TransCon IL-2 β/γ: a novel long-acting prodrug with sustained release of an IL-2Rβ/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-2Rβ/γ$^+$ natural killer (NK) cells and CD8$^+$, CD4$^+$ and gamma delta (γδ) T cells. However, IL-2 also potently activates immunosuppressive IL-2Rα$^+$ regulatory T cells (Tregs) and IL-2Rα$^+$ 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-2Rα$^+$ 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-2Rα binding yet retain strong IL-2Rβ/γ activity, IL-2 β/γ was created by permanently attaching a small methoxy polyethylene glycol (mPEG) moiety in the IL-2Rα 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-2Rβ/γ binding and activation without IL-2Rα 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

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 γδ T cells, relative to CD4$^+$

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Half-life extended IL-2Rβ/γ-selective interleukin-2 (IL-2) variants have shown promise for reducing IL-2Rα-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-2Rβ/γ-selective IL-2 variant (IL-2 β/γ) containing a small polymer to block IL-2Rα binding. Importantly, IL-2 β/γ resembles endogenous IL-2 in size, sequence and IL-2Rβ/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-2Rα$^+$ eosinophils and with no signs of vascular leak syndrome or cytokine release syndrome in cynomolgus monkeys.

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 γδ T cells.

Summary TransCon IL-2 β/γ is a novel long-acting prodrug with sustained release of an IL-2Rβ/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 (γδ) T cells and CD4^{+} and CD8^{+} effector T cells, but also IL-2Rα^{+} immunosuppressive regulatory T cells (Tregs) that likely limit efficacy.[1 6] IL-2 can signal with picomolar affinity to trimeric IL-2 receptor IL-2Rα/β/γ or with nanomolar affinity through dimeric IL-2Rβ/γ.[1] T cells and NK cells can upregulate IL-2Rα on activation, while Tregs constitutively express high IL-2Rα levels.