

TransCon IL-2 β/γ : a novel long-acting prodrug with sustained release of an IL-2R β/γ -selective IL-2 variant with improved pharmacokinetics and potent activation of cytotoxic immune cells for the treatment of cancer

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ABSTRACT

Background Recombinant interleukin-2 (IL-2, aldesleukin) is an approved cancer immunotherapy but causes severe toxicities including cytokine storm and vascular leak syndrome (VLS). IL-2 promotes antitumor function of IL-2R β/γ ⁺ natural killer (NK) cells and CD8⁺, CD4⁺ and gamma delta ($\gamma\delta$) T cells. However, IL-2 also potently activates immunosuppressive IL-2R α ⁺ regulatory T cells (Tregs) and IL-2R α ⁺ eosinophils and endothelial cells, which may promote VLS. Aldesleukin is rapidly cleared requiring frequent dosing, resulting in high C_{max} likely potentiating toxicity. Thus, IL-2 cancer immunotherapy has two critical drawbacks: potent activation of undesired IL-2R α ⁺ cells and suboptimal pharmacokinetics with high C_{max} and short half-life.

Methods TransCon IL-2 β/γ was designed to optimally address these drawbacks. To abolish IL-2R α binding yet retain strong IL-2R β/γ activity, IL-2 β/γ was created by permanently attaching a small methoxy polyethylene glycol (mPEG) moiety in the IL-2R α binding site. To improve pharmacokinetics, IL-2 β/γ was transiently attached to a 40 kDa mPEG carrier via a TransCon (transient conjugation) linker creating a prodrug, TransCon IL-2 β/γ , with sustained release of IL-2 β/γ . IL-2 β/γ was characterized in binding and primary cell assays while TransCon IL-2 β/γ was studied in tumor-bearing mice and cynomolgus monkeys.

Results IL-2 β/γ demonstrated selective and potent human IL-2R β/γ binding and activation without IL-2R α interactions. TransCon IL-2 β/γ showed slow-release pharmacokinetics with a low C_{max} and a long (>30 hours) effective half-life for IL-2 β/γ in monkeys. In mouse

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Half-life extended IL-2R β/γ -selective interleukin-2 (IL-2) variants have shown promise for reducing IL-2R α -mediated toxicities, but may be limited by high C_{max} -mediated effects of fully bioactive agents. Furthermore, permanent fusion to large half-life extension domains could affect IL-2 potency and/or ability for deep tumor penetration.

WHAT THIS STUDY ADDS

⇒ This study utilizes a novel prodrug technology that provides sustained release of an IL-2R β/γ -selective IL-2 variant (IL-2 β/γ) containing a small polymer to block IL-2R α binding. Importantly, IL-2 β/γ resembles endogenous IL-2 in size, sequence and IL-2R β/γ potency.

⇒ On administration, the TransCon IL-2 β/γ prodrug ensures sustained release of IL-2 β/γ levels in a bioactive range for an extended period of time and with a low C_{max} . This prolonged exposure of IL-2 β/γ induces durable and remarkable expansions of multiple cytotoxic immune cell types, to higher magnitudes than seen with other approaches, with minimal increases in IL-2R α ⁺ eosinophils and with no signs of vascular leak syndrome or cytokine release syndrome in cynomolgus monkeys.

tumor models, TransCon IL-2 β/γ promoted CD8⁺ T cell and NK cell activation and antitumor activity. In monkeys, TransCon IL-2 β/γ induced robust activation and expansion of CD8⁺ T cells, NK cells and $\gamma\delta$ T cells, relative to CD4⁺

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This work demonstrates that TransCon IL-2 β/γ has the potential to substantially improve the therapeutic index beyond what has been achieved by aldesleukin and could bode well for combination therapies with complementary mechanisms.

T cells, Tregs and eosinophils, with no evidence of cytokine storm or VLS. Similarly, IL-2 β/γ enhanced proliferation and cytotoxicity of primary human CD8⁺ T cells, NK cells and $\gamma\delta$ T cells.

Summary TransCon IL-2 β/γ is a novel long-acting prodrug with sustained release of an IL-2R β/γ -selective IL-2. It has remarkable and durable pharmacodynamic effects in monkeys and potential for improved clinical efficacy and tolerability compared with aldesleukin. TransCon IL-2 β/γ is currently being evaluated in a Phase 1/2 clinical trial (NCT05081609).

BACKGROUND

Recombinant interleukin-2 (IL-2, aldesleukin) therapy is approved for treatment of metastatic melanoma and renal cell carcinoma and elicits durable clinical responses in a subset of patients.¹ However, major toxicities with life-threatening complications, including vascular leak syndrome (VLS), eosinophilic infiltration of cardiac and pulmonary tissues and cytokine release syndrome (CRS), have limited its scope of treatment.² VLS is characterized by vascular damage, increased vascular permeability, leakage of fluids, proteins and electrolytes into interstitial spaces, tissue edema, hypotension and ultimately multi-organ failure that can be fatal.^{3–5} IL-2 activates not only cells with antitumor function, including natural killer (NK) cells, gamma delta ($\gamma\delta$) T cells and CD4⁺ and CD8⁺ effector T cells, but also IL-2R α ⁺ immunosuppressive regulatory T cells (Tregs) that likely limit efficacy.^{1,6} IL-2 can signal with picomolar affinity to trimeric IL-2 receptor IL-2R $\alpha/\beta/\gamma$ or with nanomolar affinity through dimeric IL-2R β/γ .¹ T cells and NK cells can upregulate IL-2R α on activation, while Tregs constitutively express high IL-2R α levels.⁷ Therefore, IL-2 potently activates Tregs compared with IL-2R β/γ ⁺ CD8⁺ T cells, $\gamma\delta$ T cells and NK cells that possess antitumor function. Also, IL-2 potently activates IL-2R α ⁺ eosinophils, type 2 innate lymphoid cells and endothelial cells, which may promote VLS and limit the therapeutic window.^{8–11} In addition to IL-2R α engagement, another major drawback of aldesleukin therapy for cancer is its suboptimal pharmacokinetic (PK) properties with a short (85 min) elimination half-life requiring frequent, high dose administrations resulting in high C_{max} values that likely potentiate toxicity.¹²

While several IL-2R β/γ -biased IL-2 molecules designed to more preferentially activate NK cells and CD8⁺ T cells have been described, most approaches still have limitations such as incomplete IL-2R β/γ selectivity,^{13–14} reduced IL-2R β/γ potency¹⁵ or high C_{max}-mediated effects from delivery of fully bioactive agents.^{15–21} Here we describe the creation of TransCon IL-2 β/γ , a novel long-acting prodrug with sustained release of a novel receptor-selective IL-2 variant

(IL-2 β/γ). Thus, TransCon IL-2 β/γ was designed using two independent approaches to optimally address the limitations of IL-2 in cancer immunotherapy, namely avoiding activation of undesired IL-2R α ⁺ cell types and poor PK properties (high C_{max} and short half-life), by achieving controlled and prolonged exposure of IL-2 β/γ to safely and robustly activate lymphocytes with antitumor function including CD8⁺ T cells, $\gamma\delta$ T cells and NK cells.

Here, we describe the generation and preclinical characterization of IL-2 β/γ and the TransCon IL-2 β/γ prodrug. Active IL-2 β/γ was tested in vitro for IL-2R binding, IL-2 pathway signaling and in functional assays to assess proliferation and cytotoxic activity using primary human cells. TransCon IL-2 β/γ was evaluated in vivo in tumor-bearing mice alone and in combination with TransCon TLR7/8 Agonist, an investigational prodrug for sustained intratumoral (IT) release of the toll-like receptor (TLR)7/8 agonist resiquimod,²² currently in Phase 1/2 testing (NCT04799054). Lastly, the safety and pharmacodynamic (PD) profile of TransCon IL-2 β/γ was assessed in cynomolgus monkeys.

METHODS

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

IL-2 containing an engineered cysteine within the IL-2R α binding region and a C125S stabilizing mutation was expressed and purified from *Escherichia coli* or Chinese hamster ovary cells. Permanently PEGylated IL-2 variants (including IL-2 β/γ which contains a 5 kDa methoxy polyethylene glycol (mPEG) group) were constructed by selective and permanent conjugation of mPEG-maleimides of varying size to the engineered cysteine. IL-2 β/γ was further conjugated to a branched 40 kDa mPEG-linker molecule to create TransCon IL-2 β/γ (see online supplemental methods).

Characterization of binding to human IL-2R α or IL-2R β

Biacore analysis was performed by injecting recombinant human IL-2 (hereafter referred to as control IL-2), or permanently mPEG-conjugated IL-2 variants onto human IL-2R α -Fc (Symansis) or human IL-2R β -Fc (Symansis) coated sensor chips via an immobilized mouse anti-human IgG1-Fc antibody (Cytiva Life Sciences). Sensorgram data were double reference subtracted prior to affinity analyses.

Characterization of pSTAT5 induction in primary blood cell subsets

Cynomolgus monkey or human whole blood or human peripheral blood mononuclear cells (PBMCs), from at least two donors each, were incubated with IL-2 variants for 30 min at 37°C and then fixed (using Lyse/Fix for whole blood and 1.6% paraformaldehyde for PBMCs). Samples were analyzed by flow cytometry for signal transducer and activator of transcription 5 phosphorylation (pSTAT5) in IL-2R $\alpha/\beta/\gamma$ ⁺ Tregs and IL-2R β/γ ⁺ CD8⁺ T cells and NK cells using methanol permeabilization

techniques. Detailed descriptions can be found in the online supplemental methods table SM 1A-F.

In vivo mouse tumor models

Female BALB/c mice were inoculated subcutaneously (SC) in the right rear flank with CT26 colon carcinoma cells (American Type Culture Collection (ATCC)). Mice with established (average 80–85 mm³) tumors were untreated or treated with buffer control or TransCon IL-2 β/γ intravenously (IV) alone or in combination with IT TransCon TLR7/8 Agonist, a prodrug designed for sustained release of a TLR7/8 agonist, resiquimod^{22–24} as indicated in figure legends. All TransCon IL-2 β/γ dose levels are expressed in IL-2 equivalents, and all dose levels of TransCon TLR7/8 Agonist correspond to the amount of resiquimod. Additionally, mice were bled 96 hours after TransCon IL-2 β/γ dosing for flow cytometry analysis (see online supplemental methods). In some experiments, mice displaying complete responses (CR) were rechallenged with CT26 cells in the left rear flank and mice capable of rejecting a CT26 challenge were challenged again with 5 × 10⁵ EMT-6 tumor cells (ATCC) in the right front flank.

In the MC38 model, female C57BL/6 mice were inoculated SC with 2 × 10⁶ MC38 colon carcinoma cells (ATCC). Mice with established tumors (average 95 mm³) were untreated or treated with TransCon IL-2 β/γ IV and assessed for tumor growth as indicated in figure legends.

Tumor volumes were measured biweekly using a caliper and calculated as: $V = (\text{length} \times \text{width}^2) / 2$.²⁵ For graphs and analyses of mean tumor volumes, the last measured tumor volume data from mice taken off study (eg, reaching tumor volume limits of 3000 mm³) are included in subsequent time points after euthanasia while raw data are displayed as spider plots of individual animal responses.

Pharmacodynamic and pharmacokinetic analyses in cynomolgus monkeys

In a first study, male cynomolgus monkeys (n=4/group) received either daily aldesleukin (0.4 mg/day on Days 1–5; average 0.044 mg/kg/day) or a single 1.0 mg TransCon IL-2 β/γ dose (average 0.122 mg/kg) IV. All TransCon IL-2 β/γ dose levels are expressed in IL-2 equivalents. As no control group was included, all data were compared with predose levels for each animal. Animals were bled at various time points for PD assessments and then returned to the stock after the in-life portion of the study was completed (no terminal endpoints or histopathology was performed). Serum levels of IL-5, IL-6, E-selectin and VCAM-1 were analyzed by electrochemiluminescence using the Meso Scale Diagnostics (MSD) platform. Hematology was analyzed using a Beckman Coulter DxH600. For PK analysis, serum IL-2 levels were quantified by MSD. IL-2 β/γ plasma levels were quantified by removing TransCon IL-2 β/γ using anti-PEG antibody (ANP Technologies) conjugated agarose beads followed by ELISA using a monoclonal anti-IL-2 capture antibody (Thermo Fisher Scientific) and a biotinylated polyclonal rabbit anti-IL-2 (Thermo Fisher Scientific) detection antibody. Non-compartmental analysis was applied to calculate C_{max}, area under the curve (AUC) and terminal half-life (t_{1/2}) for IL-2 β/γ or for aldesleukin.

In a second study, 0.1 to 0.9 mg/kg TransCon IL-2 β/γ was administered IV (slow bolus) to male and female monkeys (n=1 animal/sex/group) on Days 1 and 15. As both cycles gave similar PK/PD results, only data from the first cycle are presented. No control group was included so all data were compared with predose levels or to historical control data for anatomical pathology results. Anatomical pathology was performed on Day 29 at necropsy. Organ weights and macroscopic observations were recorded, and histopathology was performed to assess microscopic changes (see online supplemental methods). Animals were bled at various time points for PD assessment. Hematology was analyzed using a Siemens ADVIA 2120i. Immunophenotyping was performed using a Fortessa X-20 (see online supplemental methods table SM 3A, B). Serum levels of IL-5, IL-6, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , monocyte chemoattractant protein (MCP)-1 and soluble CD25 (sCD25) were measured by immunoassays (see online supplemental methods). IL-2 β/γ plasma levels were analyzed as described above.

In vitro human PBMC proliferation assays

Healthy donor PBMCs (n=6) were cultured for 5 or 8 days with or without IL-2 β/γ (3.125–200 ng/mL). For the cells cultured for 8 days, culture medium with IL-2 β/γ was replenished on Day 5. Proliferation of CD8⁺ T cells, NK cells, $\gamma\delta$ T cells and Tregs was determined by flow cytometry based on Ki67 expression as total cell count and as frequency (% of each cell type expressing Ki67).

In vitro human lymphocyte cytotoxicity and cytokine assays

Purified CD8, NK and $\gamma\delta$ T cells were cultured in IL-2 β/γ (0, 50 and 200 ng/mL) for 48 hours prior to cytotoxicity assays. NK cytotoxicity assays used K562 target cells (ATCC) in a DELFIA dye release assay (Perkin-Elmer). CD8⁺ T cell 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. $\gamma\delta$ T cell cytotoxicity assays used Raji target cells as described above or Daudi target cells. Additionally, supernatants from the above cytotoxicity conditions were analyzed for cytokines and effector molecules via LEGENDplex (BioLegend) kits. For additional details see online supplemental methods.

Statistical analyses

The unpaired t-test was applied for data from in vivo mouse studies. No statistical analyses were performed on the monkey data due to the small number of animals per group. For in vitro conditions where the same matched samples underwent different treatment conditions, the paired t-test was applied. All statistical analyses were performed using GraphPad Prism or Microsoft Excel. A p value of <0.05 was considered statistically significant.

RESULTS

Generation of IL-2 β/γ , a potent IL-2R β/γ selective IL-2 variant with minimal IL-2R α binding

To generate an optimally biased IL-2 compound, selective for IL-2R β/γ , several strategies were pursued. In all cases we included the C125S stabilizing mutation present in aldesleukin. Initial efforts using non-directed PEGylation with a 20 kDa mPEG moiety did not achieve receptor bias and reduced potency towards IL-2R $\alpha/\beta/\gamma$ and IL-2R β/γ (data not shown). We therefore reasoned that specific inhibition of IL-2R α binding should be achieved by steric hindrance at the IL-2R α binding interface. An IL-2 mutein was generated by introducing an unpaired cysteine within the IL-2R α binding interface,²⁶ and blocking mPEGs of various sizes were then permanently conjugated to this cysteine. Specifically, 2, 5, 10, 20 and 30 kDa mPEG moieties were attached to test for IL-2R biasing and potency effects.

To first confirm lack of IL-2R α interactions, permanently PEGylated IL-2 muteins were characterized for IL-2R α versus IL-2R β subunit binding using Biacore. While an mPEG size of 2 kDa maintained some IL-2R α interactions, mPEG sizes from 5 kDa and above completely blocked the interaction with IL-2R α (online supplemental figure S1). Within the mPEG sizes that blocked IL-2R α (5, 10, and 20 kDa mPEGs), increasing mPEG size was associated with decreasing affinity for IL-2R β (online supplemental figure S1), likely due to non-specific steric hindrance from the larger mPEG moieties.^{27,28} Based on this, a 5 kDa mPEG was identified as optimal to fully block IL-2R α interactions yet maintain IL-2R β potency and used to create 'IL-2 β/γ ' (figure 1A,C).

