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Bridging therapy-induced phenotypes and genetic immune dysregulation to study interleukin-2-induced immunotoxicology

Charline Sommer [a], Sophie Jacob [a], Tonia Bargmann [a], Muhammad Shoaib [b], Basel Alshaikhdeeb [b], Venkata P. Satagopam [b], Susann Dehmel [a], Vanessa Neuhaus [a], Armin Braun [a,c,1], Katherina Sewald [a,1,*]

[a] Fraunhofer Institute for Toxicology and Experimental Medicine ITEM, Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), Member of the German Center for Lung Research (DZL), Member of the Fraunhofer Cluster of Excellence Immune-Mediated Diseases CIMD, Hannover, Germany
[b] Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Luxembourg
[c] Institute of Immunology, Hannover Medical School, Hannover, Germany

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ABSTRACT

Interleukin-2 (IL-2) holds promise for the treatment of cancer and autoimmune diseases, but its high-dose usage is associated with systemic immunotoxicity. Differential IL-2 receptor (IL-2R) regulation might impact function of cells upon IL-2 stimulation, possibly inducing cellular changes similar to patients with hypomorphic *IL2RB* mutations, presenting with multiorgan autoimmunity. Here, we show that sustained high-dose IL-2 stimulation of human lymphocytes drastically reduces IL-2Rβ surface expression especially on T cells, resulting in impaired IL-2R signaling which correlates with high IL-2Rα baseline expression. IL-2R signaling in NK cells is maintained. CD4+ T cells, especially regulatory T cells are more broadly affected than CD8+ T cells, consistent with lineage-specific differences in IL-2 responsiveness. Given the resemblance of cellular characteristics of high-dose IL-2-stimulated cells and cells from patients with IL-2Rβ defects, impact of continuous IL-2 stimulation on IL-2R signaling should be considered in the onset of clinical adverse events during IL-2 therapy.

1. Introduction

IL-2, a key cytokine for T cell growth, differentiation, survival, and function [1], reviewed in [2], is a promising treatment option for both cancer and autoimmune diseases by dose-dependently activating different immune cell subsets. Low-dose IL-2 therapy aims to selectively expand regulatory T cells (Tregs), favoring immunosuppression to treat inflammatory diseases [3–5]. Conversely, IL-2 at higher doses additionally activates effector cells such as CD8+ T cells or natural killer (NK) cells, leading to elimination of tumor cells [6]. However, high-dose IL-2 therapy is associated with severe immune-related side effects on vasculature (leading to vascular leakage), skin, intestine, and other organs [7–10]. Current studies aim to refine specificity of IL-2 molecules for more efficient immunotherapy [11,12]. Yet, ensuring safety of IL-2-based therapies demands a better understanding of molecular mechanisms behind IL-2-induced side effects.

Cell-specific differences in IL-2 affinity are in part mediated by differential expression of IL-2 receptor (IL-2R) subunits. IL-2Rβ (CD122), together with the common γ chain γc (IL-2Rγ; CD132), forms the intermediate-affinity IL-2Rβγ, shared by IL-15 [13] and expressed on resting T effector cells (Teffs) and NK cells [14]. IL-2Rγ is further shared by IL-4, IL-7, IL-9, and IL-21 [15–18]. The high-affinity trimeric IL-2Rαβγ is formed by additional expression of IL-2Rα (CD25), which is specific for IL-2 [19] and highly expressed on Tregs [14,20] and activated αβ and γδ Teffs, and NK cells [21–23]. Upon IL-2 binding, STAT5 commonly is phosphorylated [24] and the IL-2-IL-2R complexes are internalized, leading to the degradation of IL-2Rβ and IL-2Rγ, while IL-2Rα is recycled to the cell surface [25].

Rare mutations in the IL-2R subunits can lead to severe immune disfunction [reviewed in [26]]. Deficiencies in IL-2Rγ lead to X-linked

Abbreviations: FMO, fluorescence minus one; GDs, γδ T cells; gMFI, geometric mean fluorescence intensity; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; NK, natural killer; PBMCs, peripheral blood mononuclear cells; PIB, peripheral blood mononuclear cell isolation buffer; pSTAT5, phosphorylated signal transducer and activator of transcription 5; Teffs, T effector cells; Tregs, regulatory T cells; X-SCID, X-linked severe combined immune deficiency.

* Corresponding author at: Fraunhofer ITEM, Preclinical Pharmacology and In-Vitro Toxicology, Nikolai-Fuchs-Str. 1, 30625 Hannover, Katherina, Germany.
E-mail address: katherina.sewald@item.fraunhofer.de (K. Sewald).

[1] Shared last authorship.

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severe combined immune deficiency (X-SCID). On the other hand, *IL2RA* and *IL2RB* mutations are associated with autoimmunity, likely due to reduced numbers and impaired function of Tregs and increased levels of serum cytokines such as IL-2 [27–30]. These conditions can lead to symptoms similar to those observed in patients undergoing IL-2 therapy, such as widespread inflammation, affecting multiple organs and inducing dermatitis or colitis [27–30]. Particularly, in patients with hypomorphic *IL2RB* mutations, IL-2Rβ is drastically reduced on CD4+ and CD8+ T cells, impairing IL-2R signaling of cells. Interestingly, this dysregulated IL-2R signaling does not extend to NK cells, where IL-2Rβ expression remains relatively high [27,30]. In mouse models of *Il2rb* knockout, Tregs are similarly reduced, and systemic inflammation is observed [31]. However, in contrast to patients with deficient IL-2Rβ, knockout mice do not show signs of colitis or skin abnormalities, indicating differences in how these mutations manifest across species [31,32].

Considering the intracellular degradation of IL-2Rβ after IL-2 binding and the similarities of organ symptoms in *IL2RB* mutations, we hypothesize that continuous systemic IL-2 stimulation – similar to what occurs during IL-2 therapy – might induce cellular characteristics similar to those seen in hypomorphic *IL2RB* mutations. This could include 1) decreases in IL-2Rβ surface expression especially on T cells but less pronounced on NK cells; and 2) a corresponding decrease in IL-2Rβ signaling capacity in T cells only. To explore the effects of systemic IL-2 exposure, we treated human peripheral blood mononuclear cells (PBMCs) with varying IL-2 concentrations for up to 7 days. Subsequently, IL-2R surface expression, intracellular receptor abundance, and IL-2R signaling responsiveness were assessed.

2. Materials and methods

2.1. PBMC isolation

PBMCs were isolated from buffy coats obtained from Blutspendedienst NSTOB. All donors or their next of kin, caretakers, or guardians gave written informed consent to the Blutspendedienst NSTOB and only anonymized samples from healthy donors were received. Buffy coats were mixed with PBMC isolation buffer (PIB) (PBS + 2 mM EDTA (Invitrogen) + 0.1% BSA (Sigma-Aldrich)) 1:2, layered on top of Ficoll-Paque™ PLUS (Cytiva), and centrifuged at 700 ×*g* for 20 min. PBMC interface was carefully removed, washed with PIB and centrifuged (300 ×*g*, 10 min). Hemolysis was performed and cells were again washed with PIB. Cell numbers and viability were determined using a hemocytometer before usage of cells.