[7] Therefore, IL-2 potently activates Tregs compared with IL-2Rβ/γ^{+} CD8^{+} T cells, γδ T cells and NK cells that possess antitumor function. Also, IL-2 potently activates IL-2Rα^{+} eosinophils, type 2 innate lymphoid cells and endothelial cells, which may promote VLS and limit the therapeutic window.[8–11] In addition to IL-2Rα 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-2Rβ/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-2Rβ/γ selectivity,[13 14] reduced IL-2Rβ/γ potency[15] 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-2Rα[+] 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, γδ 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-2Rα 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-2Rα or IL-2Rβ

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-2Rα-Fc (Symansis) or human IL-2Rβ-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-2Rα/β/γ^{+} Tregs and IL-2Rβ/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 mm^3) 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 rechallenge were challenged again with $5×10^5$ EMT-6 tumor cells (ATCC) in the right front flank.

In the MC38 model, female C57BL/6 mice were inoculated SC with $2×10^6$ MC38 colon carcinoma cells (ATCC). Mice with established tumors (average 95 mm^3) 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=(length×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 mm^3) 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, γδ 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 γδ 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. γδ 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-2Rβ/γ selective IL-2 variant with minimal IL-2Rα binding

To generate an optimally biased IL-2 compound, selective for IL-2Rβ/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-2Rα/β/γ and IL-2Rβ/γ (data not shown). We therefore reasoned that specific inhibition of IL-2Rα binding should be achieved by steric hindrance at the IL-2Rα binding interface. An IL-2 mutein was generated by introducing an unpaired cysteine within the IL-2Rα 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-2Rα interactions, permanently PEGylated IL-2 muteins were characterized for IL-2Rα versus IL-2Rβ subunit binding using Biacore. While an mPEG size of 2 kDa maintained some IL-2Rα interactions, mPEG sizes from 5 kDa and above completely blocked the interaction with IL-2Rα (online supplemental figure S1). Within the mPEG sizes that blocked IL-2Rα (5, 10, and 20 kDa mPEGs), increasing mPEG size was associated with decreasing affinity for IL-2Rβ (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-2Rα interactions yet maintain IL-2Rβ 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 EC_{50} of 34.5 vs 0.1 ng/mL; CD8^{+} EC_{50} of 62.2 vs 25.7 ng/mL; and NK cell EC_{50} 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-2Rα/β/γ^{+} Tregs with well-maintained potency in IL-2Rβ/γ^{+} CD8^{+} T cells and NK cells is consistent with loss of IL-2Rα binding and IL-2Rβ/γ selectivity for IL-2 β/γ (figure 1D). In agreement with IL-2Rβ 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-2Rβ/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 technology[27] (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 mm[3] vs 2653 mm[3], 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 mm[3] vs 1032 mm[3], 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 mm[3] vs 2562 mm[3], 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
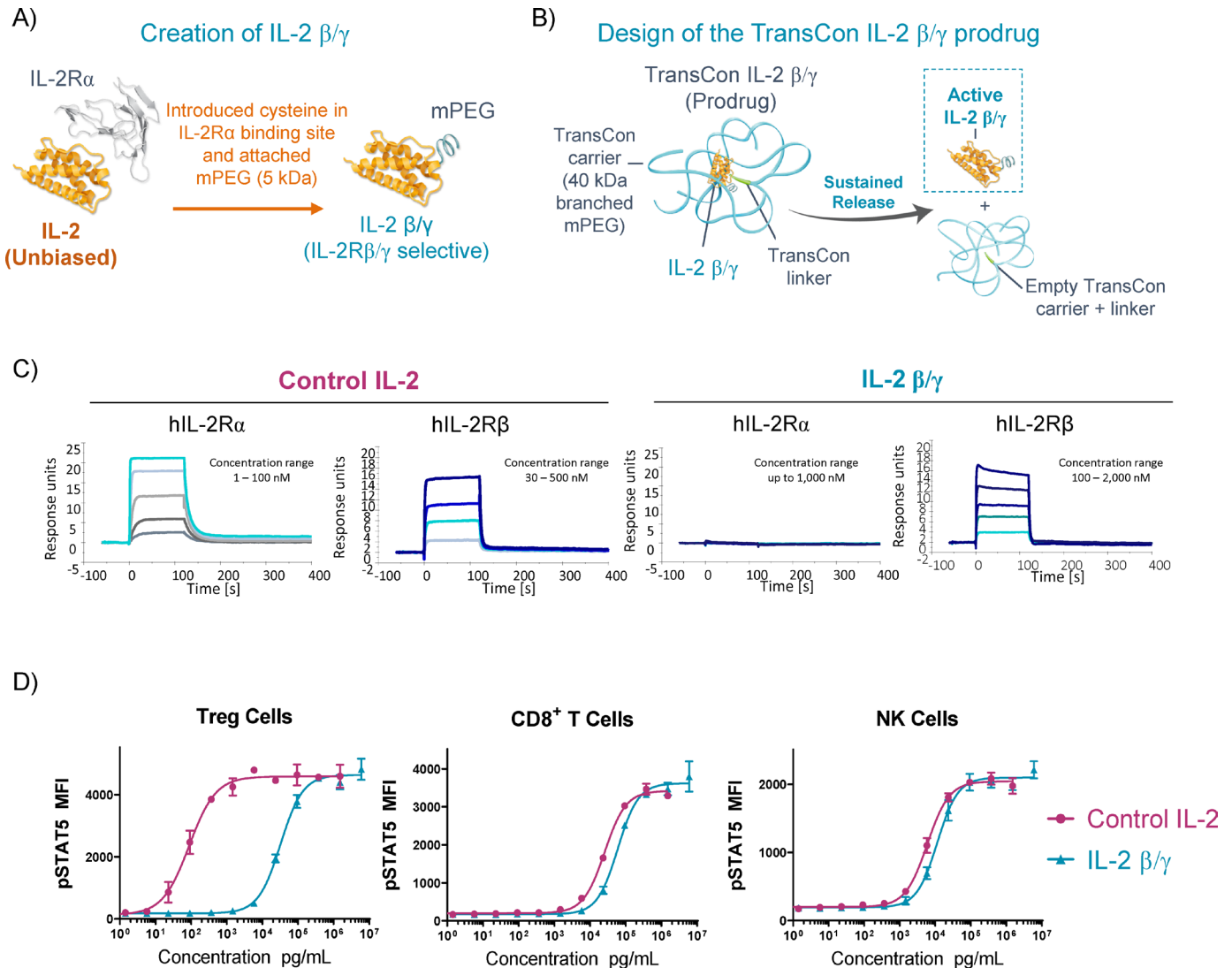
Figure 1 TransCon IL-2 β/γ is a novel IL-2 variant designed for IL-2Rβ/γ receptor selectivity and improved PK properties. (A) Design of IL-2 β/γ via introduction of a permanently attached 5 kDa mPEG to an engineered cysteine at the IL-2Rα binding site of IL-2. (B) Design of the TransCon IL-2 β/γ prodrug 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-2Rα