Next, to evaluate receptor bias of IL-2 β/γ in primary human lymphocytes, pSTAT5 potency experiments were performed using whole blood or PBMCs. Compared with control IL-2, IL-2 β/γ demonstrated substantially reduced potency in Tregs while maintaining similar or slightly reduced potency in CD8⁺ T cells and NK cells (Treg EC₅₀ of 34.5 vs 0.1 ng/mL; CD8⁺ EC₅₀ of 62.2 vs 25.7 ng/mL; and NK cell EC₅₀ of 11.8 vs 6.0 ng/mL for IL-2 β/γ vs control IL-2). The large potency loss seen with IL-2 β/γ in IL-2R $\alpha/\beta/\gamma$ ⁺ Tregs with well-maintained potency in IL-2R β/γ ⁺ CD8⁺ T cells and NK cells is consistent with loss of IL-2R α binding and IL-2R β/γ selectivity for IL-2 β/γ (figure 1D). In agreement with IL-2R β binding data, increasing mPEG size beyond 5 kDa resulted in decreasing potency for induction of pSTAT5 (online supplemental figure S2) and cytotoxicity (online supplemental figure S3) in human IL-2R β/γ ⁺ NK cells and CD8⁺ T cells.

Generation of TransCon IL-2 β/γ , a long-acting prodrug with sustained release of IL-2 β/γ

To improve PK properties, the TransCon IL-2 β/γ prodrug was created by transiently conjugating a branched 40 kDa mPEG carrier to the receptor-selective IL-2 β/γ via the TransCon linker technology²⁷ (figure 1B). This technology is similar to what is used in the long-acting TransCon Growth Hormone product, lonapegsomatropin-tcgd,^{29,30}

and results in shielding of receptor binding and reduced renal clearance, blocking bioactivity while extending prodrug half-life.²⁷ Under physiological pH and temperature, TransCon IL-2 β/γ is designed to slowly release active IL-2 β/γ from the carrier via autocleavage in a controlled manner that follows first-order kinetics.

TransCon IL-2 β/γ induced CD8⁺ T cell and NK cell activation and promoted antitumor efficacy in mice

Female BALB/c mice with established CT26 tumors were treated with TransCon IL-2 β/γ IV and/or TransCon TLR7/8 Agonist IT and evaluated for tumor growth inhibition and PD effects. Treatment with TransCon IL-2 β/γ alone reduced tumor volumes compared with control animals at Day 18 (1948 mm³ vs 2653 mm³, t-test p value: 0.011, figure 2A–C). At Day 14, clear antitumor efficacy was observed for treatments with TransCon TLR7/8 Agonist alone and in combination with TransCon IL-2 β/γ . At later time points the combination with TransCon IL-2 β/γ demonstrated superior efficacy compared with TransCon TLR7/8 Agonist alone (Day 25: 2226 mm³ vs 1032 mm³, t-test p value: 0.003). Similarly, treatment with the combination of TransCon IL-2 β/γ and TransCon TLR7/8 Agonist more than doubled the number of CRs (8/18) compared with TransCon TLR7/8 Agonist alone (3/18). To assess antitumor immune memory, mice with CRs from the first study were re-challenged with CT26 tumor cells. While CT26 tumors grew as expected in naïve mice, all of the CR mice demonstrated rejection of the subsequent CT26 rechallenge (figure 2D). Next, these mice who rejected the CT26 rechallenge were challenged with EMT6, a distinct tumor cell line with shared neoantigens.³¹ While all mice that previously rejected CT26 rechallenge showed reduced EMT6 tumor growth compared with naïve mice, mice previously treated with TransCon IL-2 β/γ and TransCon TLR7/8 Agonist completely rejected the challenge with EMT6 (figure 2E).

The antitumor effect of TransCon IL-2 β/γ was also assessed in C57BL/6 mice with established MC38 tumors. Similar to the CT26 model, a significant reduction in tumor growth was observed following treatment with TransCon IL-2 β/γ compared with control (Day 20: 1678 mm³ vs 2562 mm³, t-test p value: 0.0099, figure 2F, online supplemental figure S4).

In CT26-bearing mice, treatments containing TransCon IL-2 β/γ induced robust proliferation and expansion of NK cells and, especially after the second dose (data not shown), CD8⁺ T cells, as measured by Ki67 induction and total counts in the blood and spleen (figure 3A,B). Additionally, these changes corresponded with increases in the CD8/CD4 and CD8/Treg ratios (online supplemental figure S5A). Lastly, no substantial induction of PD-1 on CD4⁺ or CD8⁺ T cells was seen in the blood or spleen (data not shown).

In functional studies using in vitro stimulation with PMA and ionomycin to illustrate effector molecule potential, blood CD8⁺ T cells and NK cells from TransCon IL-2 β/γ treated animals showed enhanced granzyme B expression

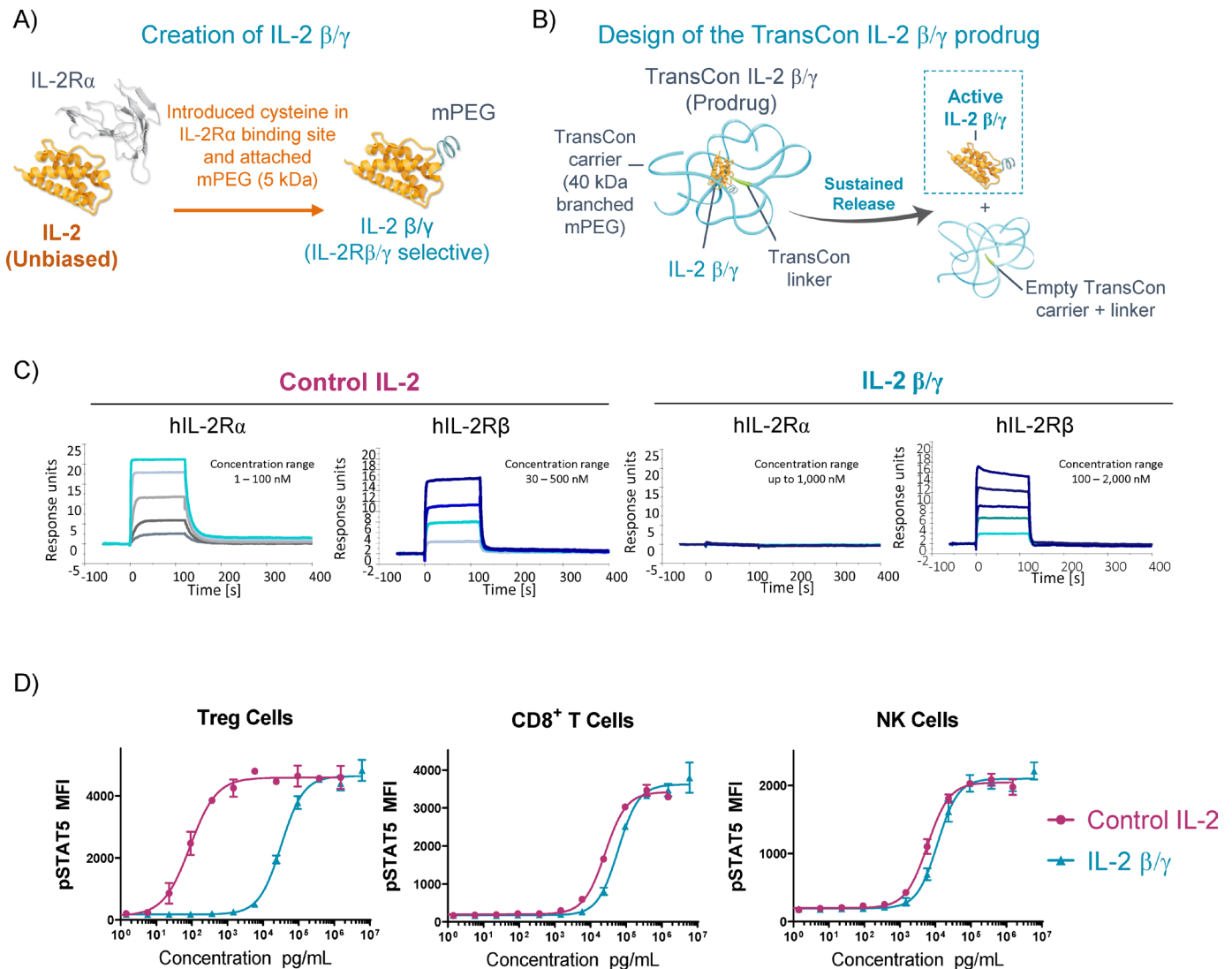


Figure 1 TransCon IL-2 β/γ is a novel IL-2 variant designed for IL-2 β/γ receptor selectivity and improved PK properties. (A) Design of IL-2 β/γ via introduction of a permanently attached 5 kDa mPEG to an engineered cysteine at the IL-2 α binding site of IL-2. (B) Design of the TransCon IL-2 β/γ prodrug with sustained release of active IL-2 β/γ under physiological conditions. (C) Biacore sensorgrams from binding experiments with human control IL-2 or IL-2 β/γ to human IL-2 α or human IL-2 β . (D) Human blood was stimulated with control IL-2 or IL-2 β/γ for 30 min at indicated concentrations then analyzed by flow cytometry for pSTAT5 in the indicated cell populations. Data are shown as median fluorescence intensity (MFI) of pSTAT5 and shown as mean \pm SEM for two donors. IL, interleukin; mPEG, methoxy polyethylene glycol; NK, natural killer; PK, pharmacokinetic; pSTAT5, signal transducer and activator of transcription 5 phosphorylation; Treg, regulatory T.

and IFN- γ production with highest levels mostly observed in mice treated with the combination of TransCon IL-2 β/γ and TransCon TLR7/8 Agonist (figure 3C and online supplemental figure S5B). Of note, TransCon IL-2 β/γ -mediated granzyme B induction was also seen in freshly isolated (without in vitro stimulation) peripheral NK cells and CD8 $^+$ T cells from tumor-free animals (data not shown).

TransCon IL-2 β/γ elicited robust lymphocyte expansion in cynomolgus monkeys without inducing eosinophils or systemic biomarkers for VLS in contrast to high-dose IL-2

The pharmacology of TransCon IL-2 β/γ was next investigated in cynomolgus monkeys. In vitro, cynomolgus monkey lymphocytes demonstrated almost identical

IL-2 β/γ selectivity and pSTAT5 potency results as seen with human cells (online supplemental figure S6). To confirm IL-2 β/γ selectivity in vivo, monkeys were administered IV with either a daily dose of 0.4 mg (average 0.044 mg/kg) aldesleukin for 5 days or a single 1.0 mg dose of TransCon IL-2 β/γ (average 0.122 mg/kg) and assayed for hematology and blood analytes associated with systemic inflammation and vascular damage. While treatment with both TransCon IL-2 β/γ and aldesleukin increased absolute lymphocyte counts (ALC), treatment with aldesleukin, but not TransCon IL-2 β/γ , increased eosinophil counts (figure 4A). Accordingly, minimal induction of systemic inflammatory markers such as IL-5 and IL-6 were observed after treatment with TransCon IL-2

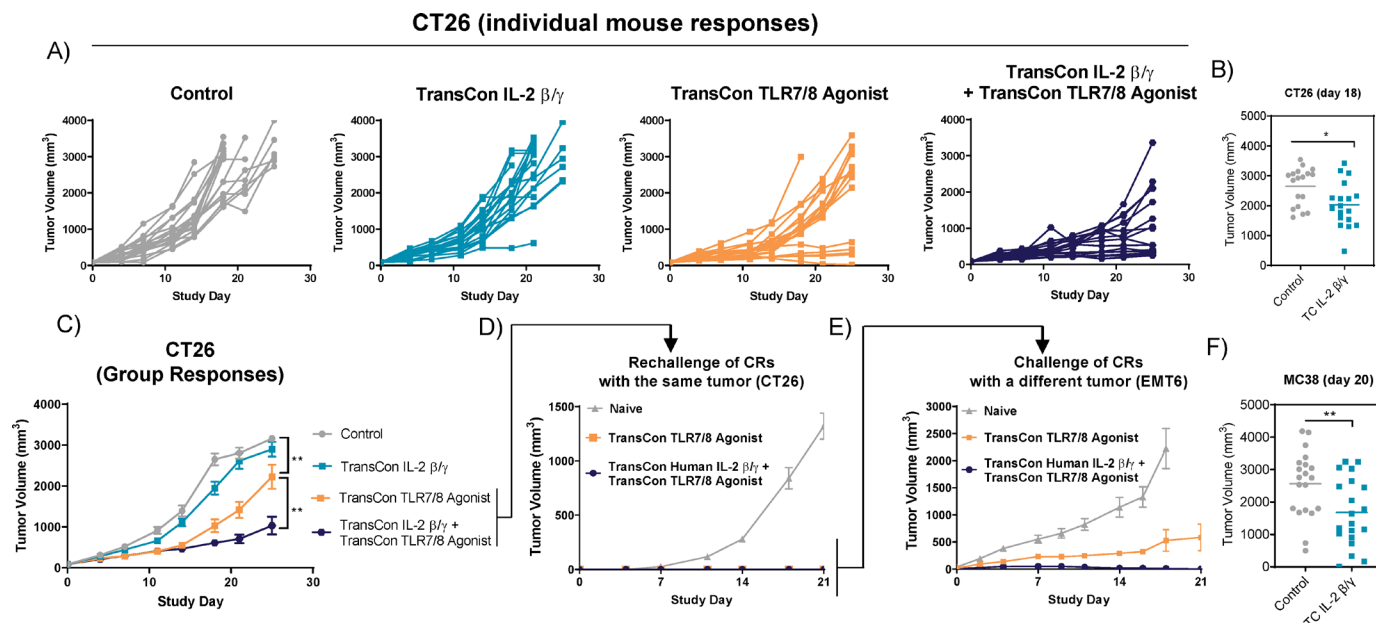


Figure 2 TransCon IL-2 β/γ shows antitumor effects in vivo and enhances TransCon TLR7/8 Agonist therapy and establishment of immune memory. (A–C) Female BALB/c mice were inoculated with either (i) 0.5 or (ii) 2.0×10^6 CT26 cells. Mice with established tumors were either (i) treated on Days 0, 6, and 16 with buffer or $60 \mu\text{g}$ (approximately 3 mg/kg) TransCon IL-2 β/γ intravenously and/or a single $134 \mu\text{g}$ (approximately 6.7 mg/kg) intratumoral dose (on Day 0) of TransCon TLR7/8 Agonist ($n=8/\text{group}$), or (ii) untreated or treated on Days 0, 7, and 14 with $72 \mu\text{g}$ (approximately 3.6 mg/kg) TransCon IL-2 β/γ intravenously and/or a single $100 \mu\text{g}$ (approximately 5 mg/kg) intratumoral dose (on Day 0) of TransCon TLR7/8 Agonist ($n=10$), respectively, followed by measurements of tumor volume ($n=18$ mice/group total). Because of the similarity in conditions between (i) and (ii) above, tumor volume data from both are presented together. (A) Individual animal tumor volumes. (B) Mean tumor volumes on Day 18, and (C) over time ($\pm \text{SEM}$). (D) Mice from (i) in (A–C) ($n=2$ from TransCon TLR7/8 Agonist treatment and $n=6$ from TransCon TLR7/8 Agonist plus TransCon IL-2 β/γ treatment) demonstrating complete responses (CR) were rechallenged, along with naïve BALB/c mice ($n=8$), with CT26 in the opposite flank with mean tumor volumes $\pm \text{SEM}$ shown. (E) Mice from (D), all of which showed complete rejection of CT26 rechallenge, along with additional naïve BALB/c mice ($n=8$), were challenged with a distinct tumor cell line EMT6 at a contralateral site. Mean tumor volume $\pm \text{SEM}$ are shown. (F) Female C57BL/6 mice with established MC38 tumors were treated with buffer control or $60 \mu\text{g}$ TransCon IL-2 β/γ on Days 0, 7 and 14 ($n=20$ mice/group). Tumor volumes Day 20 are shown as mean $\pm \text{SEM}$. Statistical analyses were performed using unpaired t-test. *, $p<0.05$; **, $p<0.01$. IL, interleukin; TLR, toll-like receptor

β/γ as opposed to treatment with aldesleukin (figure 4B). Similarly, in contrast to aldesleukin treatment, minimal induction of endothelial cell injury markers (E-selectin and VCAM-1) were observed in serum after treatment with TransCon IL-2 β/γ (figure 4C). PK analysis showed that while daily aldesleukin treatment for 5 days resulted in pulsatile spikes and drops in IL-2 serum levels (mean $t_{1/2}$: 1.45 hours), a single dose of TransCon IL-2 β/γ led to prolonged levels of systemic IL-2 β/γ (mean $t_{1/2}$: 32 hours) (online supplemental figure S7).

In a second study, monkeys were administered higher doses of TransCon IL-2 β/γ . While ALC increases over predose were robust (average 3.6-fold, 27.4-fold and 19.6-fold increases at 168 hours for 0.1, 0.3 and 0.9 mg/kg, respectively), eosinophil counts were minimally changed (average 1.4-fold, 1.3-fold and 3.1-fold increases at 216 hours for 0.1, 0.3 and 0.9 mg/kg, respectively) (online supplemental figure S8A). Moreover, levels of IL-5, IL-6, IFN- γ and TNF- α remained low in animals administered with up to 0.9 mg/kg of TransCon IL-2 β/γ , whereas a dose-dependent induction of MCP-1 and sCD25 was seen (online supplemental figure S8B). Doses up to 0.3 mg/kg

were well tolerated with no adverse findings, but animals receiving 0.9 mg/kg showed signs of exaggerated pharmacology with, for example, subdued behavior and body weight loss. Histopathology did not reveal any signs of vascular damage, pulmonary edema or tissue necrosis indicative of VLS in any of the groups (see online supplemental results).