2.2. PBMC culture

After isolation, 5×10^5 PBMCs were cultured in 96 u-well plates in 100 μL full medium (RPMI 1640 (Gibco), 1% penicillin/streptomycin (Gibco), 5% human serum (Invitrogen), 1% L-glutamine (Sigma-Aldrich), 1% MEM NEAA (100×) (Gibco), 25 mM HEPES buffer (Biowest), 1% sodium pyruvate (Gibco)) and 100 μL of IL-2 (Proleukin® S, aldesleukin, Clinigen) were added to reach final concentrations of 1–10,000 IU/mL in technical duplicates. To investigate proliferation, cells were stained with 1 μM CMFDA (Invitrogen) for 15 min at 37 °C in RPMI without serum and subsequently washed with full medium prior to culture. Cells were cultured under normal cell culture conditions and half of medium was replaced with new medium containing IL-2 in the respective concentration on day 3. For measurements on day 0 (15 min time point), cells were rested in full medium at 37 °C, 5% CO_2 for 1 h before stimuli were added. After stimulation for 15 min, 18 h, or 5 or 7 days, cells were centrifuged at 350 ×*g*, 5 min, supernatants were carefully removed and cell pellets were used for flow cytometrical analysis of cell subsets, IL-2R expression, and pSTAT5.

2.3. Isolation of CD4+ Teffs

To investigate expression of common Treg markers on IL-2-stimulated CD4+ Teffs, CD4+ Teffs were isolated from human PBMCs using the CD4 + CD25+ Regulatory T Cell Isolation Kit (Miltenyi Biotec). Briefly, CD4+ cells were negatively selected and the pre-enriched CD4+ cell fraction was labeled with anti-CD25 to separate CD25+ Tregs from CD25- Teffs. The CD25- fraction was cultured for 7 days with different IL-2 concentrations as described, followed by flow cytometrical analysis.

2.4. Re-stimulation of PBMCs

PBMCs were stimulated with 1–10,000 IU/mL IL-2 for 18 h or 5 or 7 days as described. Supernatants were carefully removed and 100 μL full medium without stimulation was added. Cells were rested for 1 h at 37 °C, 5% CO_2. Afterwards, cells from all conditions were re-stimulated with 10,000 IU/mL IL-2, 100 ng/mL IL-15 (R&D Systems), or 100 ng/mL IL-7 (R&D Systems) for 15 min before analysis of pSTAT5. To correlate differences in pSTAT5 signaling with IL-2R expression, ΔpSTAT5 was calculated as the difference in pSTAT5 gMFI after IL-2 re-stimulation of unstimulated and IL-2-stimulated cells (ΔpSTAT5 = pSTAT5 gMFI (0 IU/mL) – pSTAT5 gMFI (100/1000/10,000 IU/mL)). Only αβ T cells were included in this analysis as these were the only cell types for which pSTAT5 signaling was decreased by high-dose IL-2 stimulation in every donor tested.

2.5. Flow cytometry

After culture, cell pellets of technical duplicates were pooled, Fc-blocked in 100 μL TruStain FcX™ (1:100, Biolegend) for 10 min on ice, and washed with 100 μL FACS buffer (PBS + 2 mM EDTA +1% FCS). Cell pellets were stained with surface antibodies and eBioscience™ fixable viability dye eFluor™ 506 (Invitrogen) for 20 min on ice. For experiments investigating intracellular IL-2R expression, uncoupled IL-2Rα (clone M-A251, Biolegend), IL-2Rβ (clone TU27, Biolegend), and IL-2Rγ (clone TUGh4, Biolegend) antibodies were added in 2.5× the concentrations used for surface staining to block extracellular IL-2R epitopes. Blocking of extracellular epitopes was verified by flow cytometry. Cells were washed twice with FACS buffer (350 ×g, 2 min) and either measured directly or further processed for intracellular staining using the eBioscience™ Foxp3 transcription factor staining buffer set (Invitrogen). Intracellular antibodies were added for 40 min on ice. For staining of pSTAT5, surface-stained cells were further processed using the PerFix EXPOSE kit (Beckman Coulter), and cells were stained with intracellular antibodies for 40 min at room temperature. Cells were washed, CountBright™ Absolute Counting Beads (Invitrogen) were added, and samples were measured using the Beckman Coulter Cytoflex S.

The following anti-human antibodies and clones were used for flow cytometry: CD3-FITC (clone UCHT1, Biolegend); CD3-AF700 (clone UCHT1, Biolegend); CD56-PE/Cy7 (clone HCD56, Biolegend); CD56-AF700 (clone HCD56, Biolegend); TCRγδ-BC605 (clone 11F2, BD); CD8-APC/Cy7 (clone SK1, Biolegend); CD4-BV650 (clone RPA-T4, Biolegend); FoxP3-AF647 (clone 206D, Biolegend); FoxP3-AF647 (clone 259D, Biolegend); CTLA-4-PerCP/Cy5.5 (intracellular; clone BN13, Biolegend); pSTAT5-PE (cone A17016B.Rec, Biolegend); CD25-BV421 (clone M-A251, Biolegend); CD122-PE/Cy7 (clone TU27, Biolegend); CD132-PE (clone TUGh4, Biolegend); CD45RA-BV421 (clone H100, Biolegend); CD45RO-BV650 (clone UCHL1, Biolegend); CD127-PerCP/Cy5.5 (clone A019D5, Biolegend).

Data was analyzed using FlowJo version 10.8.1. Representative gating strategy can be found in Fig. S1A. Gating for IL-2R subunits was adjusted based on isotype and fluorescence minus one (FMO) controls.

2.6. Statistics

Statistical analysis of the non-normally distributed data was performed using GraphPad Prism (version 9) and multiple Wilcoxon tests with the Holm-Šídák method to correct for multiple comparisons were applied to determine *p*-values as indicated in the figure legends. To determine differences between more than two groups, Friedman test with Dunn's multiple comparisons test was used. Differences were considered as significant for $p < 0.05$ and tendencies are depicted for $p < 0.1$.

3. Results and discussion

3.1. Continuous high-dose IL-2 stimulation induces cellular characteristics similar to IL2RB mutations

To investigate the impact of IL-2 stimulation on IL-2Rβ expression, human PBMCs were stimulated with increasing doses of IL-2 (aldesleukin) and IL-2Rβ surface abundance was quantified. Based on clinical observations of high-dose IL-2-treated patients [33,34], we defined a range of IL-2 concentrations used in our study as low-dose (1 and 10 IU/mL) and high-dose IL-2 (100–10,000 IU/mL).