or human IL-2Rβ. (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±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-2Rβ/γ selectivity and pSTAT5 potency results as seen with human cells (online supplemental figure S6). To confirm IL-2Rβ/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
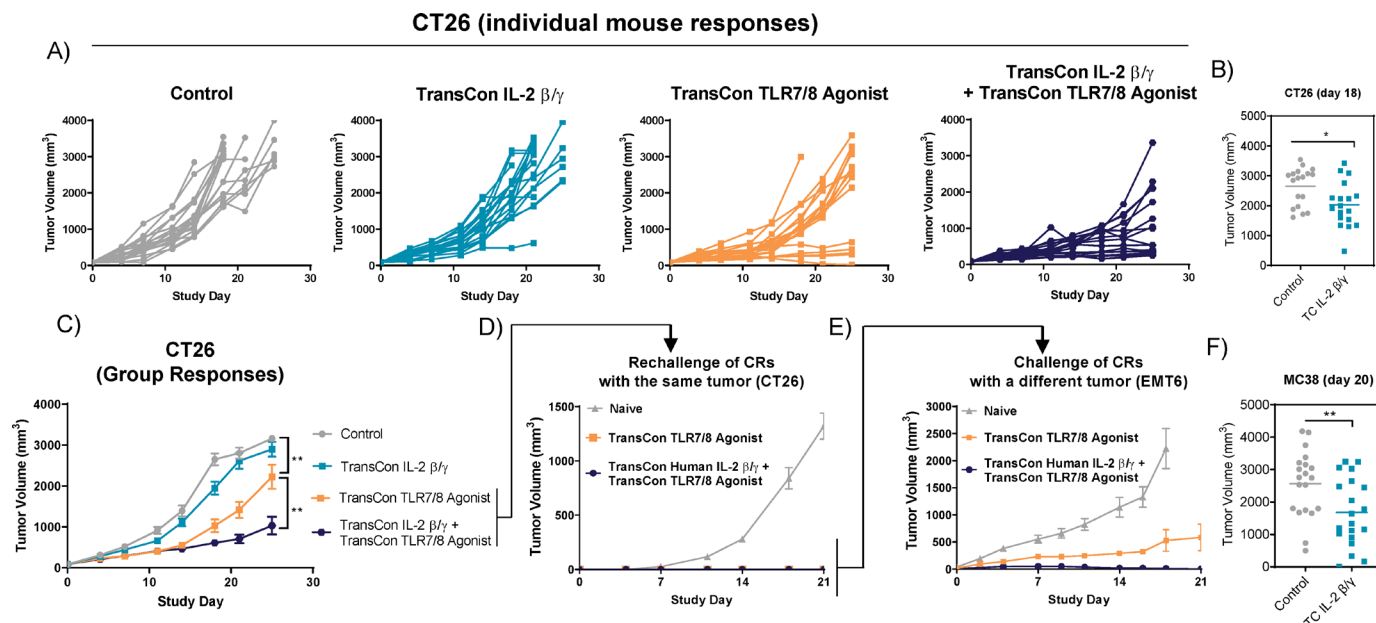
Figure 2 TransCon IL-2 β/γ shows antitumor effects in vivo and enhances TransCon TLR7/8 Agonist therapy and establishment of immune memory. (A–C) Female BALB/c mice were inoculated with either (i) 0.5 or (ii) 2.0×10⁶ CT26 cells. Mice with established tumors were either (i) treated on Days 0, 6, and 16 with buffer or 60 µg (approximately 3 mg/kg) TransCon IL-2 β/γ intravenously and/or a single 134 µg (approximately 6.7 mg/kg) intratumoral dose (on Day 0) of TransCon TLR7/8 Agonist (n=8/group), or (ii) untreated or treated on Days 0, 7, and 14 with 72 µg (approximately 3.6 mg/kg) TransCon IL-2 β/γ intravenously and/or a single 100 µg (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 (±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±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 ±SEM are shown. (F) 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). Tumor volumes Day 20 are shown as mean±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 γδ 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 γδ 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
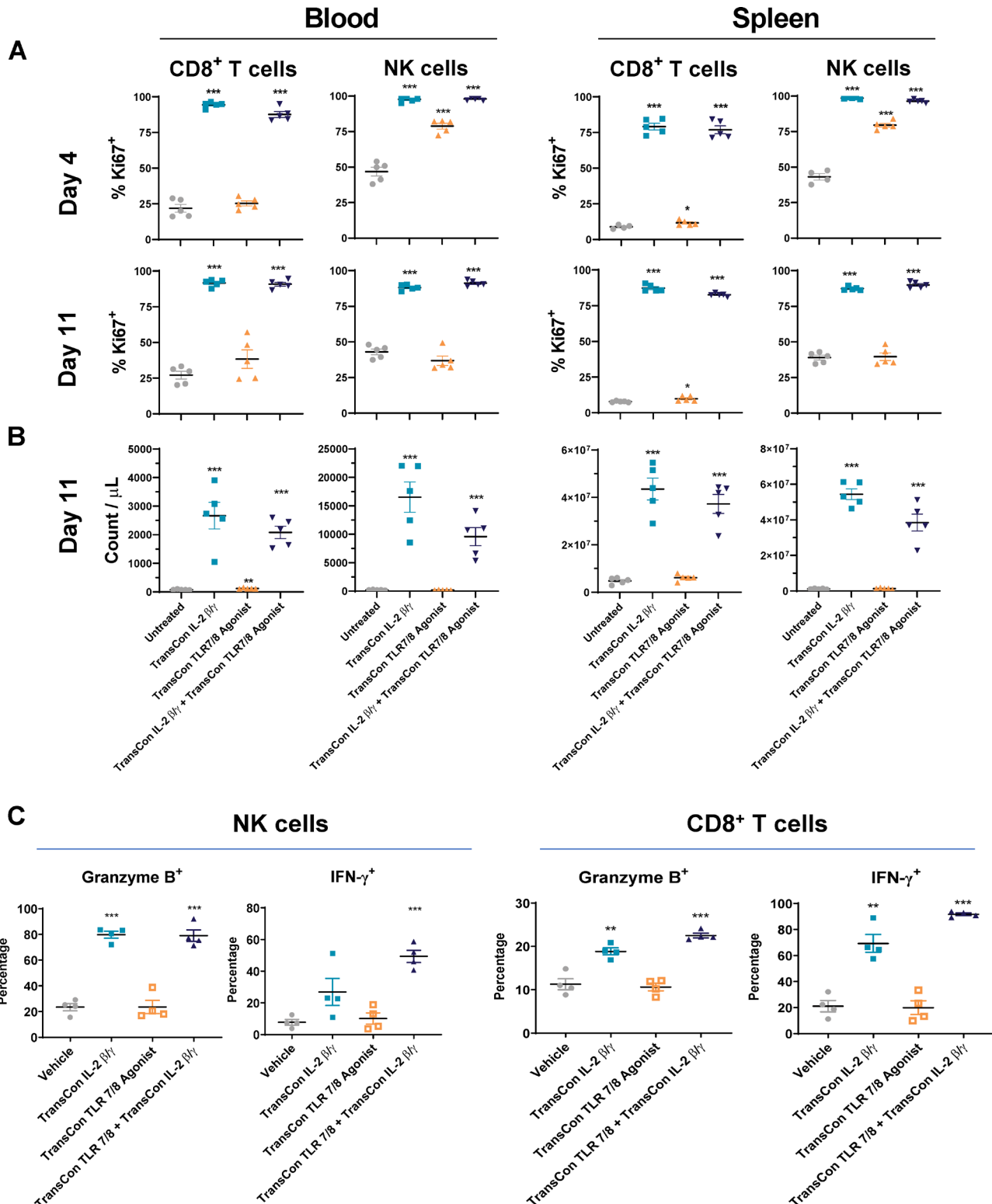
Figure 3 TransCon IL-2 β/γ robustly activates CD8+ T and NK cells in CT26 bearing BALB/C mice. (A, B) Mice were administered 72 µg (approximately 3.6 mg/kg) of TransCon IL-2 β/γ intravenously on Days 0 and 7 and/or a single 100 µg (approximately 5 mg/kg) intratumoral dose (on Day 0) of TransCon TLR7/8 Agonist. Shown (as mean±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 µg (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±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.