TransCon IL-2 β/γ induced robust expansion and proliferation of CD8⁺ T cells, NK cells and $\gamma\delta$ T cells in cynomolgus monkeys

A robust expansion of CD8⁺ T cells (up to 23.3-fold over predose), NK cells (up to 40.5-fold), and $\gamma\delta$ T cells (up to 728.6-fold) was observed in peripheral blood of monkeys after TransCon IL-2 β/γ administration, peaking 168–216 hour postdose and with maximum response already at the mid dose of 0.3 mg/kg (figure 5A). CD4⁺ T cell or Treg expansion was minimal at 0.3 mg/kg, while the 0.9 mg/kg dose induced modest increases in CD4⁺ T cells and Tregs (4.2-fold and 8.5-fold, respectively). The preferential expansion of cytotoxic cells over Tregs and CD4⁺ T cells, particularly at the 0.3 mg/kg dose, was

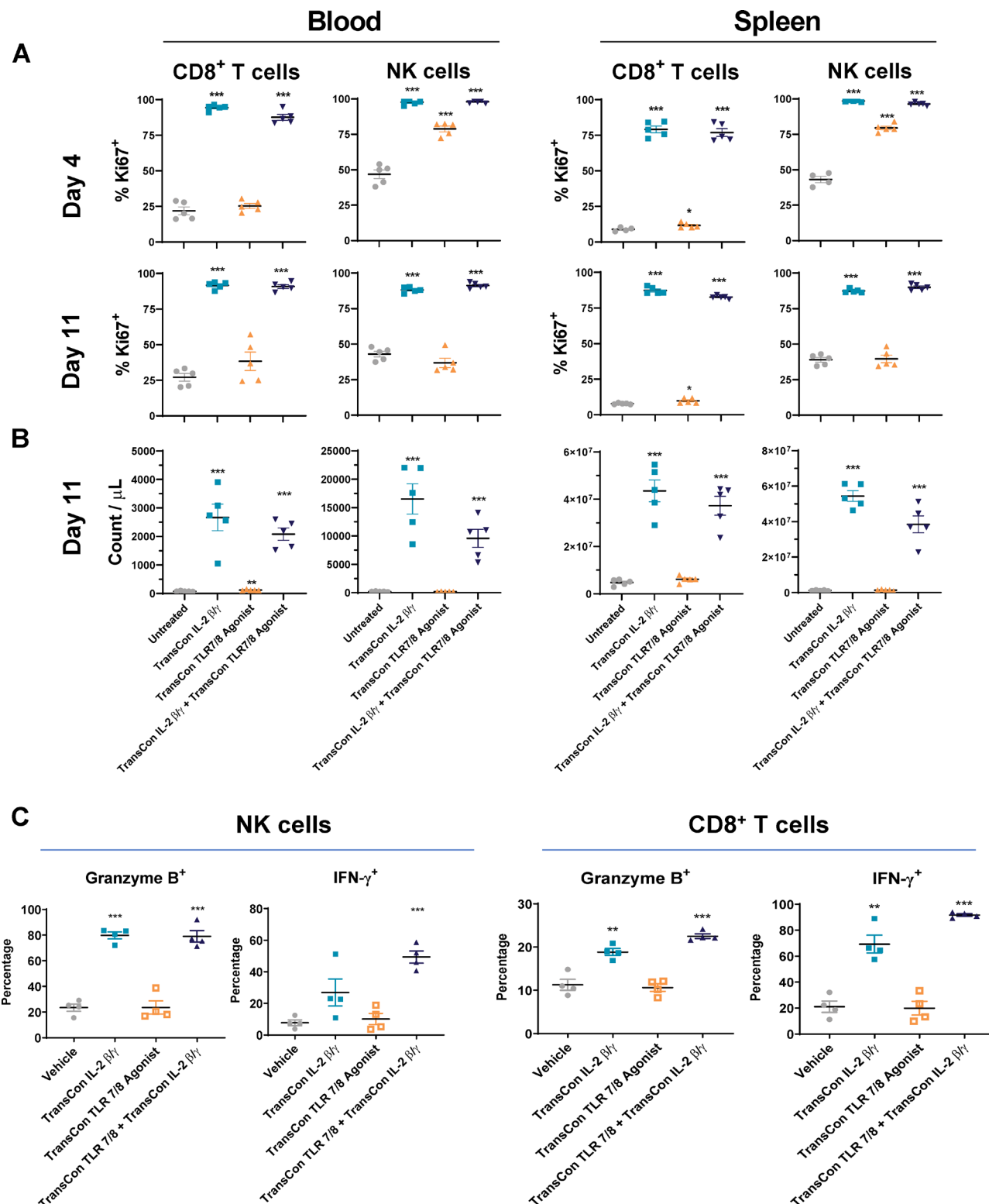


Figure 3 TransCon IL-2 β/γ robustly activates CD8⁺ T and NK cells in CT26 bearing BALB/C mice. (A, B) Mice were administered 72 μ g (approximately 3.6 mg/kg) of TransCon IL-2 β/γ intravenously on Days 0 and 7 and/or a single 100 μ g (approximately 5 mg/kg) intratumoral dose (on Day 0) of TransCon TLR7/8 Agonist. Shown (as mean \pm SEM with n=5 mice/group) are percentages of proliferating (Ki67⁺) CD8⁺ T cells and NK cells in the blood and spleen 96 hours after a single dose (Day 4) or two doses (Day 11) of TransCon IL-2 β/γ as well as (B) the total CD8⁺ T cell and NK cell counts after two doses. (C) Mice were administered 60 μ g (approximately 3 mg/kg) of TransCon IL-2 β/γ intravenously on Days 0 and 6. Peripheral blood was drawn 96 hours after the second dose and stimulated with BD Leukocyte Activation Cocktail for 4 hours to induce cytokine production before analysis by flow cytometry. Shown are plots for the proportion of granzyme B⁺ or IFN- γ ⁺ NK cells or CD8⁺ T cells (as mean \pm SEM with n=4 mice/group). Statistical analyses were performed using unpaired t-test. *, p<0.05; **, p<0.01; ***, p<0.001. IFN, interferon; IL, interleukin; NK, natural killer; TLR, toll-like receptor.

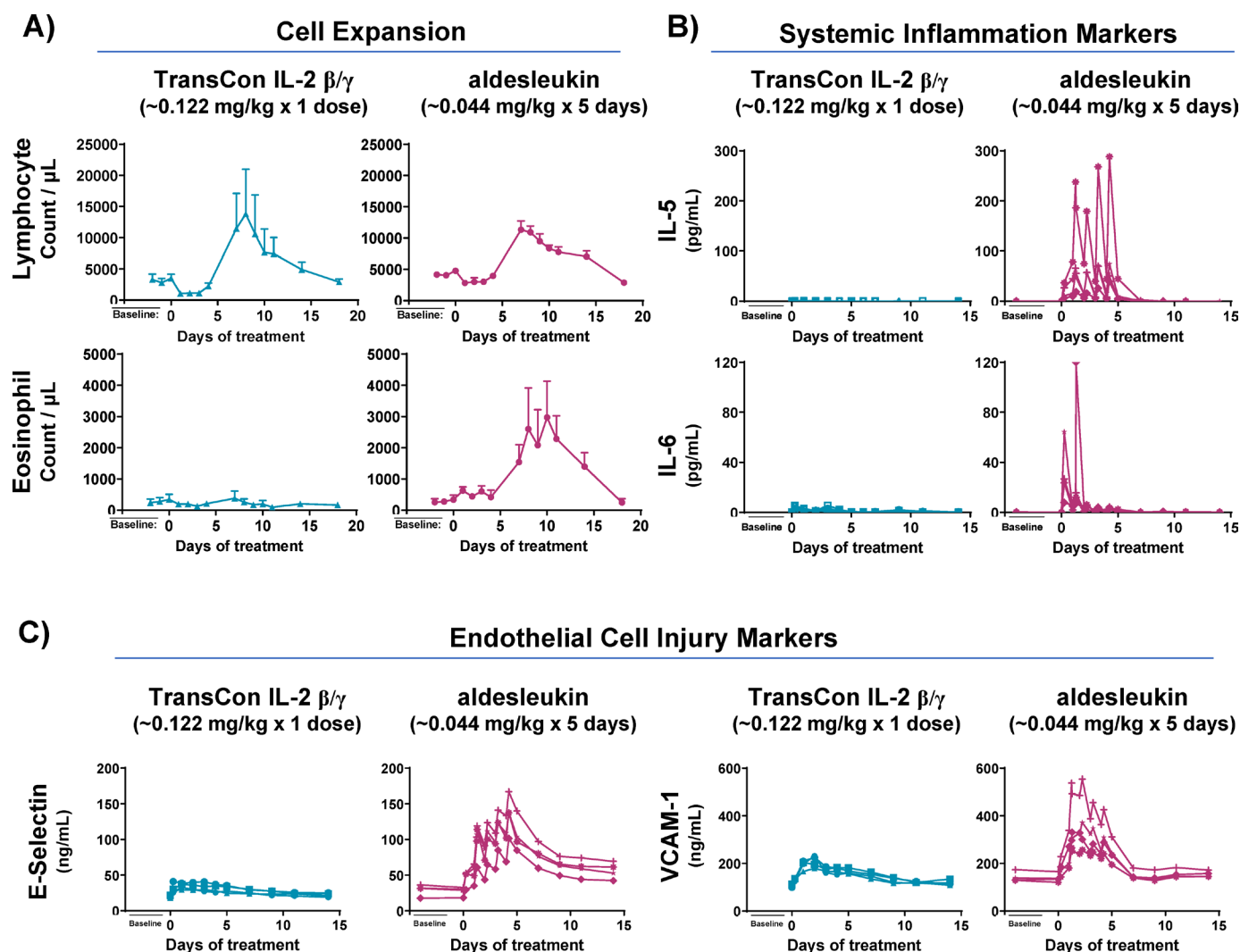


Figure 4 TransCon IL-2 β/γ promotes robust lymphocyte expansion in cynomolgus monkeys, without inducing eosinophils, systemic endothelial cell injury or inflammatory cytokines. Male monkeys ($n=4/\text{group}$) were administered daily aldesleukin (0.4 mg/day, average 0.044 mg/kg) on Days 1–5, or a single dose of 1.0 mg (average 0.122 mg/kg) TransCon IL-2 β/γ . Animals were bled throughout the study for assessment of various pharmacodynamic endpoints. (A) Total lymphocytes and eosinophils are presented as counts/ μL and shown as mean \pm SEM. (B, C) Serum concentrations of IL-5, IL-6, E-selectin and VCAM-1 presented as individual responses. IL, interleukin.

clearly observed also as robust increases in CD8⁺/Treg, NK/Treg and $\gamma\delta$ T/Treg ratios or the corresponding CD4⁺ ratios (online supplemental figure S9).

Similarly, TransCon IL-2 β/γ induced robust and durable mechanistic signs of proliferative responses (measured as % Ki67⁺ cells) in CD8⁺ T cells, NK cells and $\gamma\delta$ T cells at all dose levels. For CD4⁺ T cells and Tregs, a dose-dependent induction of Ki67 was observed, but the frequency of proliferating cells was much lower than for CD8⁺ T cells, NK cells and $\gamma\delta$ T cells (figure 5B). Impressively, the 0.3 mg/kg dose of TransCon IL-2 β/γ induced >90% Ki67⁺ cells in CD8⁺ T cells, NK cells and $\gamma\delta$ T cells while higher doses were required to achieve a similar effect in Tregs and CD4⁺ T cells. Similar expansion patterns for all populations and proliferation of cells were seen after a second administration of TransCon IL-2 β/γ (data not shown).

Further analyses demonstrated that effector memory CD8⁺ T cells were more sensitive to TransCon IL-2 β/γ for Ki67 induction and expansion responses than total CD8⁺ T cells with, for example, average 53-fold versus 19-fold expansion at 0.3 mg/kg (online supplemental figure S10). Effector CD8⁺ T cells typically express granzyme B. Accordingly, TransCon IL-2 β/γ induced robust expansion of granzyme B⁺ CD8⁺ T cells and also granzyme B⁺ NK and $\gamma\delta$ T cells (online supplemental figure S11).

Pharmacokinetics of IL-2 β/γ in cynomolgus monkeys

After intravenous dosing of TransCon IL-2 β/γ , IL-2 β/γ demonstrated low C_{max} values with average (mono-phasic) half-lives ranging from 35 to 41 hours. Between 0.1 and 0.9 mg/kg TransCon IL-2 β/γ , IL-2 β/γ exposure (both mean C_{max} and $\text{AUC}_{0-336\text{h}}$) increased generally dose proportionally (figure 5C). Of note, at 0.3 mg/kg, a dose

TransCon IL-2 β/γ dose

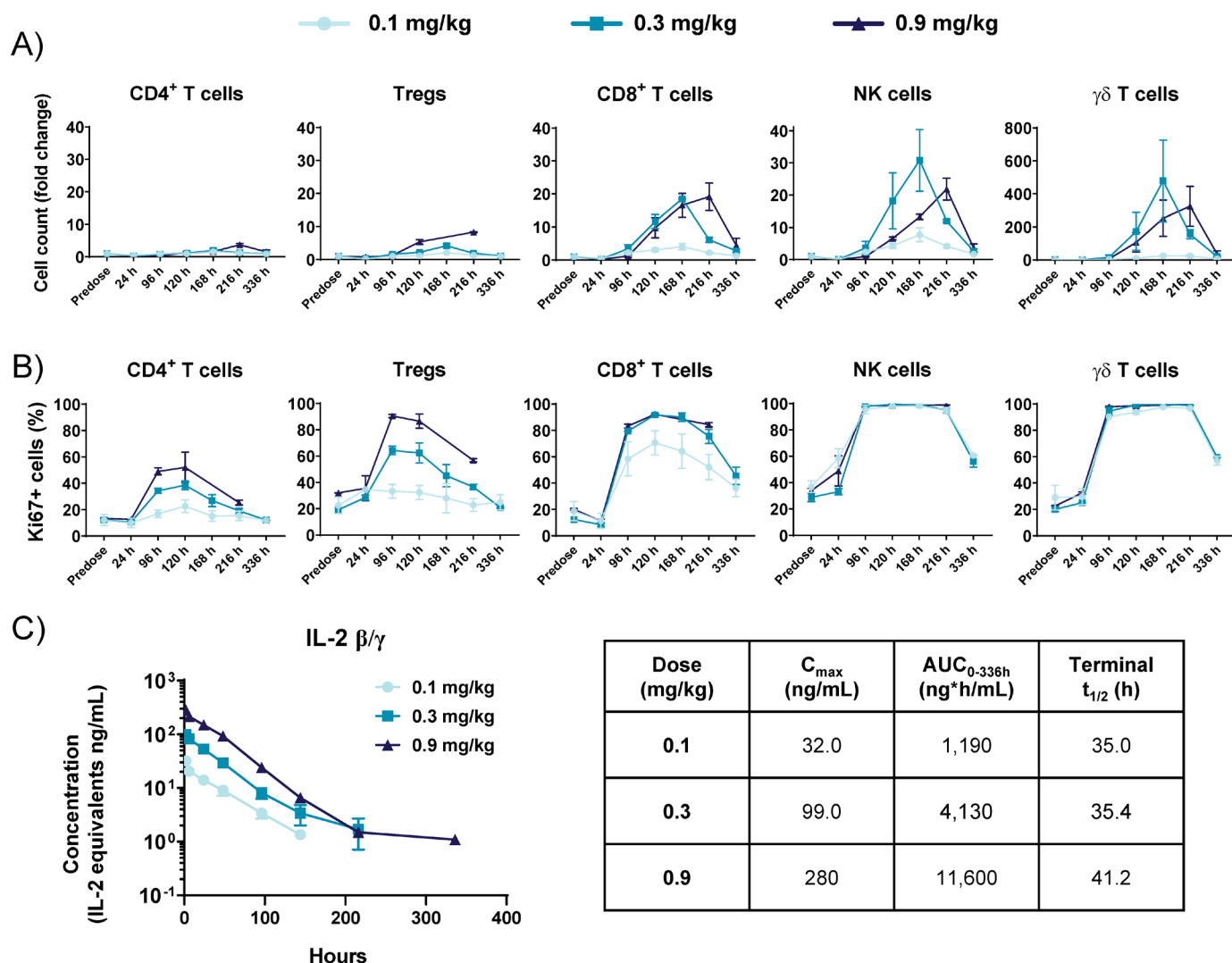


Figure 5 Pharmacodynamic and pharmacokinetic responses in cynomolgus monkeys before (predose) and at various time points after a single intravenous administration of TransCon IL-2 β/γ at 0.1, 0.3 and 0.9 mg/kg (n=1 animal/sex/group). (A) Cell count of different lymphocyte subsets as fold change over predose (mean \pm SEM). (B) Proliferation of different lymphocyte subsets as measured by % Ki67⁺ cells (mean \pm SEM). (C) Postdose plasma concentrations of IL-2 β/γ as ng/mL (mean \pm SEM) of IL-2 equivalents, and summary of pharmacokinetic parameters based on non-compartmental analysis (mean values for each dose level). AUC, area under the curve; h, hours; IL, interleukin; NK, natural killer; Treg, regulatory T; $\gamma\delta$, gamma delta; t_{1/2}, terminal half-life.

with profound PD effects, the C_{max} was only 99 ng/mL. A second dose of TransCon IL-2 β/γ administered 2 weeks later generated similar exposure (data not shown), suggesting minimal accumulation with no indication of anti-drug antibodies.