IL-2 had a clear, dose-dependent effect on reducing IL-2Rβ surface expression on lymphocytes (Fig. 1A-D). Just after 15 min of IL-2 stimulation, immediate decreases in IL-2Rβ surface expression were observed on CD4+ T cell subsets, especially prominent on Tregs at IL-2 concentrations ≥10 IU/mL (unstimulated vs. 10,000 IU/mL 22.2% vs. 0.3% IL-2Rβ + for Tregs) (Fig. 1A). This reduction was mirrored in decreasing geometric mean fluorescence intensity (gMFI) (Fig. S1B). In contrast, the effect was less pronounced on CD8+ T cells, γδ T cells, and NKT cells (Fig. 1A, S1B) and after 15 min of high-dose IL-2 stimulation 4.0% (CD8+ T cells) and >21% (γδ T cells, NKT cells) remained IL-2Rβ+. IL-2Rβ expression on NK cells remained unchanged at this early time point. After 18 h of high-dose stimulation, IL-2Rβ surface expression was found to be similar to that seen in patients with hypomorphic *IL2RB* mutations [27,30], with nearly no IL-2Rβ on T cells, including CD4+ and CD8+ T cells, γδ T cells and NKT cells and a persistent low expression throughout the culture period (Fig. 1B-E). In contrast, NK cells showed a reduction in expression, but IL-2Rβ levels remained relatively high throughout the culture period (Fig. 1B-E, Fig. S1B).

During IL-2 stimulation, we observed dose-dependent increases in frequencies of T cells with CD45RO+ memory phenotype with concomitant decreases in cells with naïve CD45RA+ phenotype (Fig. 1F). Furthermore, absolute numbers of all cell subsets – except CD4+ Teffs – increased significantly (Fig. S1C), mirrored by CMFDA dilution as a marker for proliferation (Fig. S1D). In line with previous in vitro studies and clinical reports [35–37], NK cell frequencies dose-dependently increased (Fig. 1G), and, within the NK cell population, $CD56^{bright}$ NK cells expanded (Fig. 1H). These results further align with the cellular characteristics of patients with IL-2Rβ defects [27,30]. Observed increases in NK cell frequencies are in contrast to knockout mice, where NK cells are basically absent in homozygous $Il2r^{-/-}$ animals and frequencies do not increase in heterozygous $Il2rb^{+/-}$ mice compared to wildtype mice [32], highlighting immunological differences between mice and humans.

3.2. IL-2Rα production is invariably increased, while IL-2Rγ and IL-2Rβ are differentially regulated upon IL-2 stimulation

To investigate regulation of IL-2Rα and IL-2Rγ upon prolonged IL-2 exposure, expression of both subunits was investigated after IL-2 stimulation. In line with previous clinical and in vitro reports [38–40], prolonged culture with IL-2 dose-dependently increased extracellular IL-2Rα expression (Fig. 2A-E, Fig. S1E), with statistically significant increased expression on all cell subsets investigated 18 h after IL-2 stimulation. IL-2Rα was used as an additional extracellular marker to identify Tregs because CTLA-4 and FoxP3, markers that are often used to distinguish Tregs from CD4+ Teffs, also were increased in CD4+ Teffs by high-dose IL-2 treatment. However, even though these CD4+ Teffs were CTLA-4 + FoxP3+, they remained IL-2Rα– (Fig. S2A). Therefore, only IL-2Rα gMFI is shown for extracellular IL-2Rα expression on Tregs. Extracellular IL-2Rγ expression, on the other hand, was largely unaltered with respect to frequencies and gMFI throughout the culture period (Fig. 2E-I, Fig. S2C).

Time- and cell-specific differences in IL-2R surface abundance after IL-2 stimulation could be due to differences in intracellular IL-2R abundance [41]. In accordance with IL-2Rβ surface expression, unstimulated NK cells showed the highest levels of intracellular IL-2Rβ, followed by NKT cells, γδ T cells, Tregs, CD8+ T cells, and CD4+ Teffs (Fig. 3A). These observations are in line with studies investigating intracellular IL-2Rβ abundance in murine T cells, showing that CD4+ Tregs and CD8+ T cell blasts have increased intracellular IL-2Rβ abundance compared to CD4+ Teff blasts [41]. Upon IL-2 stimulation, intracellular IL-2Rβ abundance increased over time in CD8+ T cells, γδ T cells, NKT cells, and NK cells ($p < 0.05$ when comparing IL-2Rβ MFI for 10,000 IU/mL IL-2 within one cell subsets after 15 min, 18 h, 5 days, and 7 days, tested with Friedman test), but not in CD4+ T cell subsets (Fig. 3B-E). Accordingly, *IL2RB* mRNA expression was previously shown to increase upon in vitro stimulation of CD8+ T cells and NK cells dose-dependently, while expression in differentiated type 1 and 2 T helper cells was unaltered [42]. Similar to IL-2Rβ, intracellular IL-2Rγ expression did not increase in CD4+ T cell subsets upon continuous high-dose IL-2 stimulation over time, while intracellular IL-2Rγ content in other cell subsets investigated significantly increased throughout the culture period ($p < 0.05$ when comparing IL-2Rγ MFI for 10,000 IU/mL IL-2 within one cell subset after 15 min, 18 h, 5 days, and 7 days, tested with Friedman test) (Fig. S2D). These results are in line with dose-dependent *IL2RG* expression upon IL-2 stimulation in CD8+ T cells and NK cells, but not in T helper cells [42]. On the other hand, intracellular IL-2Rα significantly increased in response to high-dose IL-2 over time in all cell subsets except CD4+ Teffs ($p < 0.05$ when comparing IL-2Rα MFI for 10,000 IU/mL IL-2 within one cell subset after 15 min, 18 h, 5 days, and 7 days, tested with Friedman test) and tended to increase across lymphocyte subsets compared to unstimulated control after 7 days of IL-2 stimulation (Fig. S2B). Similarly, *IL2RA* transcription is reported to increase in various cell subsets upon activation by IL-2 [40,42]. Together, while IL-2Rα seems to be invariably induced, these data suggest cell-specific production of IL-2Rβ and IL-2Rγ upon high-dose IL-2 stimulation in lymphocytes subsets except CD4+ T cells, possibly affecting function of cells.

3.3. High-dose IL-2 stimulation dynamically decreases IL-2R signaling capacity in T cells, especially in Tregs

Immune cells from patients suffering from hypomorphic *IL2RB* mutations display reduced IL-2R signaling in T but not in NK cells due to decreases in IL-2Rβ surface expression [27,30]. To investigate if IL-2R signaling is similarly impaired upon continuous IL-2 exposure, PBMCs were stimulated with increasing IL-2 concentrations and re-stimulated with high-dose IL-2 (10,000 IU/mL) before analyzing phosphorylated STAT5 (pSTAT5). After only 18 h of IL-2 stimulation, CD4+ Teffs showed decreases in frequencies of pSTAT5+ cell and reductions in pSTAT5 were more pronounced in CD4+ Teffs and Tregs compared to CD8+ T cells (Fig. 4A). Prolonged IL-2 stimulation led to decreased pSTAT5 signal upon re-stimulation in most cell subsets which were statistically significant for high-dose IL-2-stimulated CD4+ and CD8+ T cells, particularly Tregs (reduction in median frequency of pSTAT5+ cells of 30.8% in Tregs compared to 27.9% in CD4+ Teffs and 26.5% in CD8+ T cells, after 7 days) (Fig. 4B-D, Fig. S3A). Of note, IL-2 signaling in NK cells was not affected by prior culture with IL-2 as pSTAT5 signal remained stable around 85% (Fig. 4A-C) and gMFI was similarly unaltered (Fig. 4D, Fig. S3A).

