invitrogen), were co-incubated with various concentrations of $CD8^+$ T cells at E:T ratios from 10 to 0.16 overnight at 37°C. All cells were stained with Fixable Viability Dye eFluor 506 (Invitrogen) and analyzed by flow cytometry. The percentage of dead target cells was determined within $CTFR^+$ Raji cells.

Cytotoxicity of γδ T cells was evaluated against Raji target cells as described above and also evaluated using CTFR labeled Daudi target cells (ATCC), both at E:T ratios ranging from 8 to 0.125. The percentage of dead target cells was determined within $CTFR^+$ Raji or Daudi cells. For Daudi assays, due to some donors showing high spontaneous death, the background from conditions with Daudi alone were subtracted to visualize IL-2 β/γ induced cytotoxicity.

Additionally, supernatants from the above cytotoxicity conditions were analyzed for TNF-α, IFN-γ, IL-17A FasL, Granzyme A, Granzyme B, Granulysin and Perforin concentrations via LegendPlex (Biolegend) detection reagents using a Quanteon flow cytometer.

Supplemental Results

Anatomical Pathology

For the lungs, there were no organ weight changes, no macroscopic findings and no microscopic findings in any of the animals, indicative of no pulmonary edema. Further, histopathological examination did not reveal vascular damage or tissue necrosis in any of the tissues examined in any of the animals, supporting that TransCon IL-2 β/γ does not induce VLS.