IL-2 β/γ induced proliferation and cytolytic activity of human primary CD8⁺ T cells, NK cells and $\gamma\delta$ T cells

As TransCon IL-2 β/γ induced a robust expansion of CD8⁺ T cells, NK cells and $\gamma\delta$ T cells in monkeys, the effects of IL-2 β/γ on proliferation and cytotoxicity of human primary cells were explored. To mimic the IL-2 β/γ C_{max} in the monkey study (99 and 280 ng/mL at 0.3 and 0.9 mg/kg TransCon IL-2 β/γ , respectively), IL-2 β/γ concentrations of 50 and 200 ng/mL were tested. Human

PBMCs were stimulated with or without IL-2 β/γ for 5 or 8 days then assessed for proliferation by Ki67 expression. IL-2 β/γ induced dose-dependent and time-dependent increases in the frequency (figure 6A) and numbers (data not shown) of Ki67⁺ CD8⁺ T cells, NK cells and $\gamma\delta$ T cells. This corresponded with increases in ratios of proliferating CD8⁺ T cells, NK cells and $\gamma\delta$ T cells over proliferating Tregs (figure 6B) or CD4⁺ T cells (data not shown).

Next, the potential of human CD8⁺ T cells, NK cells and $\gamma\delta$ T cells pretreated with IL-2 β/γ to kill human tumor cells were evaluated in vitro. Initial experiments with CD8⁺ and $\gamma\delta$ T cells utilized CD20⁺ Raji B cell target cells and a suboptimal dose of a CD3–CD20 targeting bispecific T cell engager (overnight assay), while experiments with NK

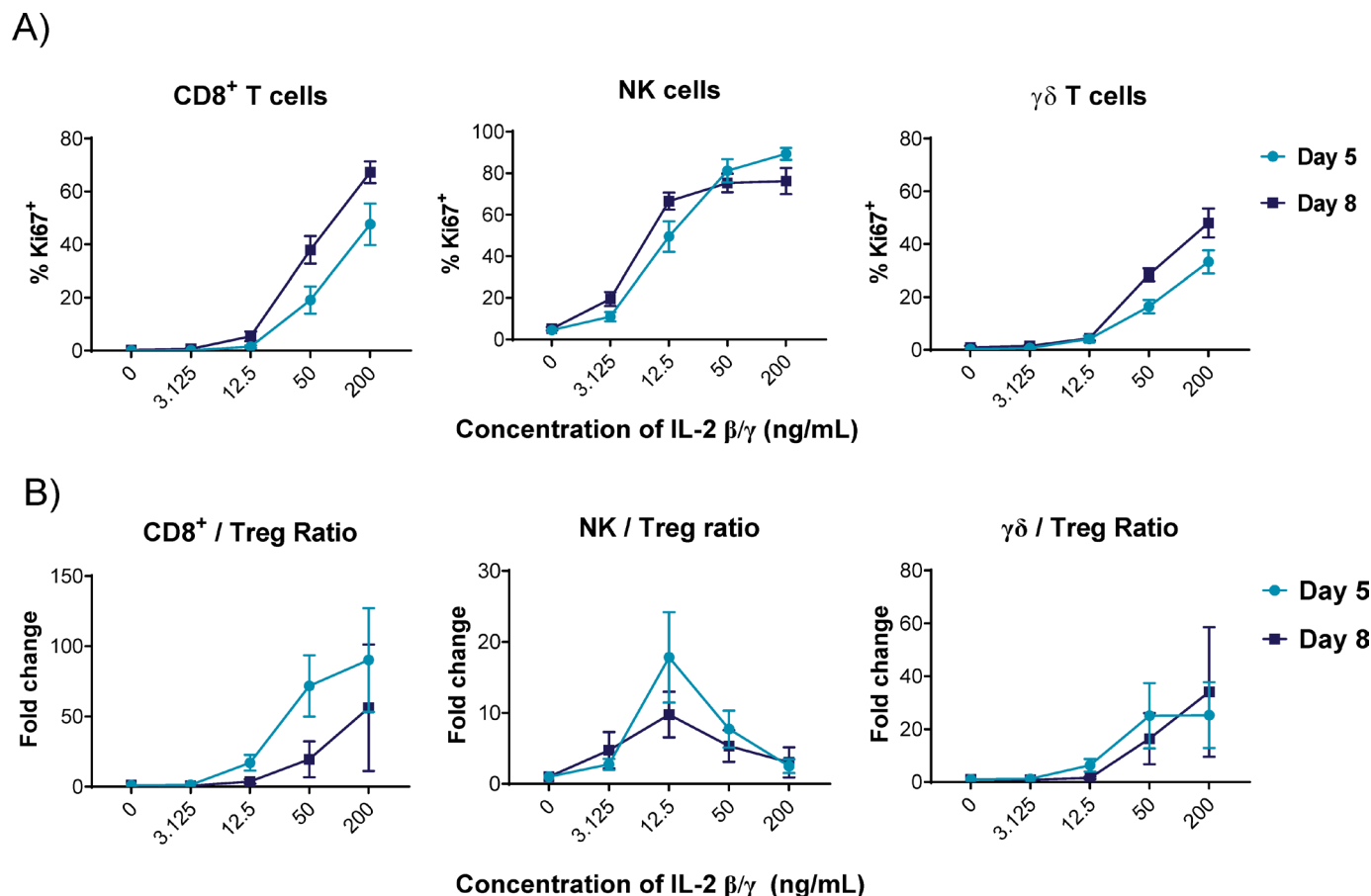


Figure 6 IL-2 β/γ induces proliferation of human primary CD8⁺ T cells, NK cells and $\gamma\delta$ T cells. (A) Proliferative response after culture for 5 and 8 days with IL-2 β/γ measured as the percentage of Ki67⁺ cells by flow cytometry. (B) Proliferative responses compared as the fold change of the ratios of proliferating (Ki67⁺) CD8⁺ T cells, NK cells and $\gamma\delta$ T cells to Tregs. Data are shown as mean \pm SEM (n=6 donors). IL, interleukin; NK, natural killer; Treg, regulatory T; $\gamma\delta$, gamma delta.

cells used K562 target cells (4 hours assay). Pretreatment with IL-2 β/γ clearly enhanced the cytotoxic potential of these cells (figure 7A). In agreement, increased levels of TNF- α , Fas ligand (FasL), granzyme A, granzyme B and perforin were observed in supernatants from the same CD8⁺ T cell, NK cell and $\gamma\delta$ T cell cytotoxicity conditions described above (figure 7B, online supplemental figures S12A-C). Additionally, IFN- γ increases were clearly seen in overnight tumor coculture supernatants with CD8⁺ and $\gamma\delta$ T cells (figure 7B), and when NK cell cocultures were extended overnight (data not shown). In contrast to the robust IFN- γ levels, no substantial IL-17A induction was observed in $\gamma\delta$ T cell cocultures treated with IL-2 β/γ (online supplemental figure S12C).

Lastly, the ability of IL-2 β/γ to enhance the effector functions of natural T cell receptor (TCR) ligand stimulated $\gamma\delta$ T cells was examined using Daudi cells, which express an endogenous $\gamma_9\delta_2$ TCR ligand.³² This is achievable because >50% of the purified $\gamma\delta$ T cells were $\gamma_9\delta_2$ cells, as expected (data not shown). IL-2 β/γ treatment substantially enhanced the cytolytic activity of endogenous TCR ligand stimulated $\gamma\delta$ T cells (online supplemental figure S13A) and enhanced levels of TNF- α , FasL, IFN- γ , granzyme A, granzyme B, perforin and granulysin in these

conditions with minimal effects on IL-17A (online supplemental figure S13B, C).

DISCUSSION

The major shortcomings of aldesleukin in cancer therapy are activation of IL-2R α^+ Tregs, endothelial cells and inflammatory eosinophils, as well as fast clearance requiring frequent high dose administrations resulting in high C_{max} values. Approaches to improve IL-2's therapeutic potential must simultaneously balance activity and tolerability. Our approach was to maintain robust, near-native IL-2R β/γ potency and selectivity while also extending circulation half-life and reducing C_{max} .

TransCon IL-2 β/γ was designed to optimize these features by two independent and complementary approaches. First, to generate an IL-2R β/γ selective agonist with potent IL-2R β/γ binding and activity, IL-2 β/γ was created by permanent conjugation of a small 5 kDa mPEG within the IL-2R α binding site of an IL-2 mutein. A 5 kDa mPEG was chosen as PEGs >5 kDa led to reduced IL-2R β/γ binding and activation, a finding also described by others,¹⁵ whereas smaller mPEGs retained some IL-2R α activity. Beyond reducing IL-2R β/γ activity,

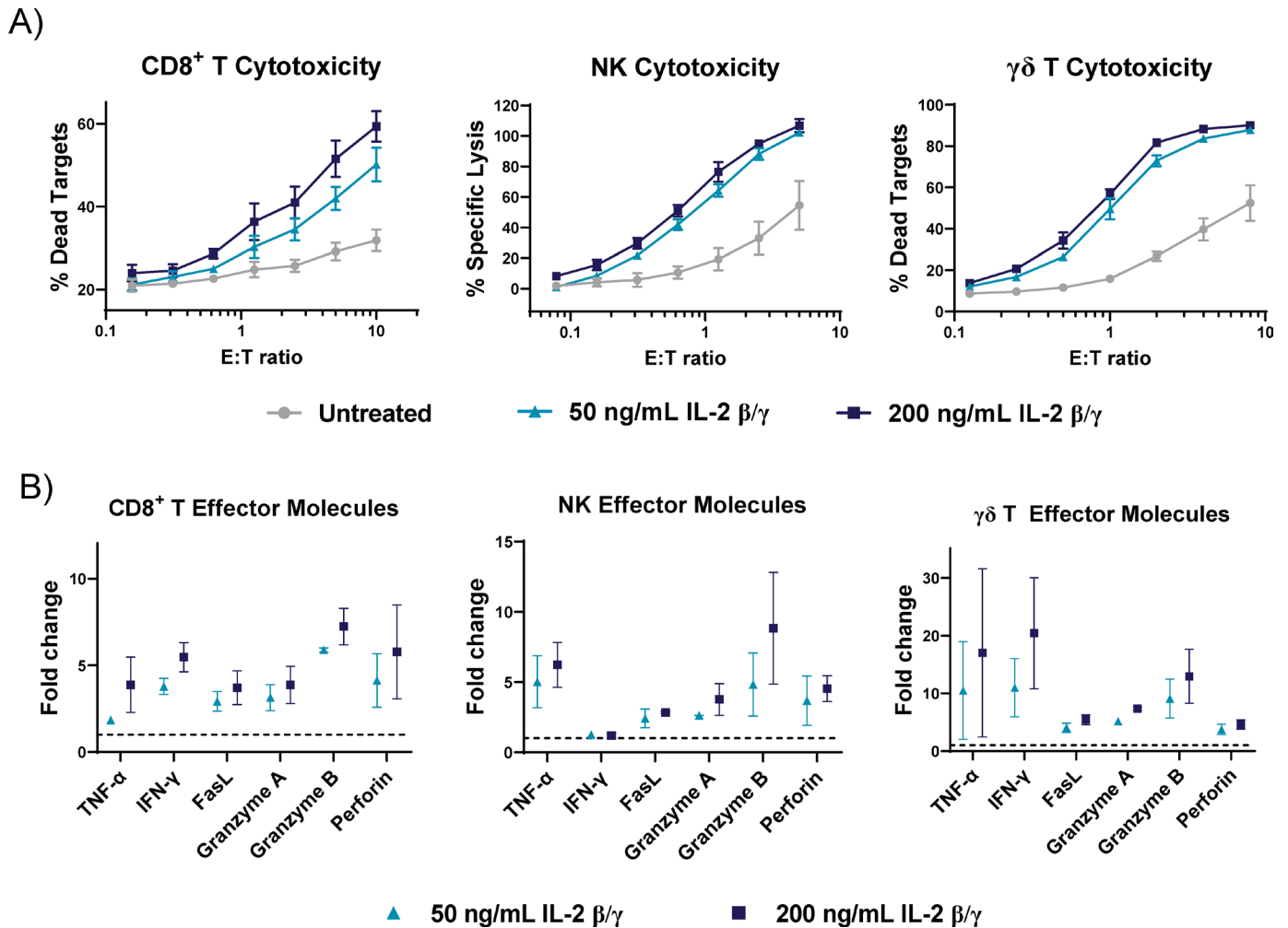


Figure 7 IL-2 β/γ induces cytotoxic and effector functions in primary human CD8⁺ T cells, NK cells and γδ T cells. (A) Human CD8⁺ T cell, NK cell and γδ T cell-mediated cytotoxicity over varying Effector to Target (E:T) ratios after 48 hours pretreatments with 0, 50 or 200 ng/mL of IL-2 β/γ. Data are represented as mean±SEM for three donors for CD8⁺ T cells and NK cells and two donors for γδ T cells. CD8⁺ and γδ T cell assays were using CD20⁺ Raji B cell lymphoma cells as target cells in the presence of 1 nM CD3-CD20 bispecific engager, while NK cell cytotoxicity assays used K562 erythroleukemia cells as target cells. (B) Supernatants were collected from cytotoxicity coculture conditions described in (A) and analyzed for effector molecules (TNF-α, IFN-γ, FasL, granzyme A, granzyme B and perforin). Data are shown as fold change compared with untreated cells at an E:T ratio of 1.25 for CD8⁺ T cells and NK cells and 1.0 for γδ T cells. FasL, Fas ligand; IFN, interferon; IL, interleukin; NK, natural killer; TNF, tumor necrosis factor; γδ, gamma delta.

larger PEG sizes could also hinder tissue distribution³³ or access to tight cell–cell interfaces including the immunological synapse.³⁴ Second, to improve PK properties, IL-2 β/γ was transiently attached to a 40 kDa mPEG carrier via a TransCon linker, shielding bioactivity and creating the TransCon IL-2 β/γ prodrug. Accordingly, at tolerable doses of TransCon IL-2 β/γ in monkeys, active IL-2 β/γ was slowly released from the mPEG carrier, leading to a low C_{max} with prolonged levels of IL-2 β/γ (effective $t_{1/2}$ > 30 hours) capable of inducing robust PD effects.

In vitro, IL-2 β/γ demonstrated no detectable binding to human IL-2Rα, while binding to human IL-2Rβ was well maintained, as intended. Accordingly, in human pSTAT5 bioassays, IL-2 β/γ demonstrated a substantially lower potency (200–600-fold) compared with control IL-2 on IL-2Rα⁺ Tregs, while maintaining activity

(within ~2–3-fold) on CD8⁺ T cells and NK cells. Furthermore, IL-2 β/γ induced proliferation and enhanced cytotoxic function in vitro in multiple human IL-2Rβ/γ⁺ cell types, including CD8⁺ T cells, NK cells and γδ T cells. As TransCon IL-2 β/γ is designed for systemic exposure of IL-2 β/γ, elimination of IL-2Rα interactions is critical for improved tolerability. In contrast, some other IL-2 prodrug approaches are designed to be activated by tumor specific proteases and still utilize wild type IL-2Rα interactions to achieve increased potency for intratumoral IL-2Rα⁺ activated lymphocytes, however this approach could also have increased potency for intratumoral Tregs.³⁵

In mice, TransCon IL-2 β/γ demonstrated antitumor efficacy and potentiated the induction of long-term immunological memory in combination with TransCon TLR7/8 Agonist. Further, TransCon IL-2 β/γ demonstrated robust

activation and proliferation of CD8⁺ T cells and NK cells over CD4⁺ T cells and Tregs both as monotherapy and in combination with TransCon TLR7/8 Agonist. These findings are consistent with reports of pharmacological effects in mice for several IL-2 therapies based on native IL-2Rβ/γ interactions but lacking IL-2Rα binding potential.^{17,18,21} These results support combining TransCon IL-2 β/γ and TransCon TLR7/8 Agonist in the clinic and are further corroborated by their complementary biology affecting different steps in the cancer-immunity cycle.³⁶ TransCon TLR7/8 Agonist activates antigen-presenting cells and enhances antigen presentation to promote activation of cytotoxic immune cells,^{22–24} which can be further expanded and activated by TransCon IL-2 β/γ. Moreover, clinical data with NKTR-262 (TLR7/8 agonist) and bempedallesleukin (NKTR-214, PEGylated aldesleukin) show that the combination is well-tolerated and has PD activity.³⁷

The antitumor efficacy of TransCon IL-2 β/γ monotherapy in mice was statistically significant, while the overall tumor growth inhibition was quite modest compared with what has been reported for other IL-2 variants.^{13, 20, 38, 39} Unlike cynomolgus IL-2Rs that bind human IL-2 with similar affinity/potency as the human counterparts, and unlike mouse IL-2Rα which maintains good affinity to human IL-2, mouse IL-2Rβ has substantially lower potency for human IL-2.^{39, 40} Thus, high doses of TransCon IL-2 β/γ were required in mice, resulting in a narrow therapeutic window for assessing efficacy in mouse models. Additionally, multiple doses of TransCon IL-2 β/γ and higher dose levels were required in mice compared with monkeys to induce robust proliferative responses and increases in CD8⁺ T cell numbers.