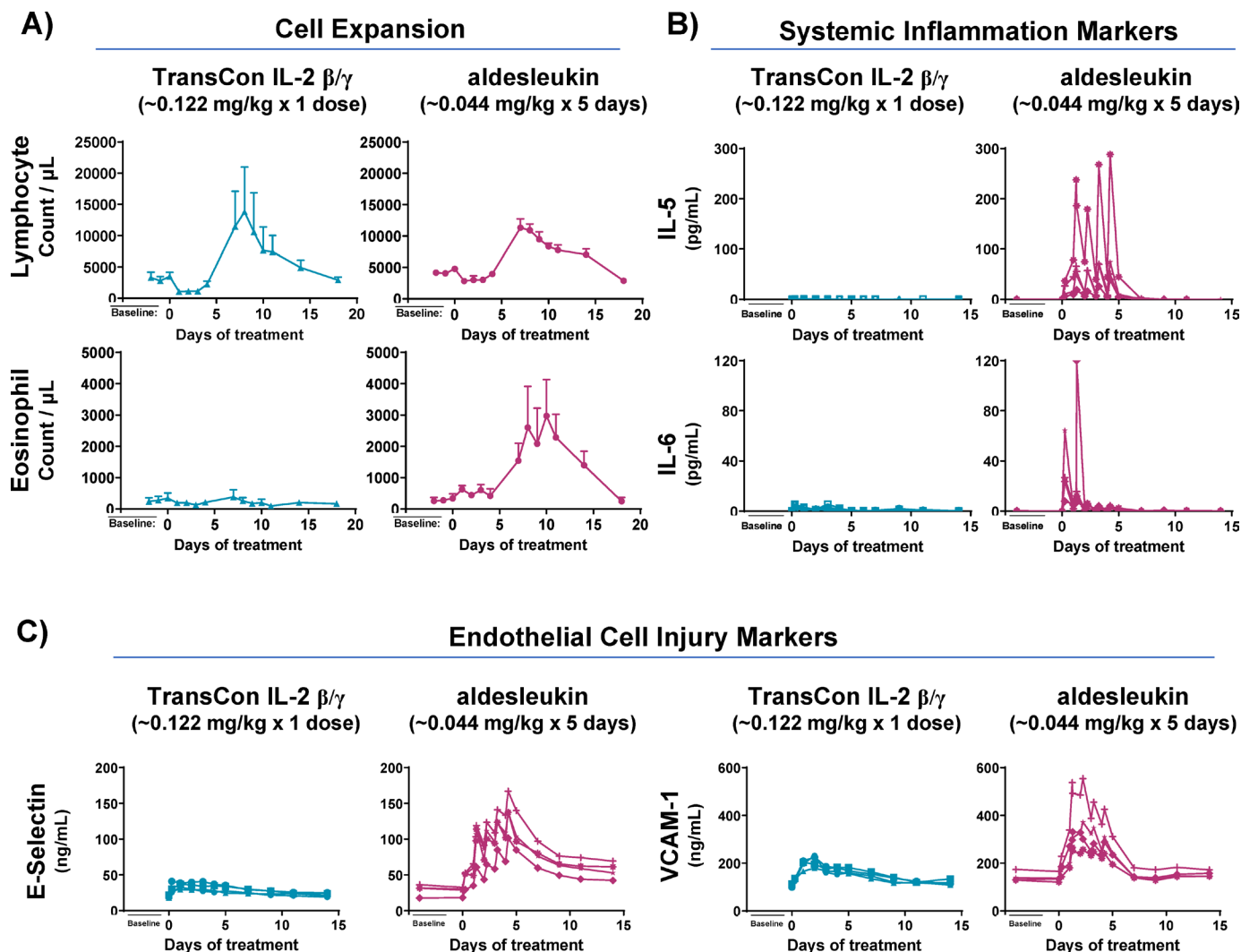
Figure 4 TransCon IL-2 β/γ promotes robust lymphocyte expansion in cynomolgus monkeys, without inducing eosinophils, systemic endothelial cell injury or inflammatory cytokines. Male monkeys (n=4/group) were administered daily aldesleukin (0.4 mg/day, average 0.044 mg/kg) on Days 1–5, or a single dose 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±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 γδ 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 γδ 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 γδ 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 γδ 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 γδ 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 (monophasic) 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 AUC_{0-336h}) increased generally dose proportionally (figure 5C). Of note, at 0.3 mg/kg, a dose
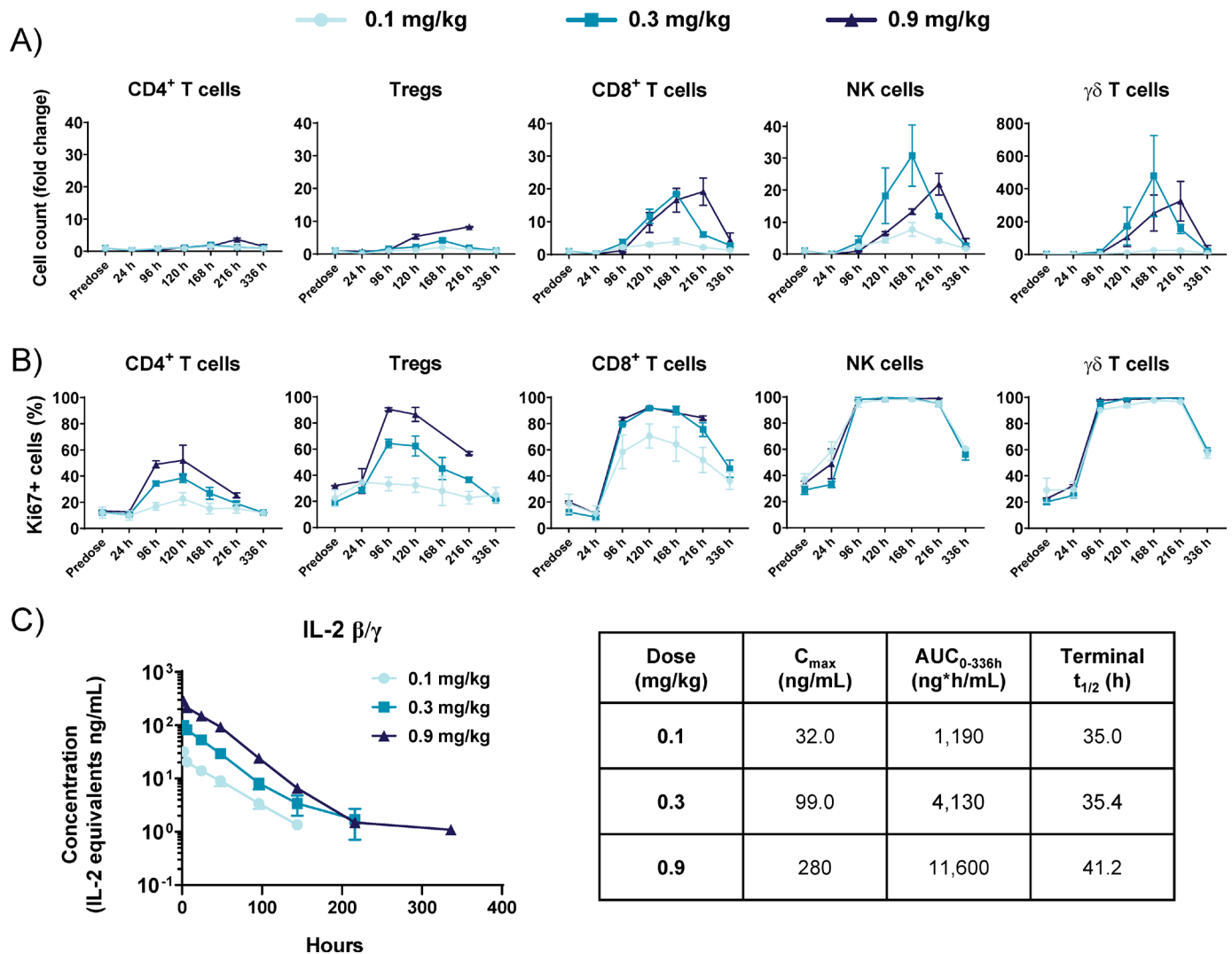
TransCon IL-2 β/γ dose