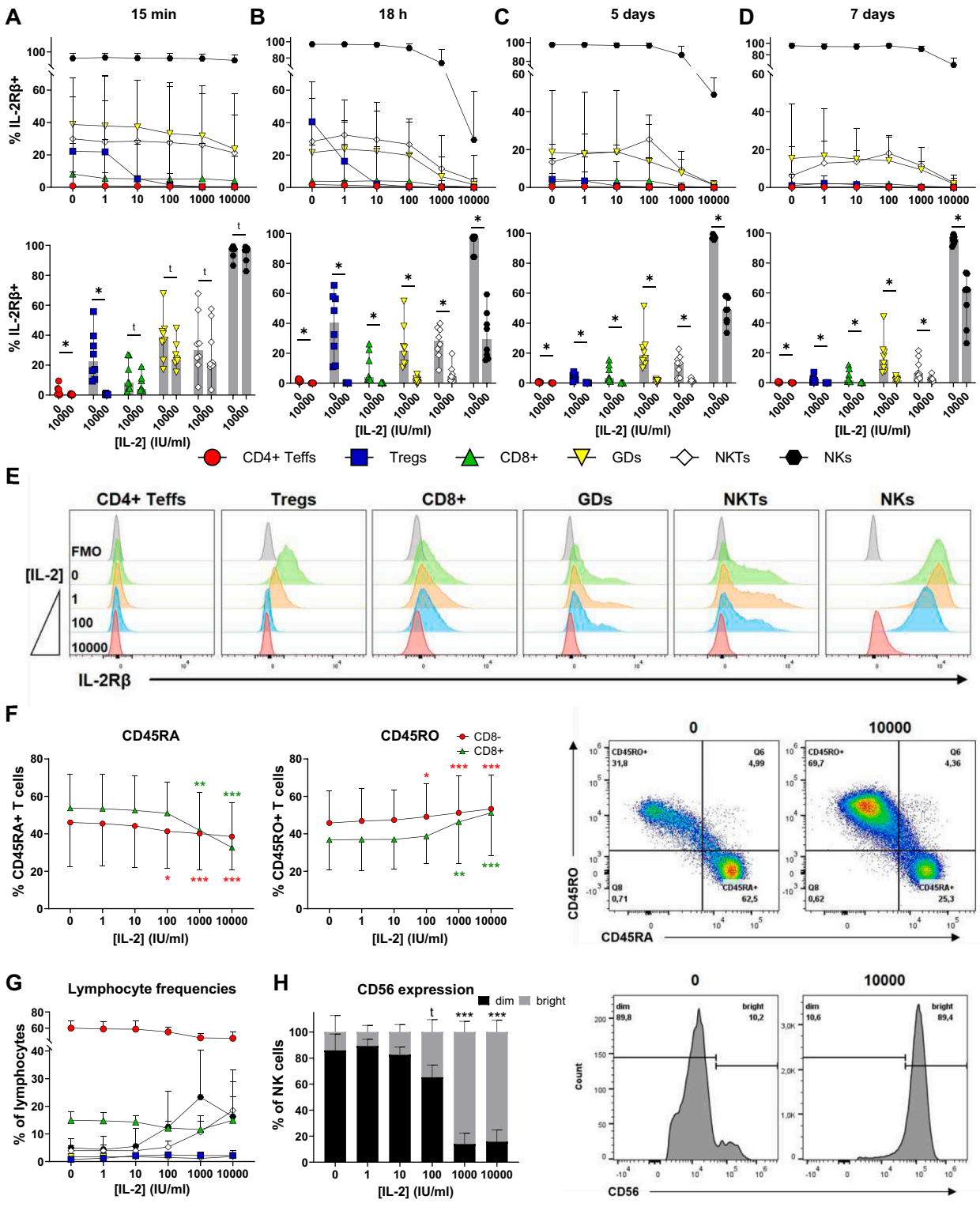

Fig. 1. Continuous hdIL-2 stimulation reduces IL-2Rβ surface expression on lymphocytes. Human PBMCs were stimulated with increasing IL-2 doses for up to 7 days prior to analysis of CD4+ Teffs, CD4+ Tregs, CD8+ T cells, γδ T cells (GDs), NKT cells, and NK cells. Mean frequencies of IL-2Rβ + cells after 15 min (A), 18 h (B), 5 days (C), or 7 days (D) of IL-2 stimulation in different concentrations (upper panel). Bar graphs (middle panel) highlight mean frequencies of IL-2Rβ + lymphocytes as shown above without IL-2 (0 IU/mL) or upon high-dose IL-2 stimulation (10,000 IU/mL). $n = 8$ donors, two independent experiments. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, all other conditions non-significant, analyzed by multiple paired *t*-test (Holm-Šídák method). E Representative histograms of IL-2Rβ expression after 18 h of stimulation with respective IL-2 concentration. FMO: Fluorescence minus one control of unstimulated cells. F Expression of CD45RA and CD45RO on CD8– and CD8+ αβ T cell subsets after 7 days of IL-2 stimulation and representative flow cytometric plots with or without IL-2 stimulation (0 vs. 10,000 IU/mL IL-2) on CD8+ T cells. G Lymphocyte frequencies after 7 days of IL-2 stimulation. H CD56 expression on NK cells and representative flow cytometric plots with or without IL-2 stimulation (0 vs. 10,000 IU/mL IL-2). n = 8 donors, two independent experiments, mean ± SD. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, all other conditions non-significant, analyzed by two-way ANOVA and Dunnett's multiple comparison test.