In monkeys, a single dose of TransCon IL-2 β/γ demonstrated profound PD effects in multiple cytotoxic lymphocyte populations that lasted for at least 10 days. TransCon IL-2 β/γ increased ALC values (up to 27-fold at 0.3 mg/kg) with minimal increases in eosinophil counts, confirming IL-2Rβ/γ selectivity, which was in stark contrast to aldesleukin that increased both. Unlike aldesleukin, TransCon IL-2 β/γ showed minimal induction of IL-5 (associated with eosinophil activation), IL-6 (classical CRS marker) and biomarkers associated with endothelial damage (E-selectin, VCAM-1) as a surrogate measure of VLS.^{4, 5} Further, TransCon IL-2 β/γ was well-tolerated up to 0.3 mg/kg. There were no signs of CRS⁴¹ and no evidence of vascular damage, pulmonary edema or tissue necrosis indicative of VLS at any dose level tested after repeated dosing.

TransCon IL-2 β/γ administration to monkeys resulted in low IL-2 β/γ C_{max} and one of the longest effective half-lives of an active IL-2 (t_{1/2} > 30 hours) reported to our knowledge. Moreover, the C_{max} of IL-2 β/γ at 0.3 mg/kg (99 ng/mL), a dose with profound PD effects, was low and in the same range as the concentration needed to stimulate IL-2Rβ/γ⁺ CD8⁺ T cells in vitro (pSTAT5 EC₅₀: 62.2 ng/mL, online supplemental figure S6). This low C_{max} and long half-life enabled uniquely prolonged bioactive IL-2

β/γ levels which likely explain why such robust PD effects at tolerable doses were observed in monkeys compared with what has been reported for other IL-2 variants. Specifically, while max PD doses of bempedallesleukin (0.1 mg/kg) and THOR-707 (0.3 mg/kg) increased ALCs by approximately 5-fold¹³ and 4-fold,⁴⁰ respectively, TransCon IL-2 β/γ (≥ 0.3 mg/kg) increased ALCs by an average 24-fold. While nemvaleukin (0.1 mg/kg/day IV × 5, or 0.5 mg/kg SC on Days 1 and 4) increased CD8⁺ T cells by 6-fold and NK cells by 4-fold,⁴² and MDNA11 (0.6 mg/kg) increased CD8⁺ T cells by ~10-fold and NK cells by ~2.5-fold,³⁹ TransCon IL-2 β/γ (≥ 0.3 mg/kg) increased CD8⁺ T cells and NK cells by on average 19-fold and 26-fold, respectively. In agreement with other IL-2Rβ/γ agonists,⁴² higher expansion was observed in effector memory compared with total CD8⁺ T cells (53-fold vs 19-fold increases at 0.3 mg/kg TransCon IL-2 β/γ, respectively), which aligns with their reported higher IL-2Rβ expression levels.^{43, 44} Lastly, while effects on monkey γδ T cells, a cell type whose proliferative and cytolytic functions are known to be augmented by IL-2Rβ/γ engagement,⁴⁵ have not been reported for other IL-2 variants, ≥ 0.3 mg/kg of TransCon IL-2 β/γ increased γδ T cells by on average 403-fold. This is an important finding not just due to the magnitude of the expansion, but because γδ T cells possess considerable antitumor function and can recognize and kill cancer cells in an MHC-independent and neoantigen-independent manner.^{45, 46} In vitro, IL-2 β/γ potentiated antitumor functions of human γδ T cells including tumor cell killing and production of cytotoxic molecules and IFN-γ with minimal IL-17A induction. This is significant as pro-tumorigenic roles of IL-17A-producing γδ T cells have been reported⁴⁶ and IL-2 β/γ showed no evidence of inducing this phenotype.

Overall, TransCon IL-2 β/γ demonstrated the targeted pharmacological and PK properties of a novel IL-2 molecule and induced profound PD responses at tolerable doses. In monkeys, these responses were characterized by durable and potent expansion of multiple cytotoxic immune cell types, including CD8⁺ T cells, NK cells and γδ T cells, with minimal increases in eosinophils and Tregs, and with no signs of VLS or CRS. This is likely due to the complete and selective IL-2Rβ/γ bias along with the greatly extended exposure and low IL-2 β/γ C_{max} enabled by the TransCon prodrug technology. In this way, at a tolerable dose with low C_{max}, TransCon IL-2 β/γ can maintain IL-2 β/γ levels in a bioactive range for an extended period of time to achieve robust and durable PD responses of cytotoxic cell types with antitumor potential. Altogether, the responses seen in monkeys are suggestive of a potentially substantial improvement in therapeutic index beyond what has been achieved by aldesleukin and could bode well for combination therapies with complementary mechanisms. In fact, IL-2 variants have been combined with anti-PD-1 therapy and have shown early encouraging clinical results.⁴⁷ In this light, TransCon IL-2 β/γ is currently being evaluated in a clinical Phase

1/2 trial both as a monotherapy and in combination with pembrolizumab or standard of care chemotherapy (NCT05081609).

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Supplemental Figure Legends

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

Figure S2. Human PBMC data show that a 5 kDa mPEG is sufficient for abolishing IL-2R α 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-2R α 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)** EC₅₀ values.

Figure S3. IL-2 β/γ demonstrates higher cytotoxic potency compared to IL-2 variants with larger IL-2R α blocking mPEGs. **(A)** NK cell cytotoxicity responses were measured against K562 targets at a fixed E:T ratio of 1.25:1 and varying concentrations of IL-2 variants attached to 5, 10 or 30 kDa IL-2R α blocking mPEGs. Data are plotted as mean \pm 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 ratio of 5:1 and varying concentrations of IL-2 muteins attached to 5, 10 or 30 kDa IL-2R α blocking mPEGs. Data are plotted as mean \pm SEM. **(C)** Pooled data from both donors for CD8⁺ T cell and NK cell cytotoxicity assays plotted as mean \pm 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 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 EC₅₀ 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 \pm 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/ μ L and shown as mean \pm SEM. **(B)** Serum concentrations of sCD25, IL-5, IL-6, MCP-1, IFN- γ and TNF- α are presented as mean \pm SEM.

Figure S9. TransCon IL-2 β/γ increases the ratio of CD8⁺ T cells, NK cells and $\gamma\delta$ 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 $\gamma\delta$ T cells to Tregs expressed as the fold change from predose ratios and shown as mean \pm SEM. **(B)** Ratios of CD8⁺ T cells, NK cells and $\gamma\delta$ T cells to CD4⁺ T cells expressed as the fold change from predose ratios and shown as mean \pm 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 \pm SEM. **(B)** Percentages of proliferating (Ki67⁺) cells within Total (left) and effector memory (right) CD8⁺ T cells shown as mean \pm SEM.

Figure S11. TransCon IL-2 β/γ induces expansion of Granzyme B expressing CD8⁺ T cells, NK cells and, $\gamma\delta$ 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 $\gamma\delta$ T cells shown as mean \pm 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 \pm 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 \pm SEM (n=3 donors).

Figure S12C. IL-2 β/γ induces cytokine and cytotoxic effector molecules in human $\gamma\delta$ T cells. Supernatants were taken from $\gamma\delta$ 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 \pm SEM (n=2 donors).

Figure S13. IL-2 β/γ induces cytokine and cytotoxic effector molecules in endogenous TCR ligand activated human $\gamma\delta$ T cells. $\gamma\delta$ T cells were cocultured overnight with Daudi lymphoma cells. **(A)** $\gamma\delta$ T cell cytotoxicity results against Daudi target cells after subtracting for background seen in conditions with targets alone. Pooled data are expressed as mean \pm SEM (n=3 donors). **(B, C)** Supernatants were taken from $\gamma\delta$ 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 \pm 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-2R α interactions yet maintaining IL-2R β / γ interactions

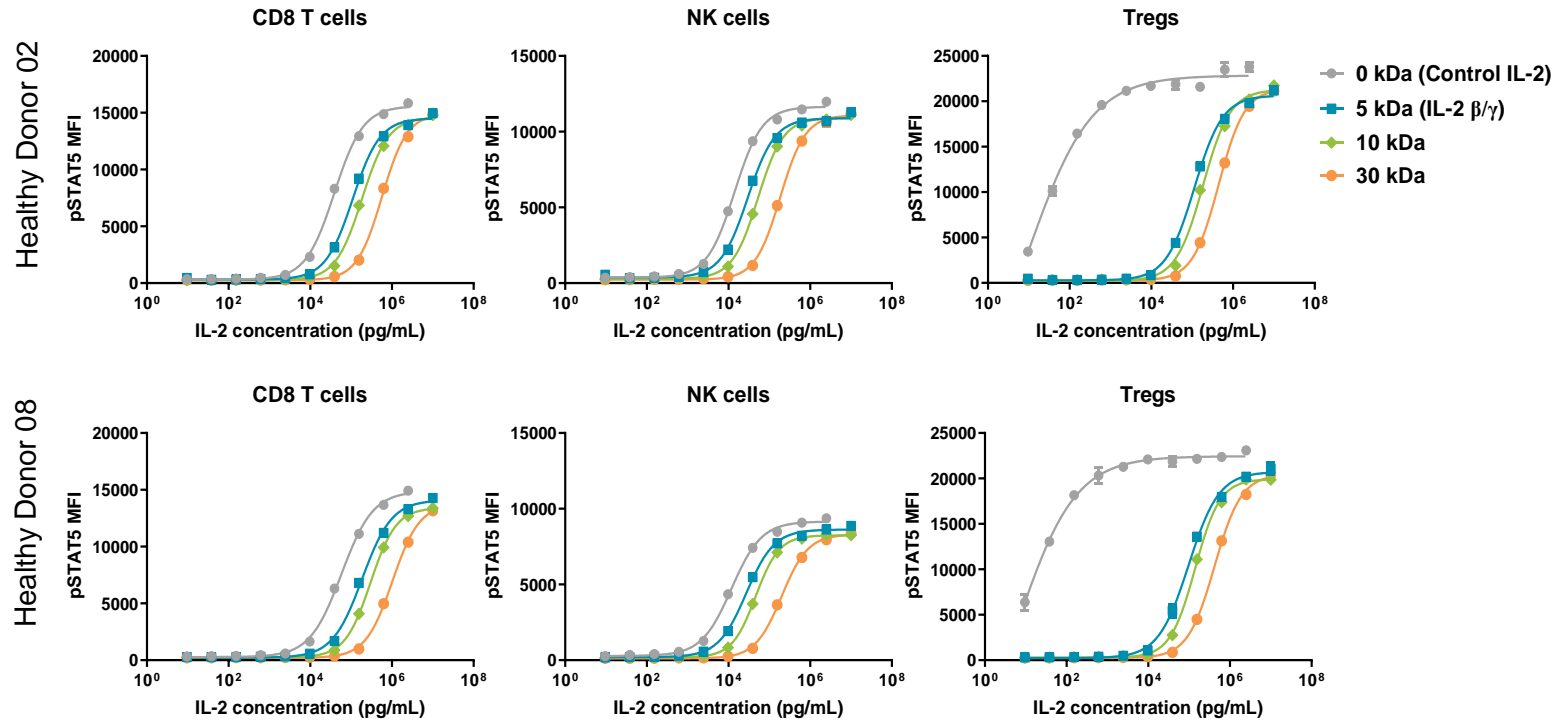
Size of biasing PEG residue	Biacore analysis (Kd values)	
	Binding towards IL-2R α	Binding towards IL-2R β
NA (IL-2 control)	9.3 nM	280 nM
2 kDa	700 nM	670 nM
5 kDa	> 2,000 nM	1,000 nM
10 kDa	> 2,000 nM	1,900 nM
20 kDa	> 3,000 nM	4,500 nM

No detectable binding towards IL-2R α



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

A)

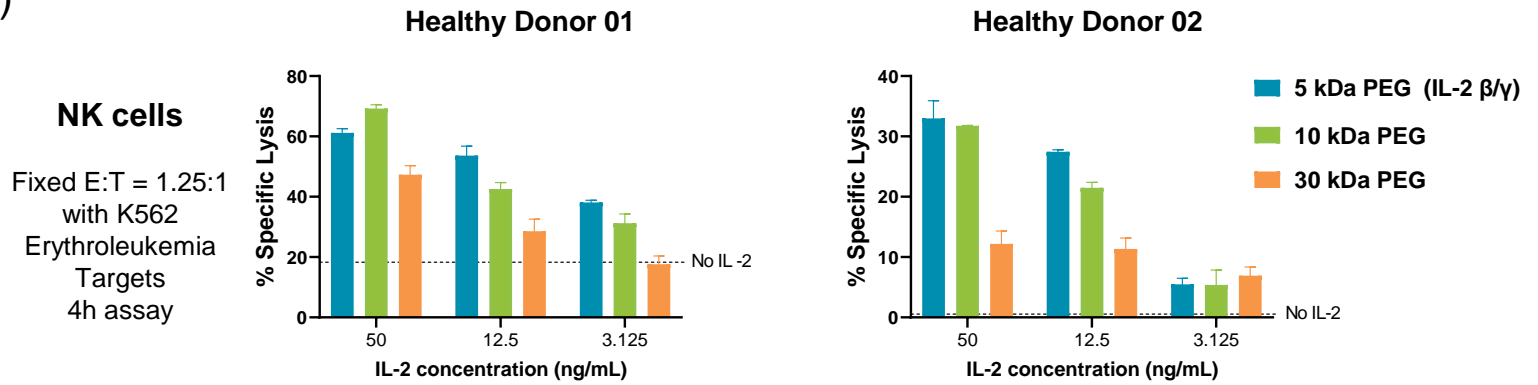


B)

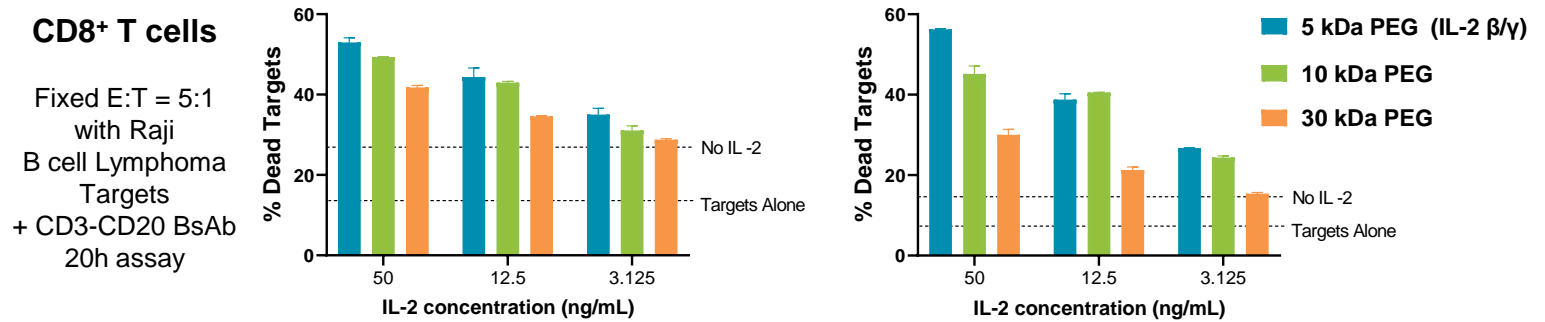
Size of biasing PEG residue	pSTAT5 EC ₅₀ potency analysis (Human PBMCs)		
	CD8 ⁺ T cells (IL-2R β/γ potency)	NK cells (IL-2R β/γ potency)	Tregs (IL-2R $\alpha/\beta/\gamma$ potency)
NA (IL-2 control)	48.75 ng/ml	13.06 ng/ml	< 0.1 ng/ml
5 kDa	148.31 ng/ml	28.41 ng/ml	105.47 ng/ml
10 kDa	242.50 ng/ml	49.41 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

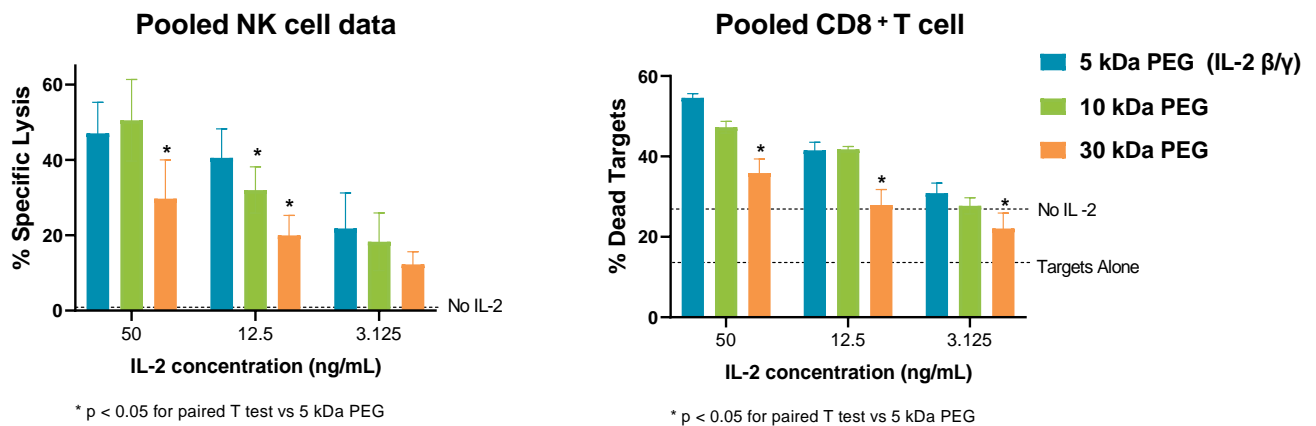
A)