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±SEM). (B) Proliferation of different lymphocyte subsets as measured by % Ki67+ cells (mean±SEM). (C) Postdose plasma concentrations of IL-2 β/γ as ng/mL (mean±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; γδ, 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 γδ T cells

As TransCon IL-2 β/γ induced a robust expansion of CD8+ T cells, NK cells and γδ 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 γδ T cells. This corresponded with increases in ratios of proliferating CD8+ T cells, NK cells and γδ 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 γδ T cells pretreated with IL-2 β/γ to kill human tumor cells were evaluated in vitro. Initial experiments with CD8+ and γδ 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
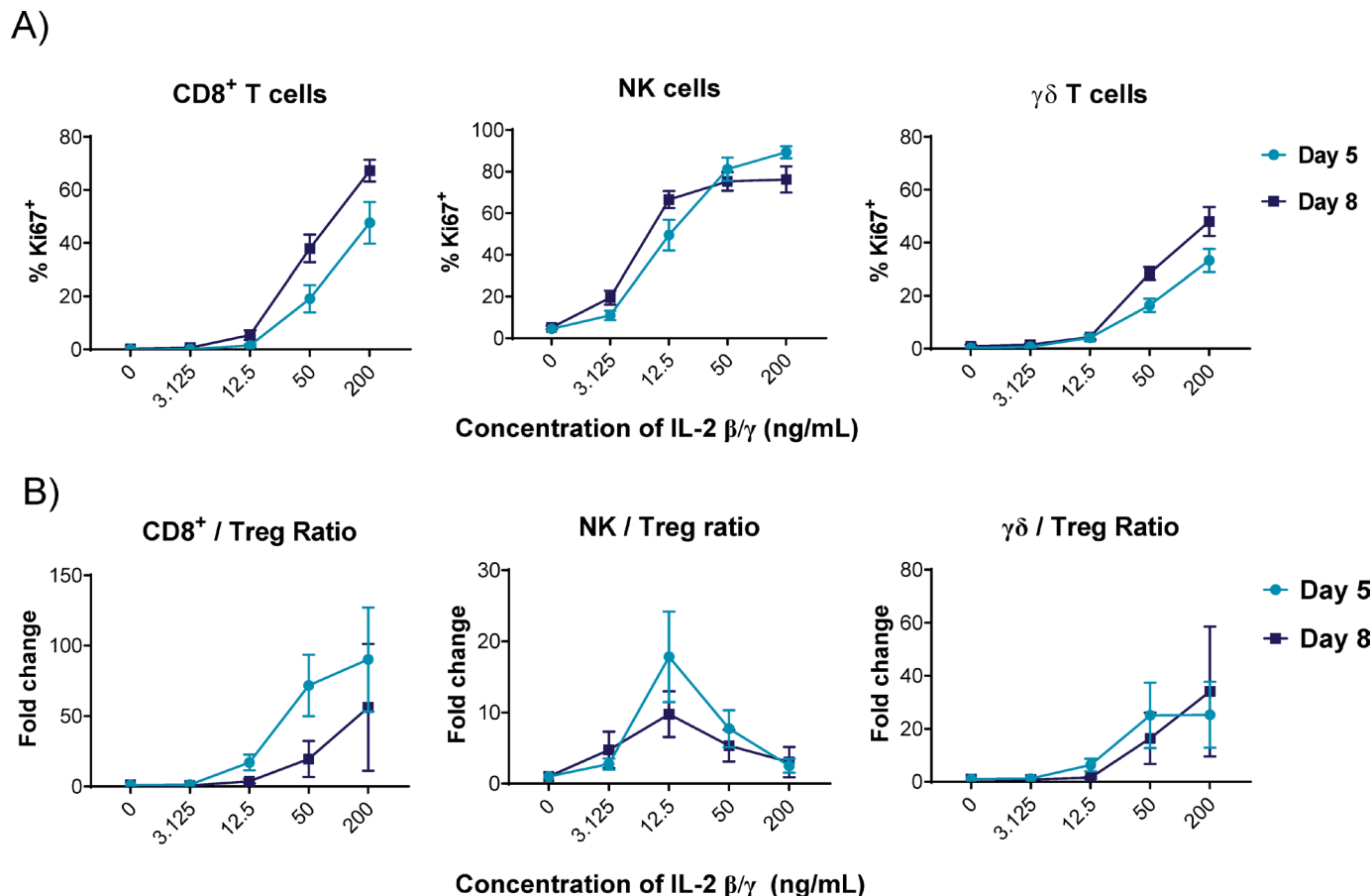
Figure 6 IL-2 β/γ induces proliferation of human primary CD8^{+} T cells, NK cells and γδ T cells. (A) Proliferative response after culture for 5 and 8 days with IL-2 β/γ measured as the percentage of Ki67^{+} cells by flow cytometry. (B) Proliferative responses compared as the fold change of the ratios of proliferating (Ki67^{+}) CD8^{+} T cells, NK