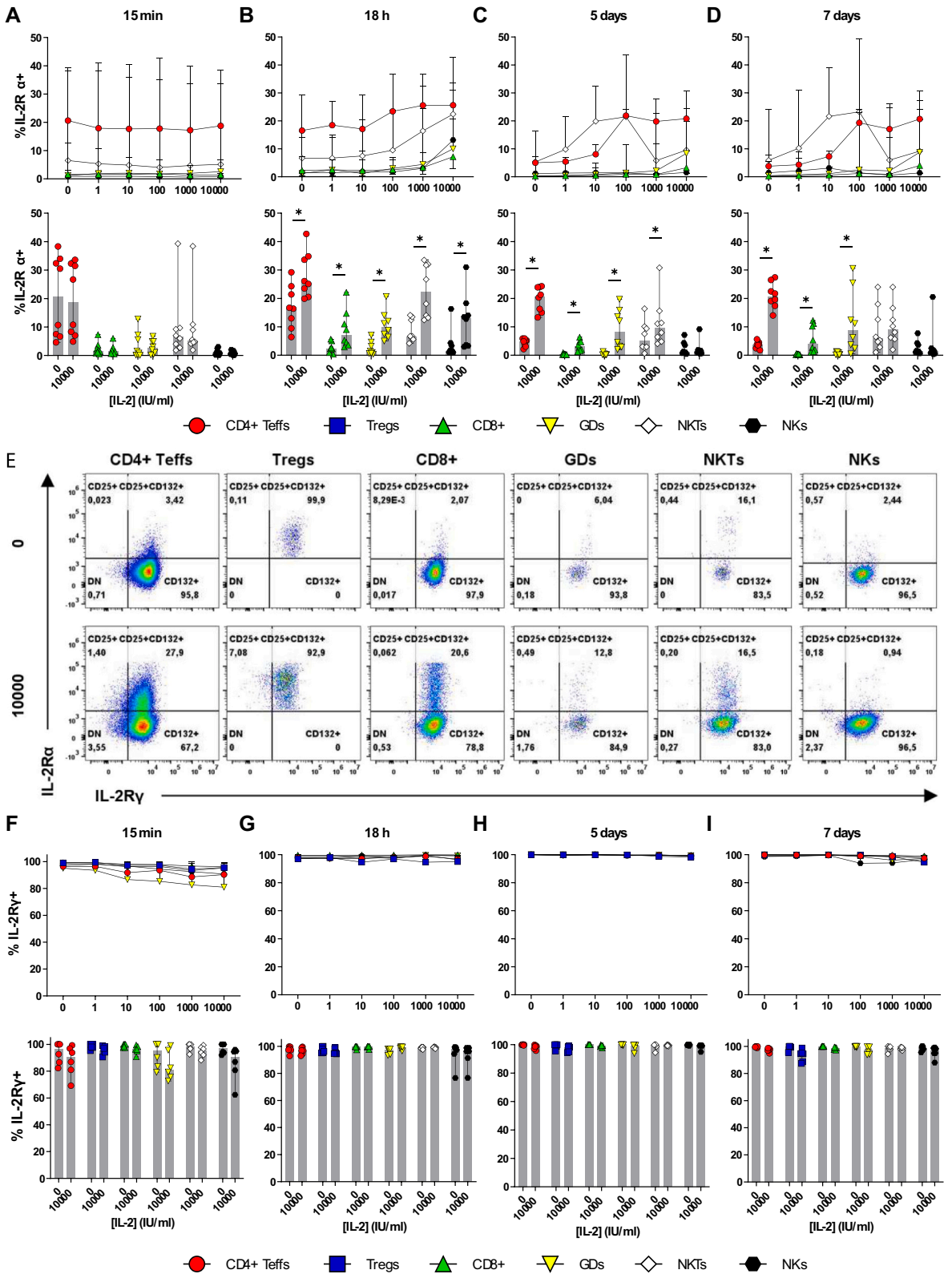

Fig. 2. Differential regulation of IL-2Rα and IL-2Rγ surface expression upon continuous IL-2 stimulation. Human PBMCs were stimulated with increasing IL-2 doses for up to 7 days prior to analysis. A-D Mean frequencies of CD4+ Teffs, CD8+ T cells, γδ T cells (GDs), NKT cells, and NK cells positive for extracellular IL-2Rα after 15 min (A), 18 h (B), 5 days (C), or 7 days (D) of IL-2 stimulation in different concentrations (upper panel). Bar graphs (lower panel) highlight mean frequencies of IL-2Rα + lymphocytes as shown above without IL-2 (0 IU/mL) or upon high-dose IL-2 stimulation (10,000 IU/mL). As IL-2Rα expression was used as an additional marker for identification of CD4+ Tregs, IL-2Rα frequencies of Tregs are not sown. E Representative flow cytometric plots of IL-2Rα and IL-2Rγ surface expression on cell subsets after 7 days of stimulation with (10,000 IU/mL) or without IL-2 (0 IU/mL). F–I Mean frequencies of cells positive for extracellular IL-2Rγ after stimulation with different IL-2 concentrations for 15 min (F), 18 h (G), 5 days (H), or 7 days (I) (upper panel) or comparatively without IL-2 vs. high-dose IL-2-stimulated lymphocytes (lower panel). $n = 4$–8 donors, two independent experiments, mean ± SD. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, all other conditions non-significant, analyzed by multiple paired t-test (Holm-Šídák method).