B)



C)



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

MC38 (individual mouse responses)

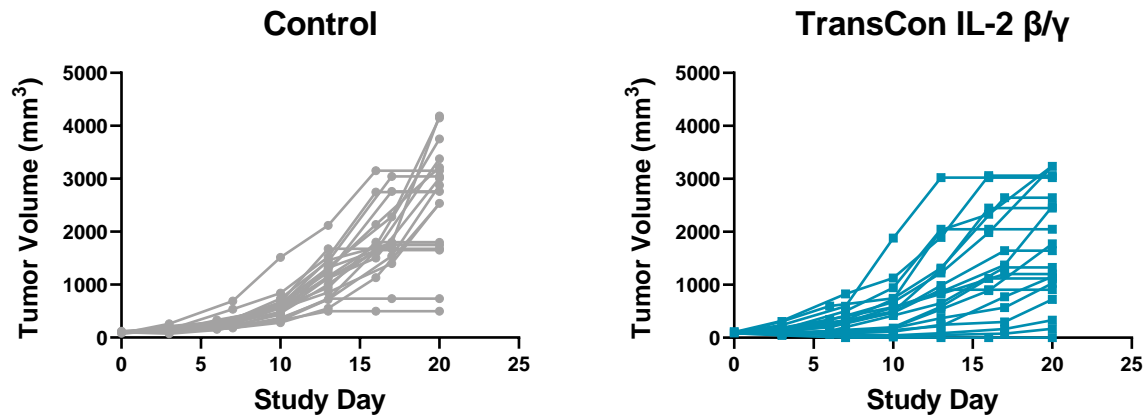
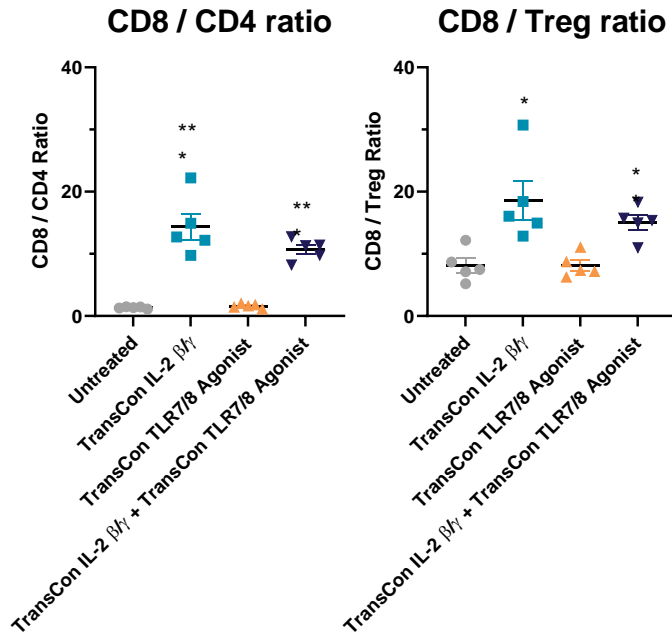


Figure S5A: TransCon IL-2 β/γ increases CD8⁺ / CD4⁺ and CD8⁺ / Treg ratios in mice

Blood



Spleen

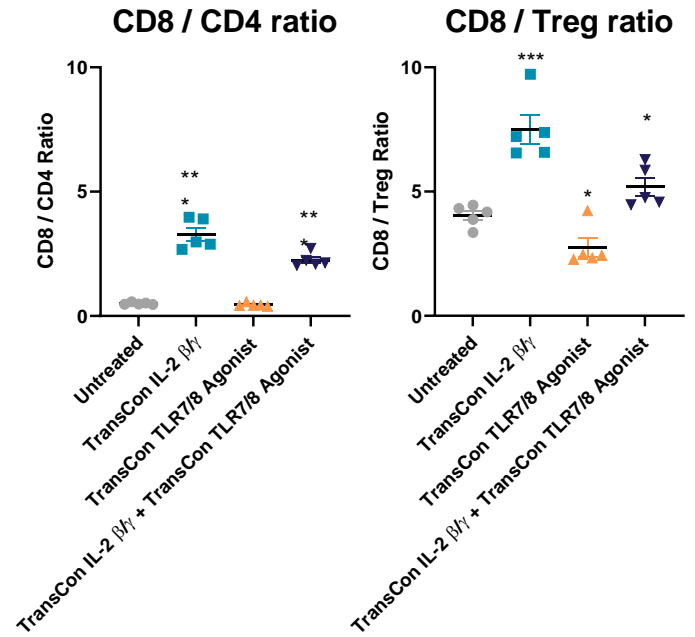
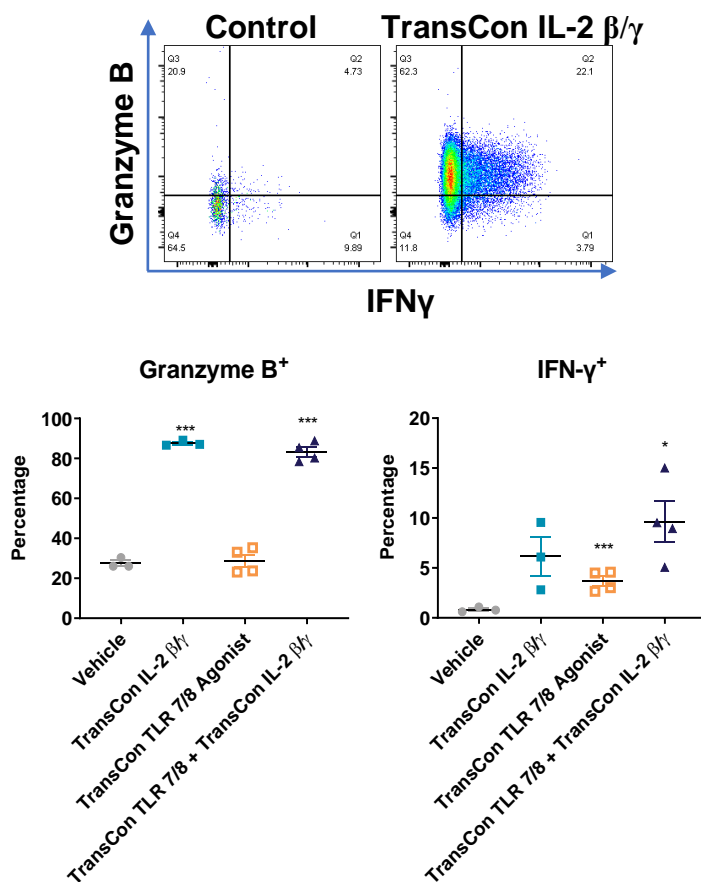


Figure S5B: TransCon IL-2 β/γ robustly activates effector function potential in CD8⁺ T and NK cells in mice

Example Gating

NK cells



CD8⁺ T cells

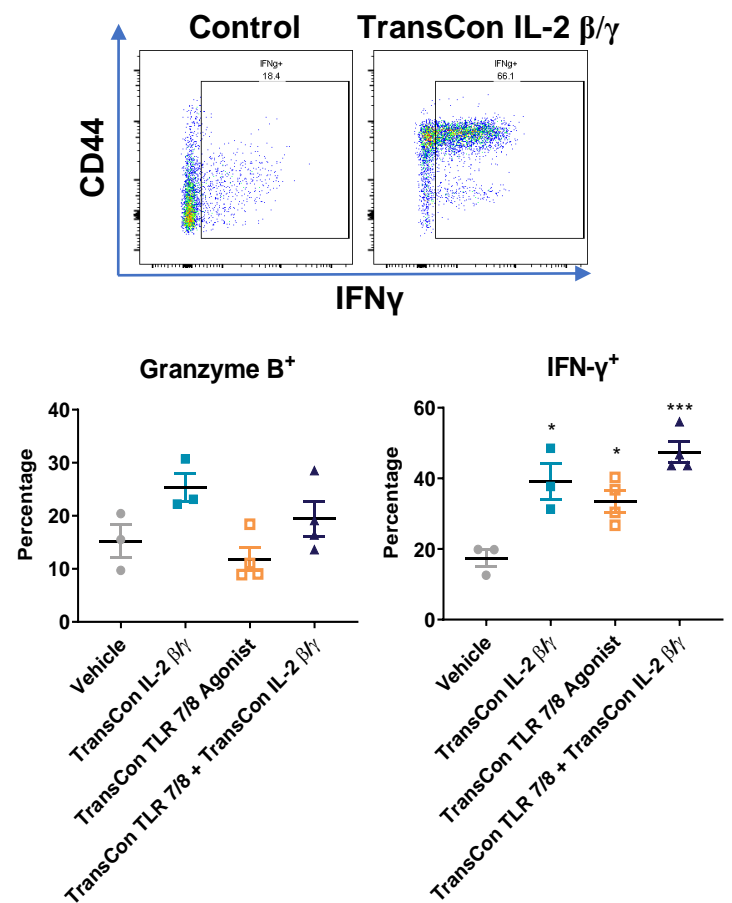
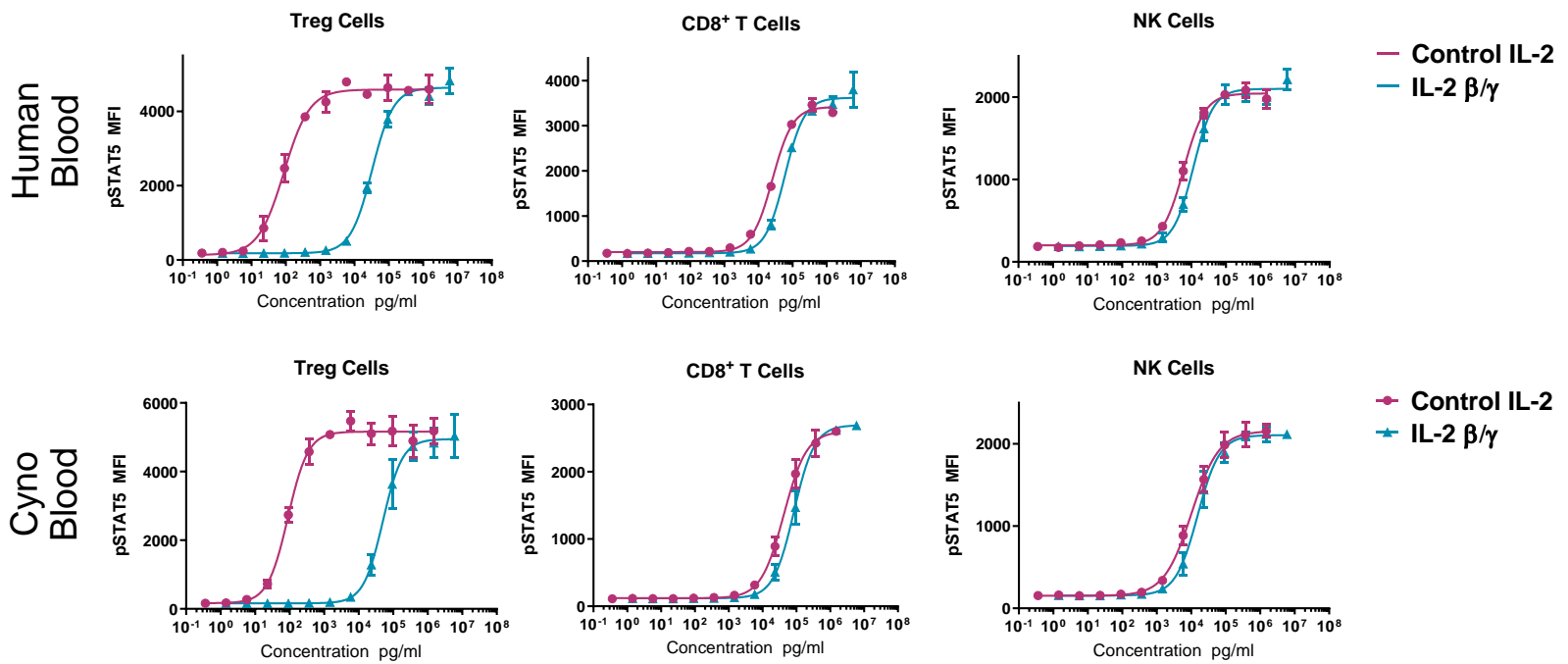


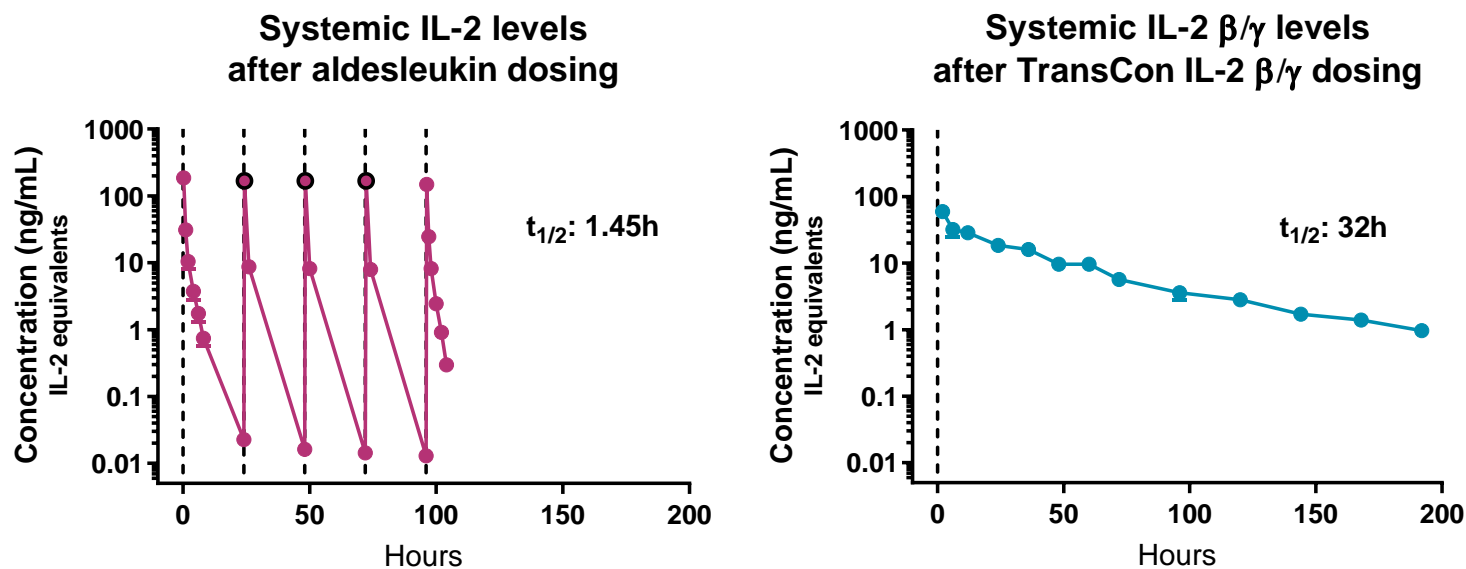
Figure S6: IL-2 β/γ is IL-2R β/γ selective in human and cynomolgus monkey cells



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 inputted 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