cells and γδ T cells to Tregs. Data are shown as mean±SEM (n=6 donors). IL, interleukin; NK, natural killer; Treg, regulatory T; γδ, 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 γδ 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 γδ 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 γδ 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 γδ 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 γδ 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 γδ 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-2Rα[+] 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-2Rβ/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-2Rβ/γ selective agonist with potent IL-2Rβ/γ binding and activity, IL-2 β/γ was created by permanent conjugation of a small 5 kDa mPEG within the IL-2Rα binding site of an IL-2 mutein. A 5 kDa mPEG was chosen as PEGs>5 kDa led to reduced IL-2Rβ/γ binding and activation, a finding also described by others,[15] whereas smaller mPEGs retained some IL-2Rα activity. Beyond reducing IL-2Rβ/γ activity,
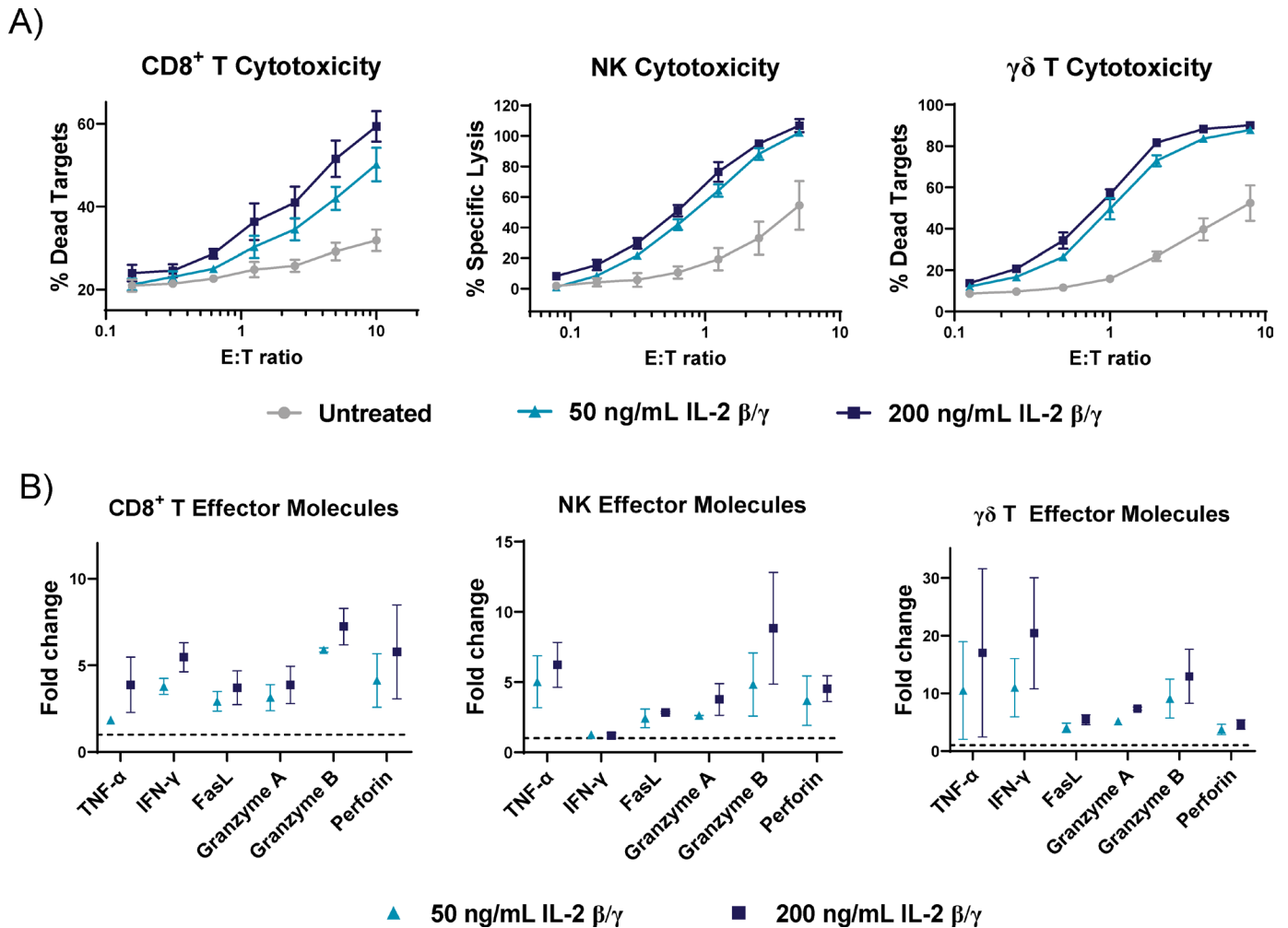
Figure 7 IL-2 β/γ induces cytotoxic and effector functions in primary human CD8^{+} T cells, NK cells and γδ T cells. (A) Human CD8^{+} T cell, NK cell and γδ T cell-mediated cytotoxicity over varying Effector to Target (E:T) ratios after 48 hours pretreatments with 0, 50 or 200 ng/mL of IL-2 β/γ. Data 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 distribution[33] 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 bempegaldesleukin (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 CRS[41] 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 EC_{50}: 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 bempegaldesleukin (0.1 mg/kg) and THOR-707 (0.3 mg/kg) increased ALCs by approximately 5-fold[13] 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 reported[46] 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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Patient consent for publication Not applicable.

Ethics approval Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

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