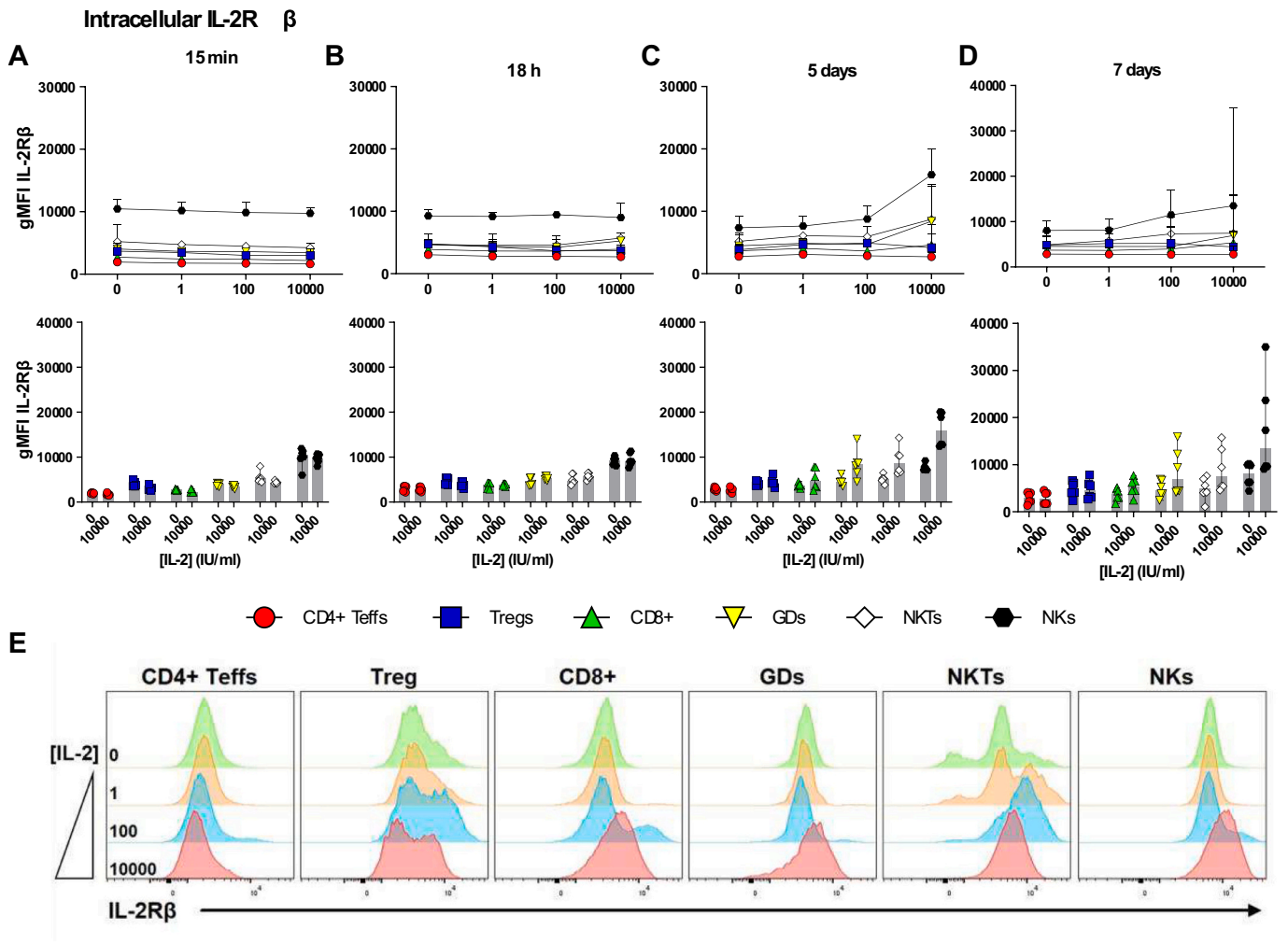

Fig. 3. Intracellular IL-2Rβ abundance is cell-specifically regulated upon IL-2 stimulation. Human PBMCs were stimulated with increasing IL-2 doses for up to 7 days prior to analysis. A-E Intracellular IL-2Rβ abundance was analyzed in CD4+ Teffs, CD4+ Tregs, CD8+ T cells, γδ T cells (GDs), NKT cells, and NK cells after 15 min (A), 18 h (B), 5 days (C), or 7 days (D) of IL-2 stimulation in different concentrations (upper panel). Bar graphs (lower panel) highlight IL-2Rβ gMFI as shown above without IL-2 (0 IU/mL) or upon high-dose IL-2 stimulation (10,000 IU/mL). $n = 6$ donors, two independent experiments. Mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, all other conditions non-significant, analyzed by paired t-test (Holm-Šídák method). E Representative histograms of intracellular IL-2Rβ in cell subsets after 7 days of IL-2 stimulation.

To identify the IL-2R subunit leading to reduced IL-2R signaling capability, cells previously stimulated with IL-2 were re-stimulated with IL-15 (also signaling through IL-2Rβγ [13]) or IL-7 (signaling through IL-7Rα and IL-2Rγ [18]) (Fig. 4D). Similar to IL-2 re-stimulation, re-stimulation with IL-15 tended to reduce frequencies of pSTAT5+ CD4+ and CD8+ T cells when previously cultured with high-dose IL-2 and reduction in IL-15 signaling was more pronounced in CD4+ T cell subsets, especially Tregs, compared to CD8+ T cells (median reduction of 42.4% pSTAT5+ cells vs. 32.2% in CD4+ Teffs and 17.4% in CD8+ T cells on day 7) (Fig. 4E+F, Fig. S3B). IL-15 signaling in NK cells was not affected by prior IL-2 stimulation (Fig. 4E+F, Fig. S3B).

Although IL-7 signaling was statistically not significantly affected by previous high-dose IL-2 stimulation (Fig. 4E+F, Fig. S3C), donor-dependent responses led to a difference in median frequency of pSTAT5+ cells of 20.7% and 28.3% for CD4+ Teffs and Tregs after 7 days of IL-2 stimulation, respectively. Specifically, in CD4+ Teffs, all tested donors showed a reduction in pSTAT5 frequency upon IL-7 re-stimulation (1 donor with reduction <5%, 5 donors ranged between reductions of 15.3–61.5%) and five out of six tested donors displayed reduced signaling in Tregs (differences ranging from 5.1 to 62.8%). Lower IL-7 signaling might be due to lack of increased IL-2Rγ production in IL-2-stimulated CD4+ T cells or because of decreased IL-7Rα (CD127) expression seen for IL-2-stimulated, activated T cells by us (Fig. S3D) and others [43,44], or both. On the other hand, IL-7 signaling was increased in NK cells after high-dose IL-2 stimulation in all donors (1 donor with increase <5%, 5 donors ranged between increases of 14.5–76.1%) (Fig. 4E+F, Fig. S3C).

Together, while IL-2R signaling in NK cells remained unaffected, T cell subsets showed pronounced reduction in IL-2R signaling after high-dose IL-2 stimulation which mostly seemed to be due to lower IL-2Rβ surface expression. Differential IL-2Rβ production upon IL-2 stimulation (Fig. 3) possibly further affects cell-specific changes in IL-2R signaling. While few publications report reduced IL-2Rβ surface expression upon in vitro IL-2 stimulation of T and NK cells [42,45,46], functional consequences have not been investigated so far, and thus, to our knowledge, this is the first study describing reduced IL-2R signaling of T cells upon continuous high-dose IL-2 stimulation. To identify published clinical reports of IL-2Rβ surface expression upon IL-2 therapy, we performed a systematic literature research using the Indra text mining tool [47]. Initially, a total of 446 publications referring to IL-2 and IL-2Rbeta or IL-2 and CD122 were identified. All these publications were first scanned using our local database of 9 million publications, followed by a manual search on selected publications. This approach yielded only one publication investigating IL-2Rβ regulation after IL-2 therapy, reporting