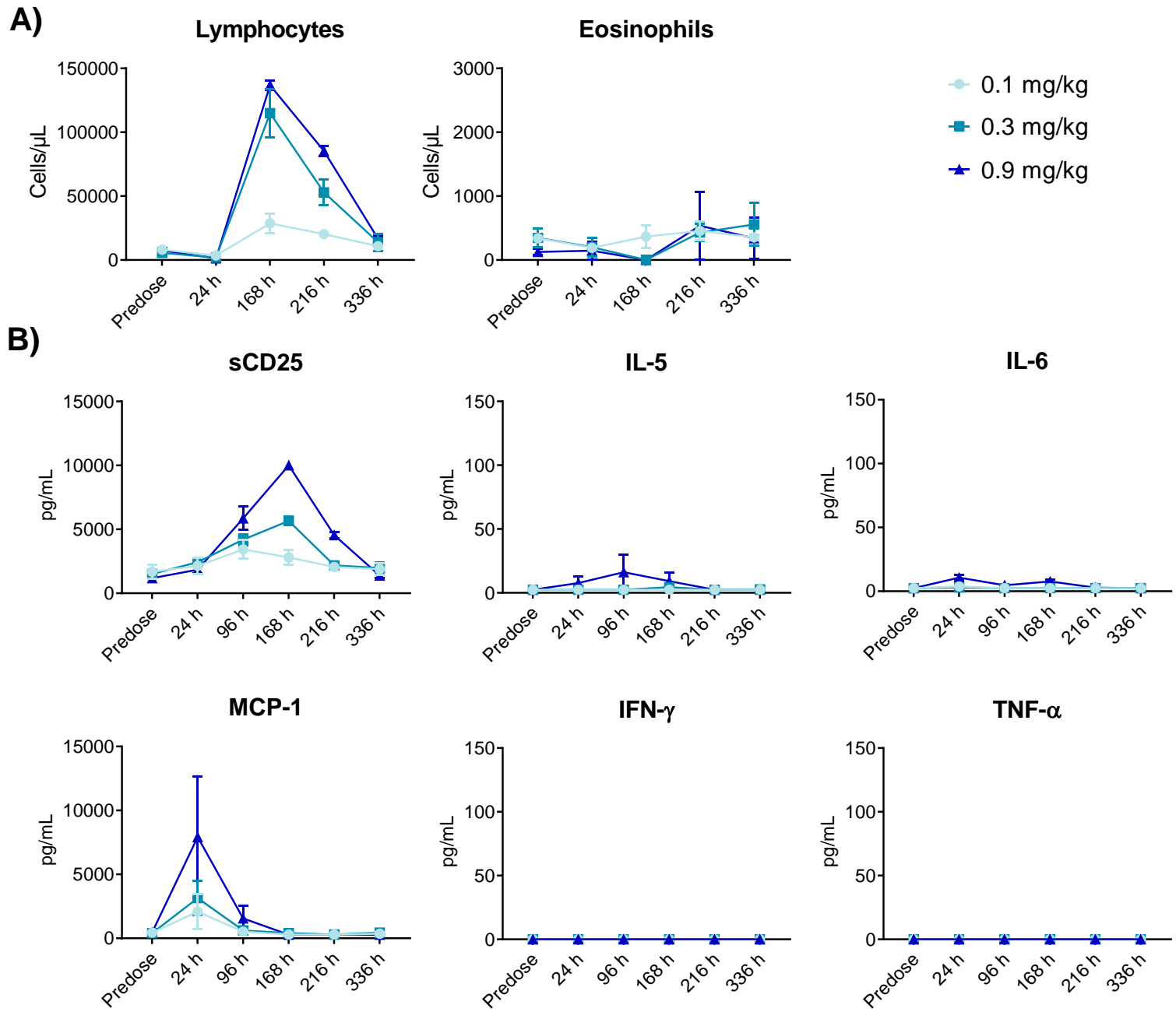
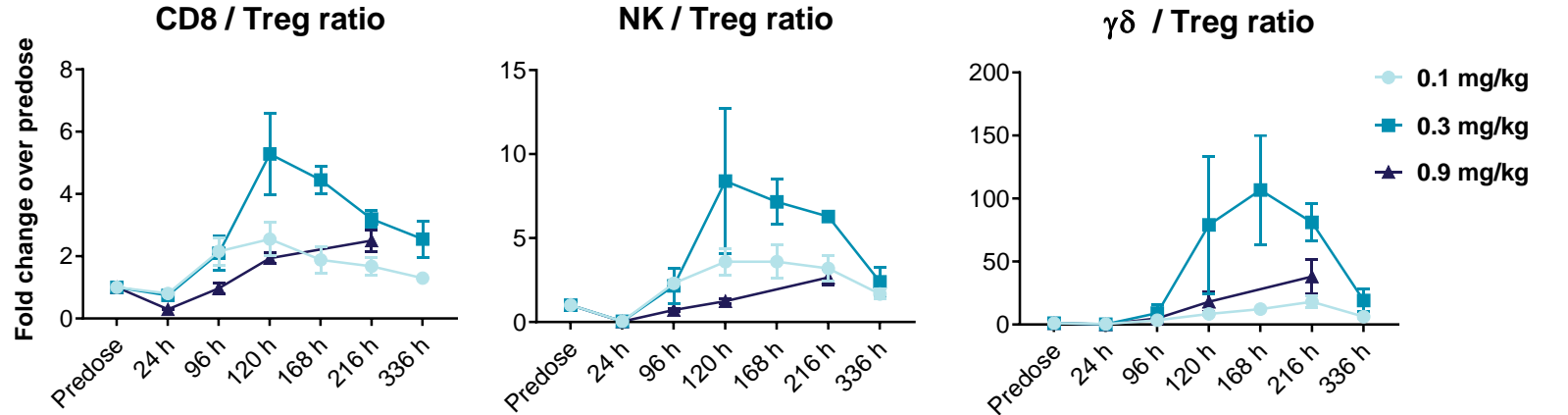


Figure S9: TransCon IL-2 β/γ increases the ratio of CD8⁺ T cells, NK cells and $\gamma\delta$ T cells to Tregs or CD4⁺ T cells in cynomolgus monkeys

A)



B)

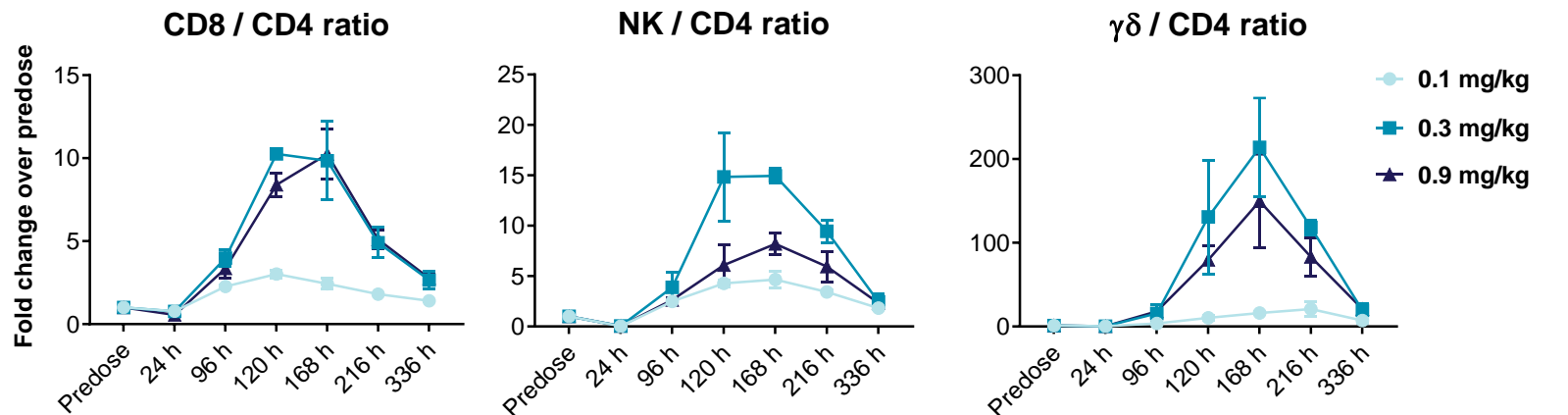
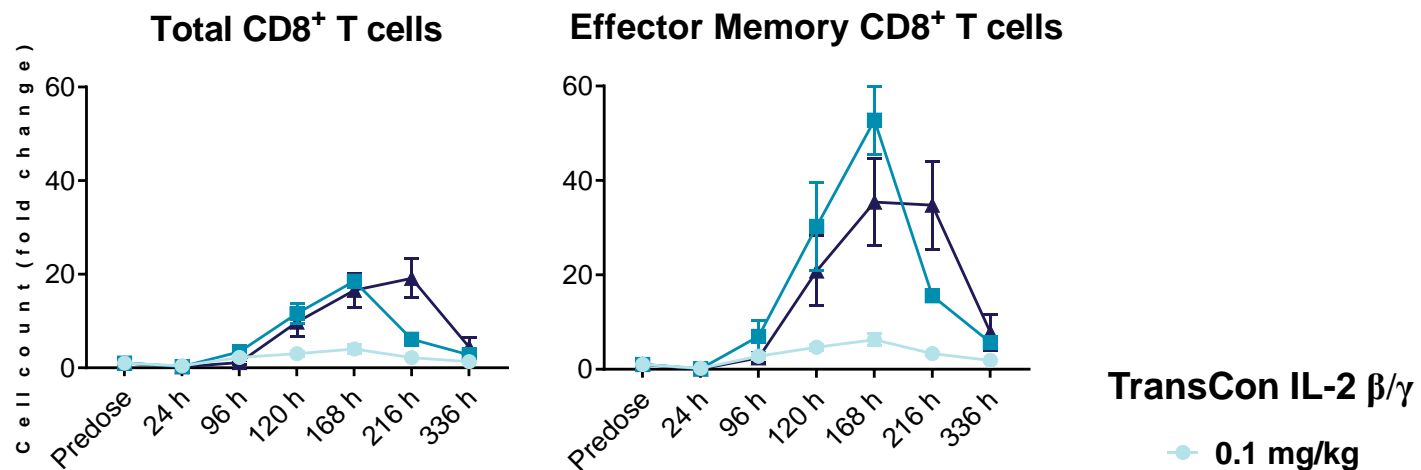


Figure S10: TransCon IL-2 β/γ induces more potent expansion of effector memory CD8⁺ T cells compared to total CD8⁺ T cells in cynomolgus monkeys

A)



B)

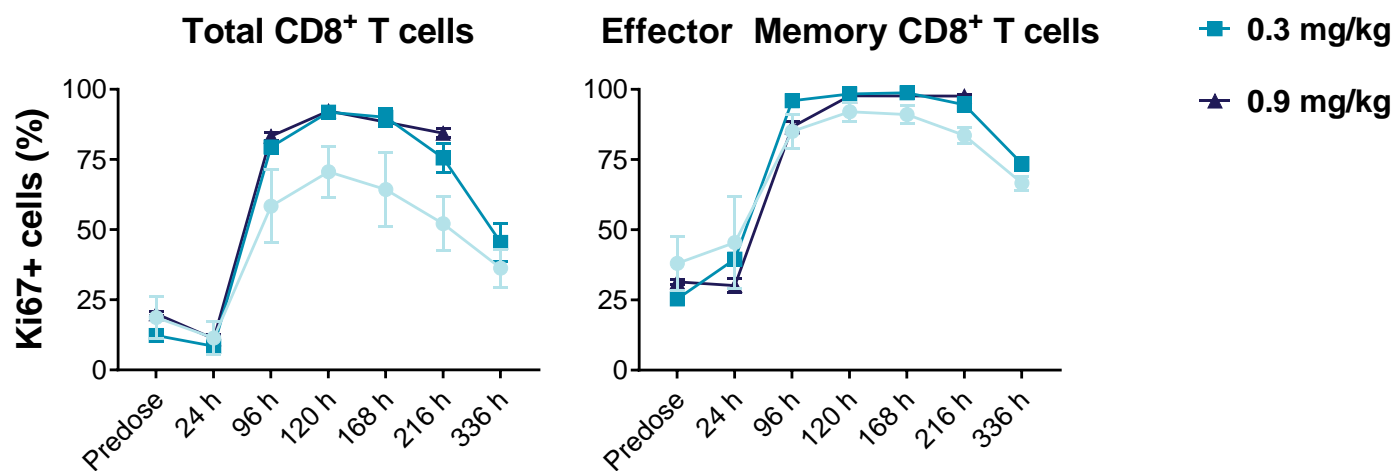


Figure S11: TransCon IL-2 β/γ induces expansion of Granzyme B expressing CD8⁺ T cells, NK cells and, $\gamma\delta$ T cells.

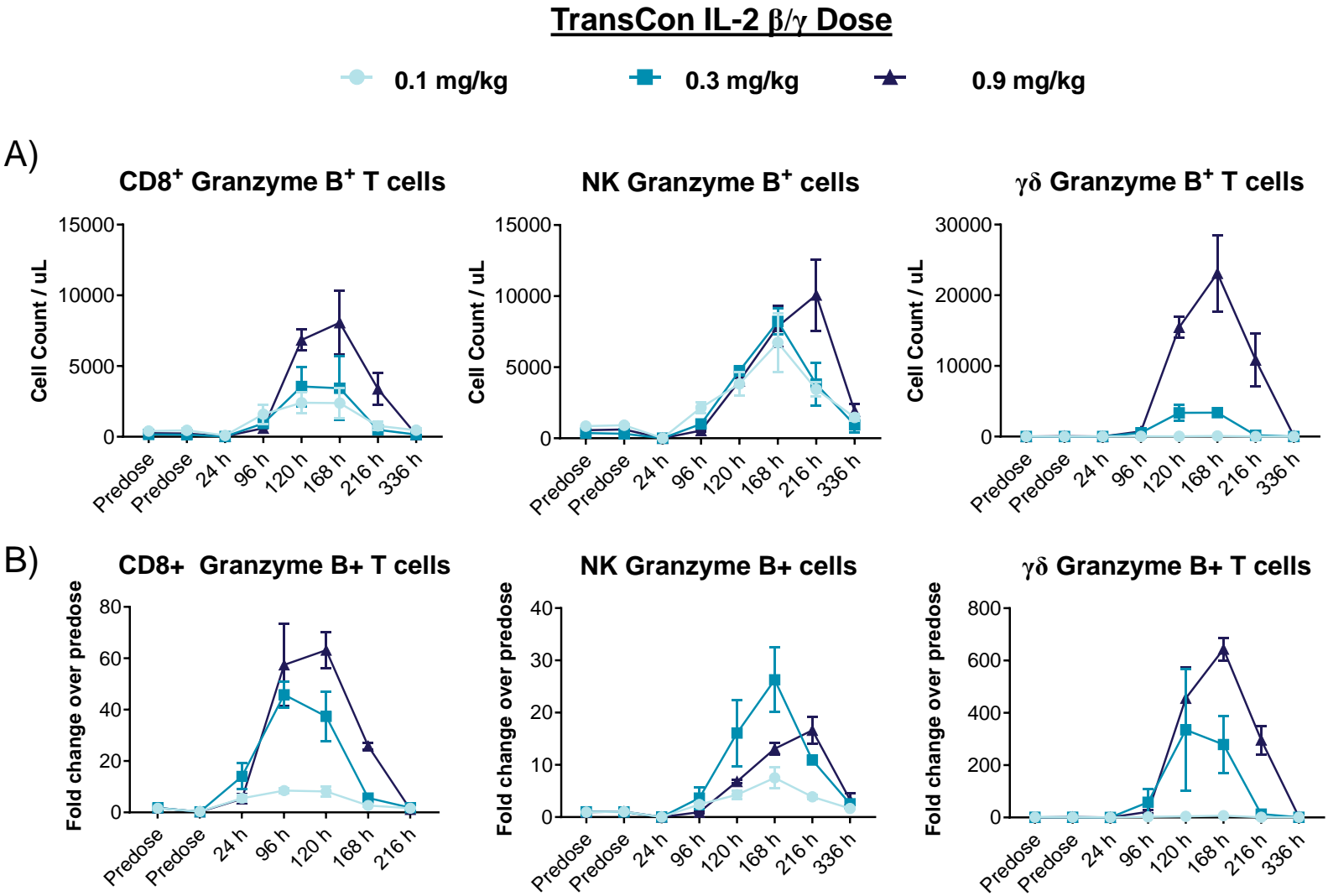


Figure S12A: IL-2 β/γ induces cytokine and cytotoxic effector molecules in human CD8+ T cells

Overnight supernatants from Raji co-culture + CD3-CD20 TCR stimulus

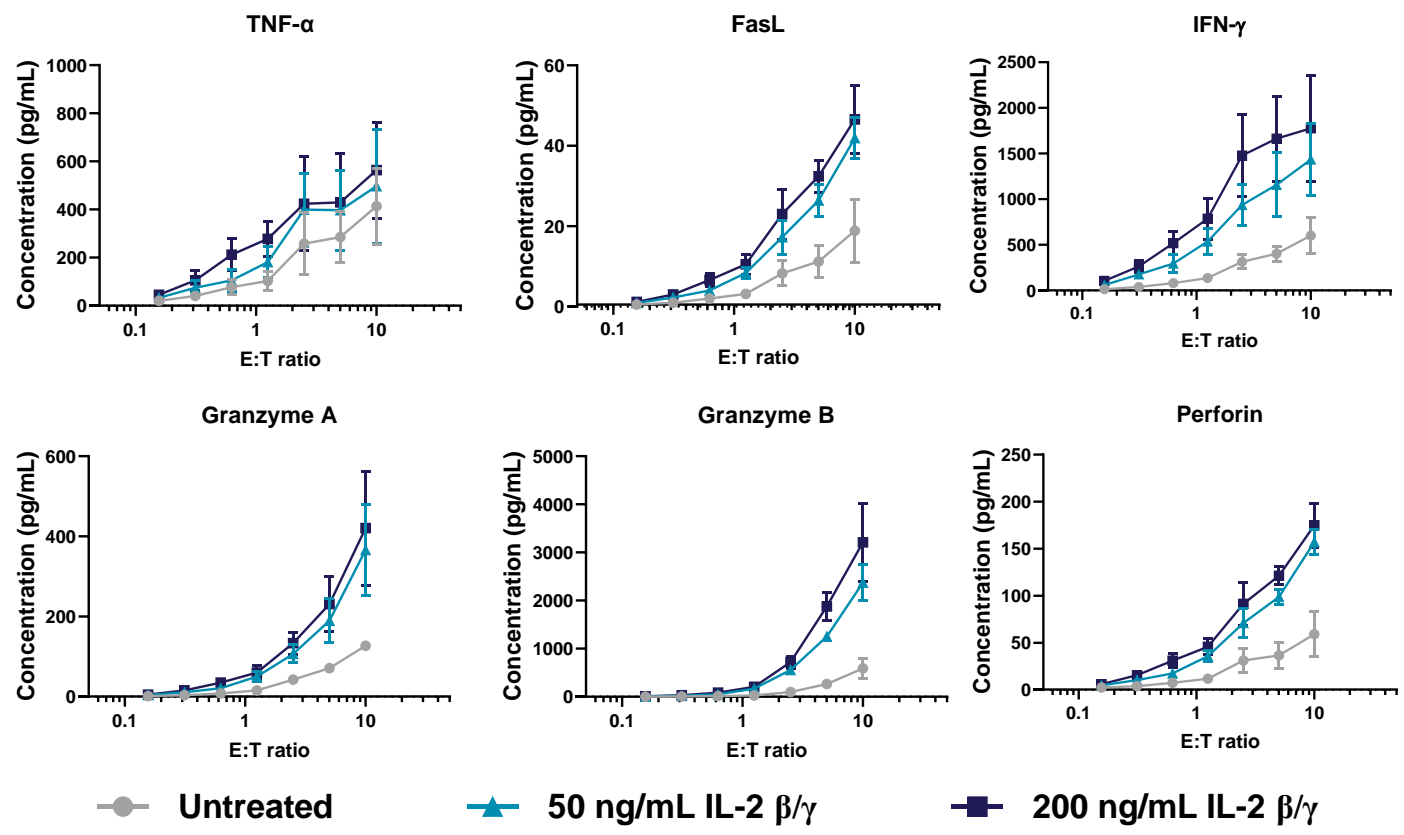


Figure S12B: IL-2 β/γ induces cytokine and cytotoxic effector molecules in human NK cells

4h supernatants from K562 co-culture

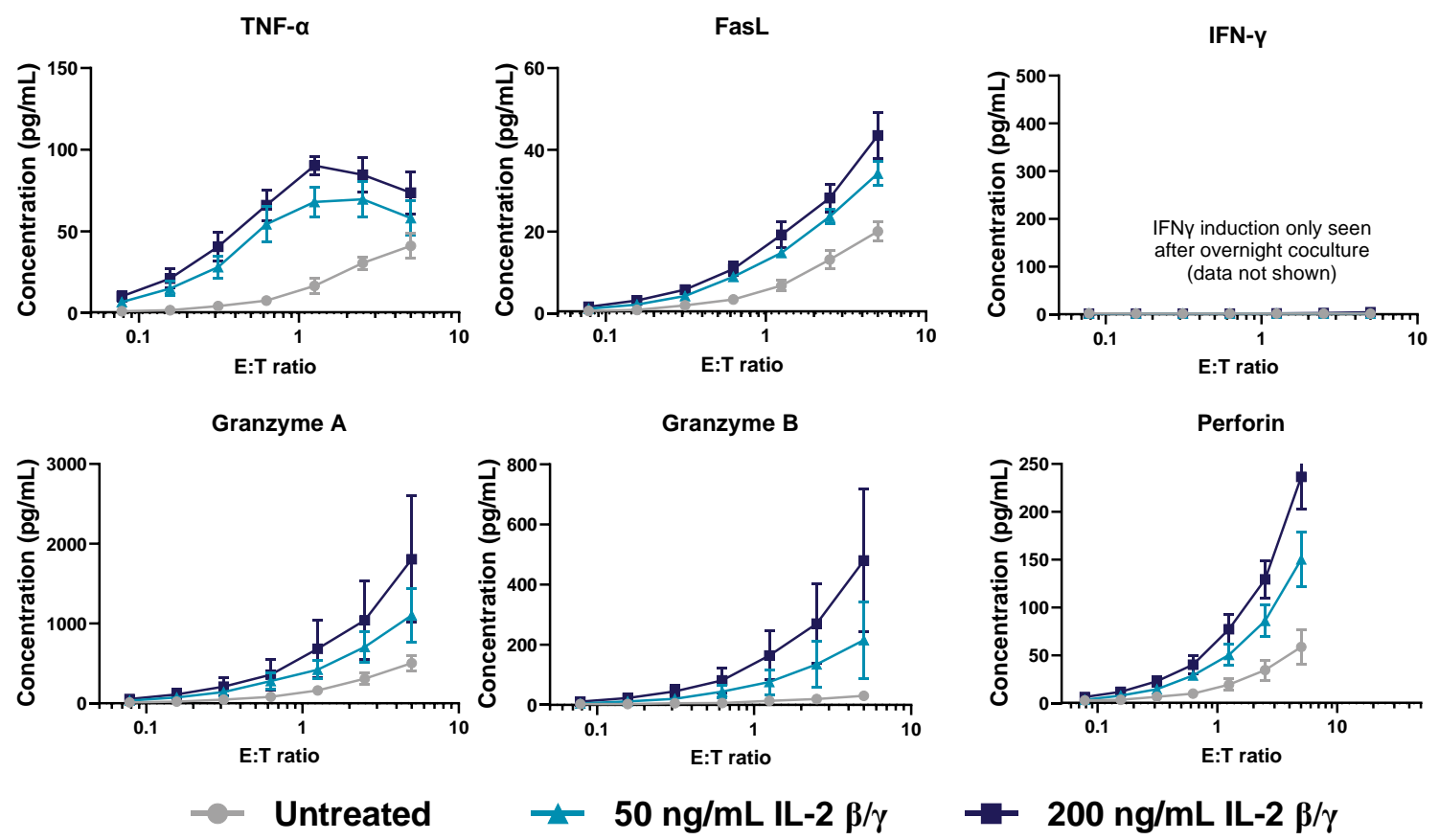


Figure S12C: IL-2 β/γ induces cytokine and cytotoxic effector molecules in human $\gamma\delta$ T cells

Overnight supernatants from Raji co-culture + CD3-CD20 TCR stimulus

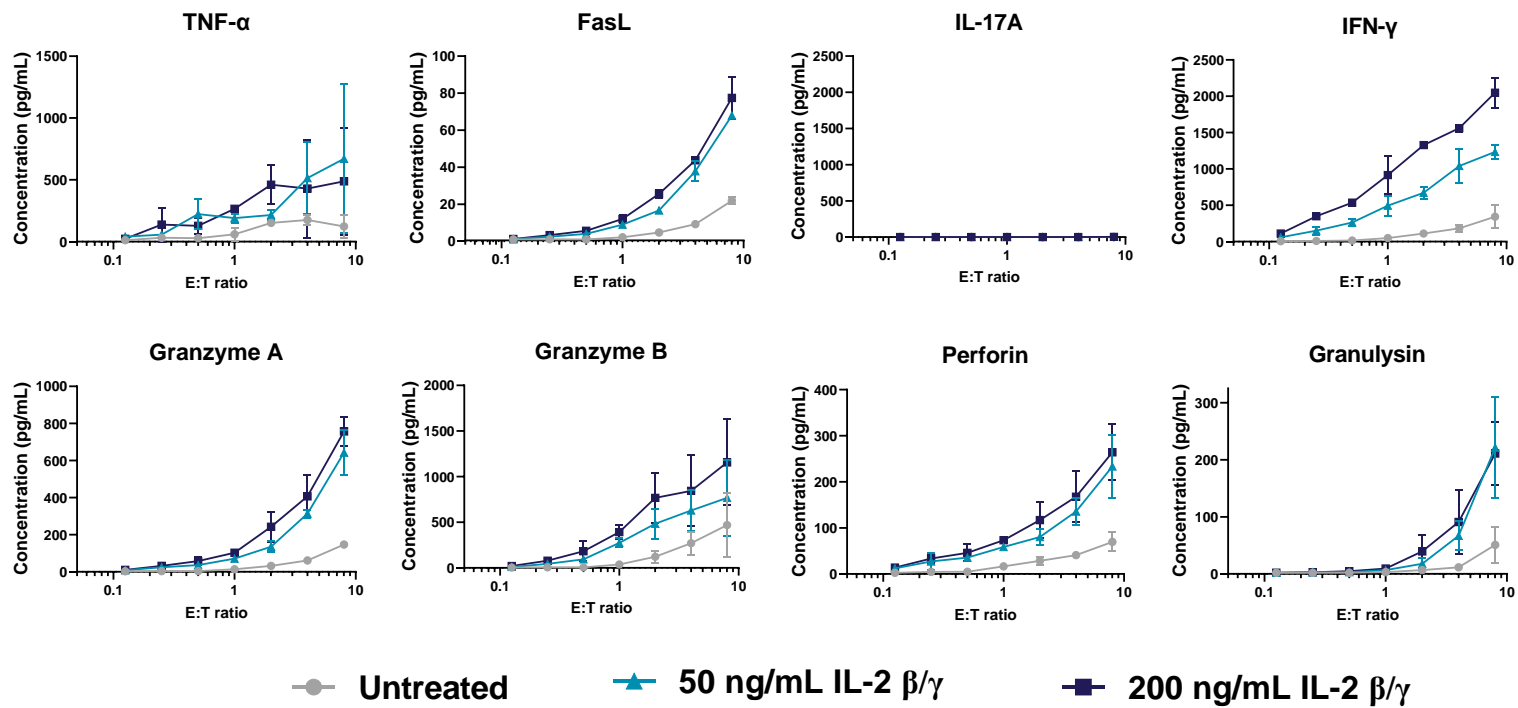
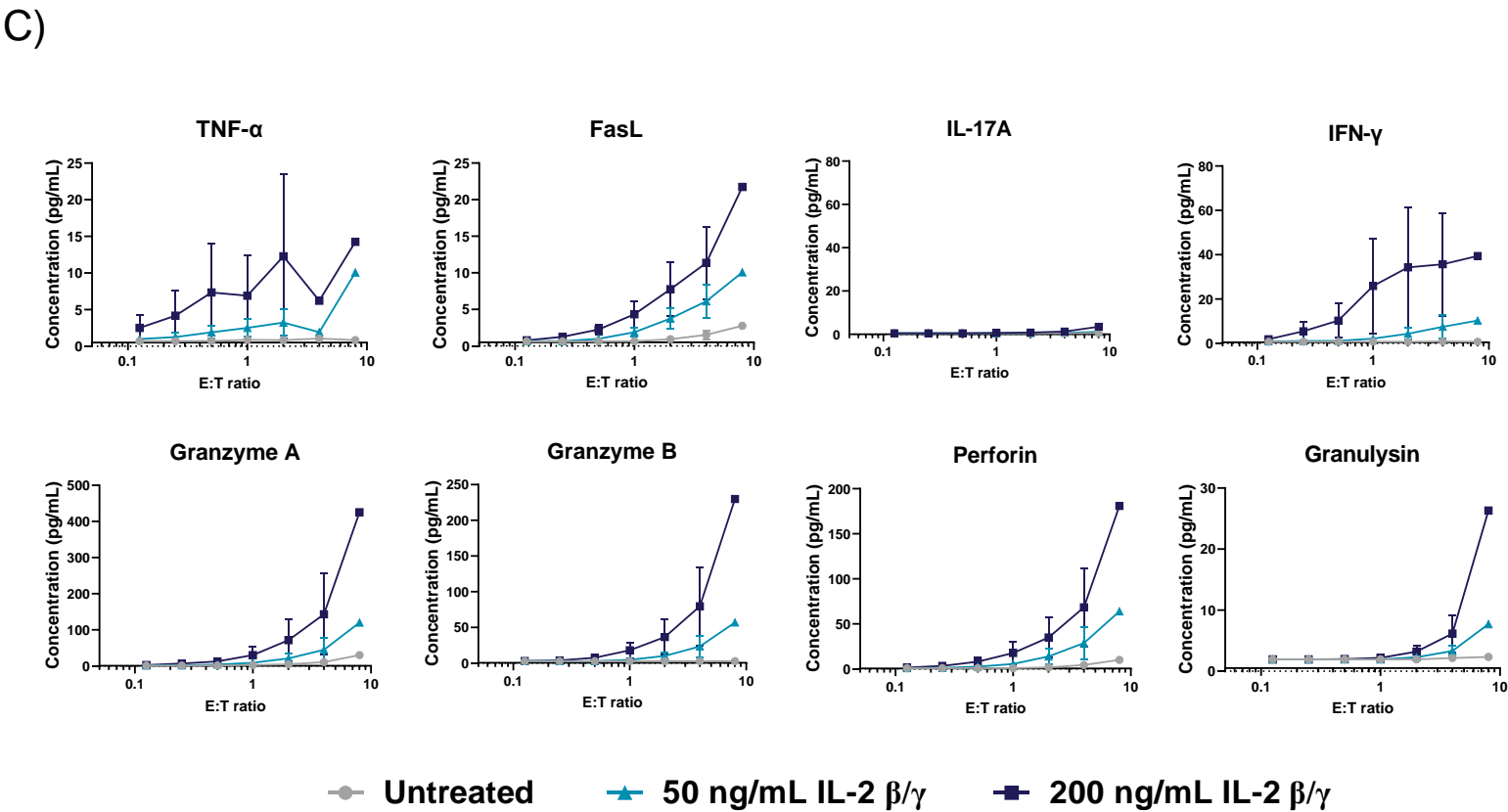
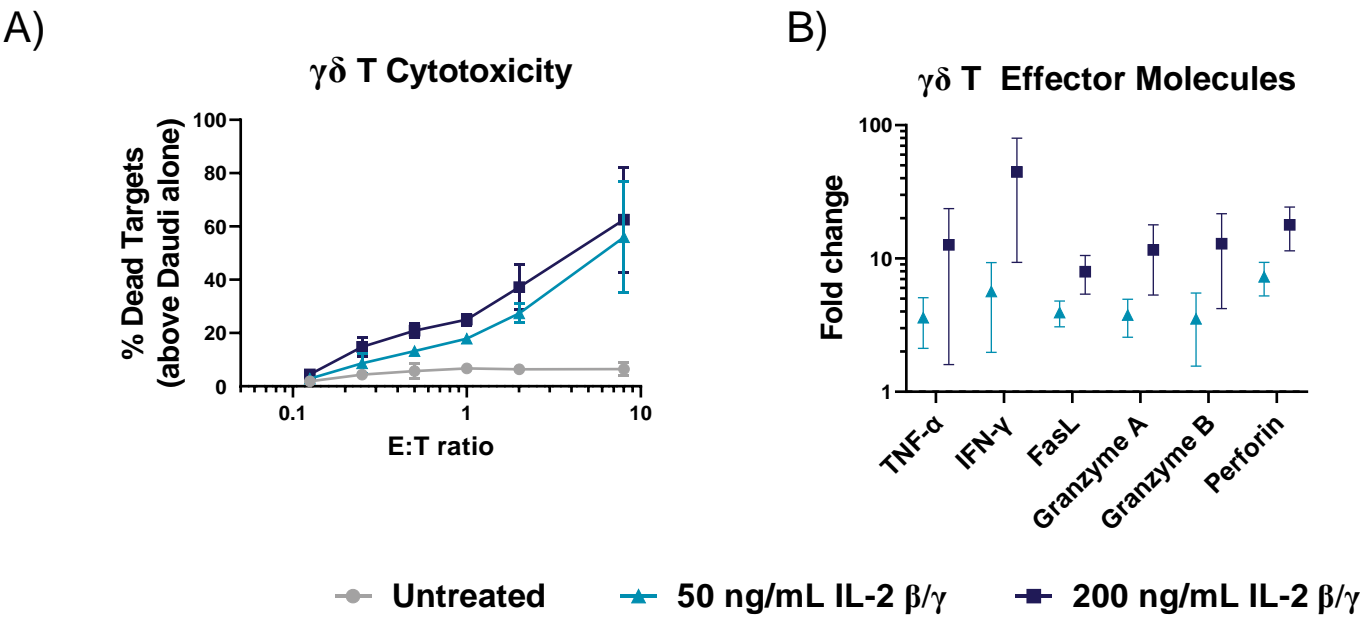


Figure S13 IL-2 β/γ induces cytotoxicity and cytokine production in endogenous TCR ligand activated human $\gamma\delta$ T cells (Daudi coculture)



Supplemental Methods (SM)

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

IL-2 containing an engineered cysteine within the IL-2R α 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-2R α 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-2R α interactions yet maintain IL-2R β/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 (EC₅₀) 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 EC₅₀ 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 EC₅₀ 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 $\gamma\delta$ 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 V δ 2	BV421	331428	B6	Biolegend
TCR V γ 9	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+
$\gamma\delta$ T cells	CD14-CD45+CD3+CD4-CD8- $\gamma\delta$ TCR+
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-2R α 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 $\gamma\delta$ T cell (average 78% purity overall with 98% purity within T cells) populations using negative selection kits (EasySepTM Human NK Cell Isolation Kit, EasySepTM Human CD8⁺ T Cell Isolation Kit, and EasySepTM Human Gamma/Delta T Cell Isolation Kit from StemCell Technologies; and TCR γ/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 $\gamma\delta$ 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, Granzyme C, 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.