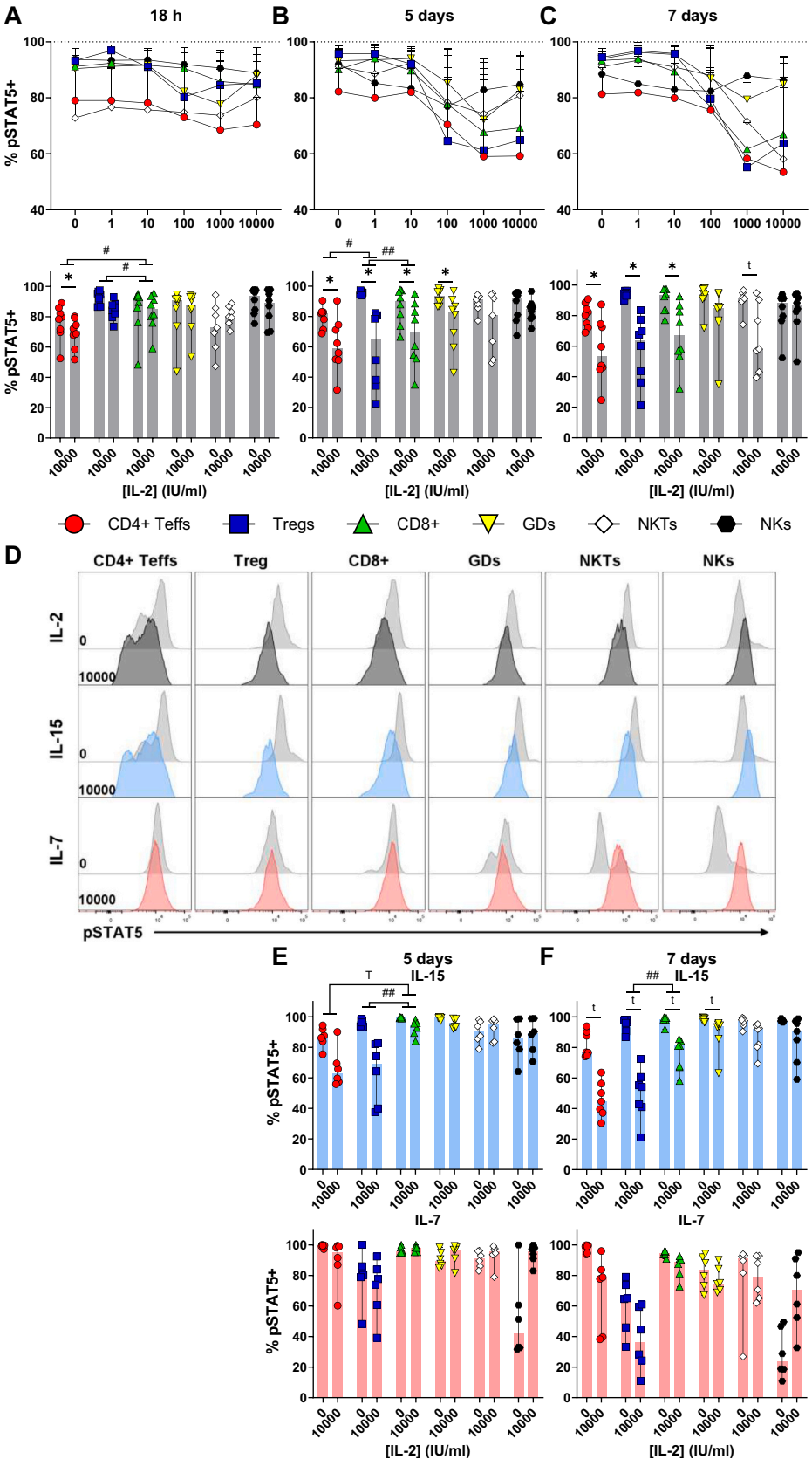

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Fig. 4. IL-2R signaling capacity is reduced in cells with previous high-dose IL-2 stimulation. Human PBMCs were stimulated with increasing IL-2 doses. After the indicated time points, cells were re-stimulated with high-dose IL-2 (10,000 IU/mL), IL-15, or IL-7 for 15 min. A-C Mean frequencies of pSTAT5+ CD4+ Teffs, CD4+ Tregs, CD8+ T cells, γδ T cells (GDs), NKT cells, and NK cells after 18 h (A), 5 days (B), or 7 days (C) of IL-2 stimulation in different concentrations, followed by re-stimulation with IL-2 (upper panel). Bar graphs (lower panel) highlight mean frequencies of pSTAT5+ lymphocytes as shown above without (0 IU/mL) or with prior high-dose IL-2 stimulation. D Representative histograms of pSTAT5 signal after IL-2, IL-15, or IL-7 re-stimulation of cells which have previously been stimulated with (lower panel) or without IL-2 (upper panel). E + F Mean frequencies of pSTAT5+ cells after 5 days (E) or 7 days (F) with or without IL-2 and subsequent re-stimulation with IL-15 (upper panels) or IL-7 (lower panels). n = 6–8 donors, two independent experiments, mean ± SD. * $p < 0.05$, ** $p < 0.01$, all other conditions non-significant, analyzed by multiple paired t-test (Holm-Šídák method).

increased expression of IL-2Rβ on lymphocytes 24 h after the last IL-2 dose [48]. However, in this study, considerably lower IL-2 doses were applied less frequently compared to conventional high-dose IL-2 therapy (1.8×10^6 IU/day daily compared to 600,000–720,000 IU/kg/dose every 8 h in high-dose IL-2 therapy), inducing minimal side effects. As the results presented here show a dynamic, dose-dependent regulation of IL-2Rβ surface expression, the distinct dosing regime and time point chosen for measurement of IL-2Rβ abundance might explain conflicting results of the clinical study compared to our results. Thus, analyzing IL-2Rβ expression and IL-2R stimulation capacity upon higher-dose IL-2 therapy or treatment with new IL-2-based compounds, characterized by increased IL-2 plasma concentrations due to extended half-life, would be crucial to correlate our findings to the clinics.

On the other hand, our results regarding reduced IL-2 and IL-15 signaling mirror observations in cells from patients with hypomorphic *IL2RB* mutations [27,30]. Additionally, in contrast to patients with defective IL-2Rβ, IL-7 signaling is reduced in high-dose IL-2-stimulated CD4+ T cell subsets of some donors. Given the importance of IL-2, IL-15, and IL-7 for T cell survival, proliferation, and homeostasis, and their particular role in maintaining tissue memory T cells in organs like the gut and skin, cell-specific reduction in sensitivity towards these cytokines could impair peripheral function and maintenance, particularly in CD4+ T cell subsets [49–54]. This potentially disturbs tissue homeostasis, even in conditions of IL-2 excess, such as during IL-2 therapy. Furthermore, reduction in IL-2R signaling capacity is more pronounced in CD4+ T cells, especially in Tregs compared to CD8+ T cells, possibly due to increased production of IL-2Rβ and IL-2Rγ in CD8+ T cells upon IL-2 stimulation. This might provide a window for increased activation of effector cells due to disturbed immune homeostasis in tissues, such as seen upon skin-specific reduction of Tregs [55] or in several autoimmune diseases [reviewed in [56]]. Ultimately, this possibly favors organ-specific phenotypes upon IL-2 therapy, comparable to patients with *IL2RB* mutations [27,30].

3.4. Decreases in IL-2 signaling capacity correlate with high IL-2Rα expression in T cells

In general, our study supports the notion of differential IL-2 responses between CD4+ and CD8+ T cells as previously shown by others [41,57,58]. This is evident by significant early decreases in IL-2Rβ surface expression, early slight reduction in IL-2R signaling capacity, and donor-specific decreases in IL-7 signaling after continuous high-dose IL-2 stimulation of CD4+ T cells. Furthermore, intracellular IL-2Rβ and IL-2Rγ abundance was increased over time in all cell subsets investigated, except CD4+ T cells. Besides differences in IL-2Rβ and IL-2Rγ production, more pronounced changes in CD4+ subsets compared to other populations could also be due to higher prevalence of IL-2Rα (Fig. 3A, S1C).

The trimeric IL-2Rαβγ has 100-fold higher affinity to IL-2 than the intermediate-affinity IL-2Rβγ [59]. Therefore, the trimeric IL-2Rαβγ tends to have a higher IL-2 occupancy than the dimeric IL-2Rβγ, irrespective of IL-2 doses given [60]. Due to the IL-2 excess in our study, we assume that the IL-2R occupancy constantly remained high. Given the decreased half-life of occupied IL-2Rs compared to free receptors [61], cells with high IL-2Rα expression might have a higher IL-2R turnover due to increased IL-2 binding which could in turn lead to more pronounced reduction in IL-2R signaling capacity. To test this hypothesis, we analyzed the possible correlation between IL-2Rα expression and reduction in pSTAT5 signal upon IL-2 re-stimulation. To investigate if high IL-2Rα expression would be a predictive marker for later decreases in IL-2R signaling, we included IL-2Rα expression at day 0 without stimulation and differences in pSTAT5 signal after re-stimulation of unstimulated cells vs. cells previously stimulated with IL-2 for 7 days.

While no correlation was found between IL-2Rα expression and reduction in pSTAT5 signal when cells were stimulated with 100 IU/mL IL-2 for 7 days, high IL-2Rα expression positively correlated with high differences in pSTAT5 signal (i.e. with a more pronounced reduction in IL-2R signaling capacity upon IL-2 re-stimulation) in higher-dose IL-2-stimulated (≥1000 IU/mL) CD4+ Teffs and Tregs (Fig. 5A+B). A similar trend was observed for CD8+ T cells (Fig. 5C). No correlation between differences in IL-2 signaling and baseline expression of IL-2Rβ or IL-2Rγ were found (Fig. S3E). These data suggest that high expression of IL-2Rα might be a prognostic factor for a pronounced reduction in IL-2R signaling capacity following high-dose IL-2 stimulation. In line with this hypothesis, we recently reported that IL-2Rα + cells are at least partly responsible for IL-2-induced skin inflammation [62] and IL-2 constructs omitting IL-2Rα binding show reduced systemic immunotoxicity [63,64].

Overall, continuous high-dose IL-2 stimulation induces cellular characteristics with several similarities to immune cells from patients with IL-2Rβ defects such as basically absent IL-2Rβ on surface of T cells, leading to impaired IL-2R signaling, while signaling in NK cells is maintained. Within the T cell compartment, CD4+ T cells and especially Tregs seem to be more broadly impaired than CD8+ T cells, supporting previous reports of lineage-specific differences in IL-2 sensitivity. As patients with hypomorphic *IL2RB* mutations present with multi-organ autoimmunity, impaired IL-2R function upon IL-2 stimulation might be a clinical biomarker for prediction of immune-related side effects during IL-2 therapy.

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CRediT authorship contribution statement

Charline Sommer: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sophie Jacob:** Writing – review & editing, Data curation. **Tonia Bargmann:** Writing – review & editing, Data curation. **Muhammad Shoaib:** Methodology, Investigation. **Basel Alshaikhdeeb:** Methodology, Investigation. **Venkata P. Satagopam:**

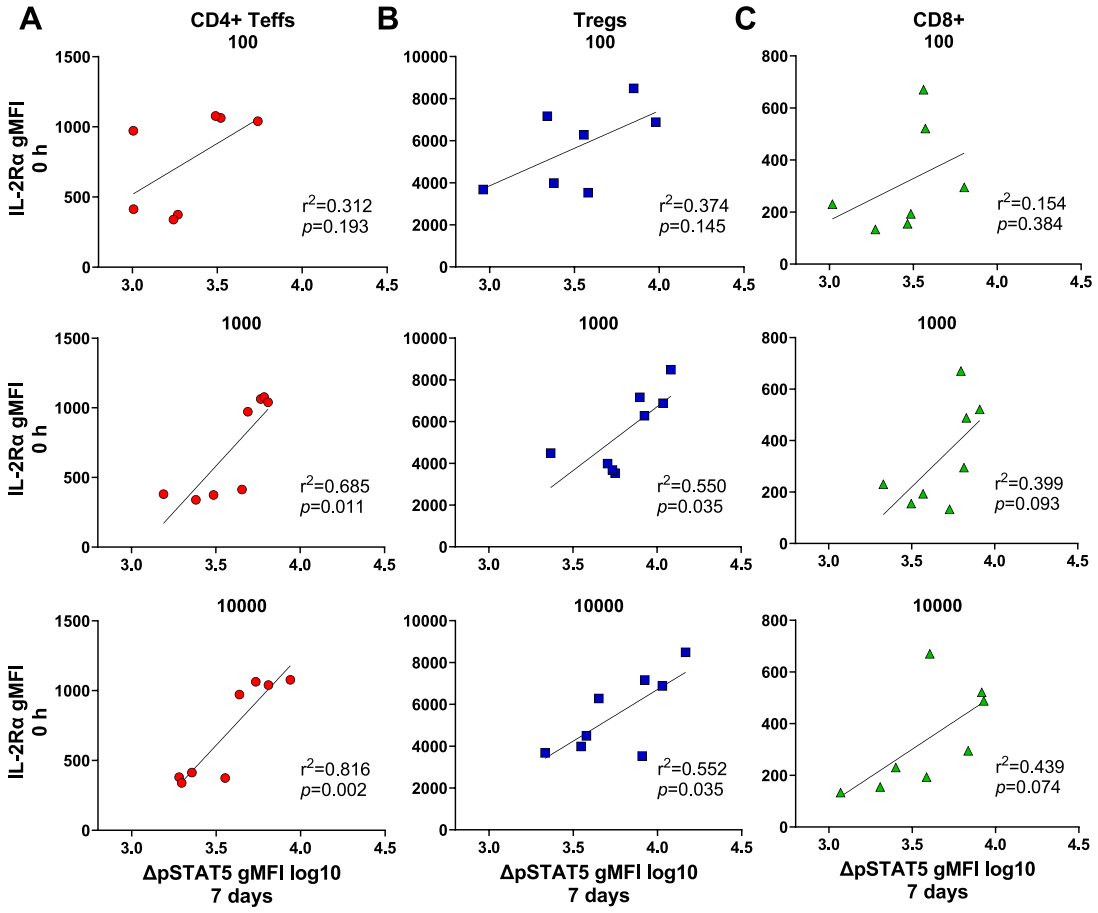

Fig. 5. IL-2Rα expression correlates with decreases in IL-2 signaling capacity of high-dose IL-2-stimulated T cells. Donor-dependent correlation of baseline IL-2Rα gMFI on CD4+ Teffs (A), CD4+ Tregs (B), and CD8+ T cells (C) without IL-2 stimulation with differences in pSTAT5 signal on day 7. For pSTAT5 signal, cells were stimulated with 100 (upper panels), 1000 (middle panels), or 10,000 IU/mL IL-2 (lower panels) or left unstimulated for 7 days and re-stimulated with high-dose IL-2 (10,000 IU/mL) for 15 min. ΔpSTAT5 is calculated as the difference in pSTAT5 gMFI of unstimulated cells and gMFI of IL-2-stimulated cells upon re-stimulation with IL-2 (ΔpSTAT5 = pSTAT5 gMFI (0 IU/mL) – pSTAT5 gMFI (100/1000/10,000 IU/mL)). The common logarithm (log10) of ΔpSTAT5 is shown. n = 8 donors, two independent experiments. r^2 and *p*-values determined using simple linear regression.

Methodology, Investigation. **Susann Dehmel:** Writing – review & editing, Supervision, Conceptualization. **Vanessa Neuhaus:** Writing – review & editing, Supervision, Conceptualization. **Armin Braun:** Writing – review & editing, Supervision, Funding acquisition. **Katherina Sewald:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Data availability

Data supporting the findings of this research article are available upon request to the corresponding author. All the data needed to evaluate the conclusions of the paper are present in the paper or the online supplemental material.

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Appendix A. Supplementary data

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