The role of interleukin-2 receptor subunits in recombinant interleukin-2-induced skin rash

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Charline Sommer, M. Sc. Biochemistry

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Acknowledged by the MD/PhD committee and head of Hannover Medical School

President: Prof. Dr. Michael P. Manns

Supervisors: Prof. Dr. Armin Braun, Dr. Katherina Sewald

Co-Supervisors: Prof. Dr. Renata Stripecke

Prof. Dr. Thomas Werfel

External expert: Prof. Dr. Elfriede Nößner

Internal expert: Prof. Dr. Sarina Ravens

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ABSTRACT

Interleukin-2 (IL-2) plays a pivotal role in immunity, supporting both immunosuppressive as well as immunostimulatory functions. This duality is partly mediated by cell-specific expression of IL-2 receptor (IL-2R) subunits, resulting in low- (IL-2Rα), intermediate- (IL-2Rβγ), or high-affinity IL-2R (IL-2Rαβγ). Regulatory T cells (T_{regs}) or group 2 innate lymphoid cells (ILC2s) with trimeric IL-2Rαβγ thus can be activated at picomolar IL-2 concentrations, whereas resting T effector cells and natural killer (NK) cells, lacking IL-2Rα, respond to higher IL-2 levels. The dual nature of IL-2 positions the cytokine in critical immune pathways, making it valuable to boost or inhibit immune reactions for the treatment of various pathologies including cancers or inflammatory diseases, respectively. However, unresolved adverse effects, including skin rashes, limit the use of recombinant IL-2 (recIL-2) in the clinics.

The objective of this thesis was to explore potential mechanisms contributing to recIL-2-induced adverse effects, with a specific emphasis on skin rashes. First, to characterize recIL-2-induced skin reactions, mice were intradermally injected with human recIL-2 (research paper 1), leading to mixed type 2/type 17 immunity through increases in IL-4- and IL-13-producing ILC2s and IL-17-producing dermal γδ T cells. While skin-specific reduction of IL-2Rα on T_{regs} significantly increased immune cell numbers, reducing IL-2Rα on all skin cells lowered immune cells in recIL-2-treated skin. These results highlight the role of innate lymphoid immune cell subsets and IL-2Rα-expressing effector cells inducing dermatitis upon recIL-2 injections. Second, to elucidate the regulation of IL-2R subunits on human immune cell subsets, human peripheral blood mononuclear cells were continuously stimulated with recIL-2 (research paper 2). High-dose recIL-2, usually applied during anti-cancer treatment, significantly decreased IL-2Rβ surface expression on T cell subsets with a corresponding reduction in IL-2 and IL-15 signaling capacity – cytokines both signaling through IL-2Rβγ. In contrast, extracellular IL-2Rβ was reduced but remained high on NK cells, thus not impairing IL-2R signaling. These cellular responses are comparable to patients suffering from hypomorphic *IL2RB* mutations which present with multiorgan autoimmunity including skin rashes. Within the T cell subsets investigated herein, $CD4^+$ T cells and especially T_{regs} seem to be more broadly impaired by high-dose recIL-2 compared to $CD8^+$ T cells or γδ T, NKT, and NK cells as IL-2R surface abundance and signaling were decreased at earlier time points which partly might be mediated by high IL-2Rα expression. Furthermore, $CD4^+$ T cell subsets showed a slight reduction in IL-7 signaling which, like IL-2 and IL-15, is a central factor for maintenance of T cells.

Overall, IL-2R subunits play central roles in inducing recIL-2-induced immune dysregulation, possibly associated with adverse effects during therapy. While activated $IL\text{-}2R\alpha^+$ effector cells drive skin inflammation during recIL-2 application, loss of IL-2Rβ surface expression upon high-dose recIL-2 stimulation seems to cell-specifically impair sensitivity towards cytokines crucial for T cell

maintenance, correlating with high IL-2Rα expression. Consequently, IL-2Rα could drive recIL-2-induced adverse effects such as skin inflammation by directly activating effector cells and indirectly impairing functionality of cells which could ultimately disturb immune balance in the skin. Aligning the results provided here with clinical data might thus help to increase the safety of future recIL-2-based or other therapies.

1 INTRODUCTION

1.1 Interleukin-2 – a cytokine with dual roles in immunity

In 1976, a new factor gained interest that induced T cell growth and survival – interleukin-2 (IL-2) was discovered.[1] Nowadays, the diverse functions of the cytokine are known to go far beyond the expansion of T cells. IL-2 is involved in immune homeostasis, activation and inhibition of immunity, T cell differentiation, and plays a central role in immune tolerance. Due to these diverse characteristics, the cytokine has great potential for immunomodulatory therapies.

Although initial studies suggested a central role of IL-2 in promoting effector functions, IL-2 knockout models did not lead to the expected immunodeficient phenotype. Instead, *Il2*$^{-/-}$ mice showed lymphadenopathy, and severe autoimmunity and quickly died after birth.[2] Similar results were found for mice treated with anti-IL-2 antibodies,[3] pointing towards a key role of the cytokine in mediating immune tolerance. In IL-2-depleted mice and IL-2 and IL-2 receptor (IL-2R) knockout mice, the numbers of regulatory T cells (T_{regs}) are significantly reduced, showing that IL-2 is crucial to developing and maintaining T_{regs} which prevent autoimmunity.[3, 4] On that note, IL-2 does not only support immune tolerance by maintaining T_{regs}, it also directly induces suppressive functions of the immune cells, independent of T cell receptor activation.[5] In line with murine studies, cells from patients with inflammatory diseases such as type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, or Crohn's disease produce less IL-2 compared to cells from healthy volunteers,[6–8] correlating with reduced activation and numbers of T_{regs}.[8, 9]

The dual roles of IL-2 – both inducing effector and regulatory functions – were further established by simultaneously deleting T_{regs} and IL-2: While mice lacking T_{regs} present with severe autoimmunity and rapidly die after birth, additional knockout of *Il2* prolonged the lifespan of these mice,[10] indicating that the function of IL-2 also includes induction of effector responses *in vivo*. Specifically, IL-2 supports the differentiation of $CD4^+$ T cells into type 2 T helper (T_H2) cells,[11] and T_H9 cells,[12] and aids in terminal differentiation of $CD8^+$ T cells,[13, 14] while inhibiting T_H17 generation.[15] Besides adaptive cells, IL-2 can impact the function of innate effector cells, inducing the production of cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, and cytotoxicity by natural killer (NK) cells, NK T (NKT) cells, and γδ T cells.[16–19] Furthermore, innate lymphoid cells (ILCs) as the innate counterparts of T_H cells and especially group 2 ILCs (ILC2s), resembling T_H2 cells, are well-described to be directly activated by IL-2, leading to proliferation and secretion of type 2 cytokines.[20–23]

Physiologically, activated T cells are well-known IL-2 producers, signaling in an autocrine and paracrine manner. Under steady-state conditions, $CD4^+$ T cells are the major IL-2 source in most secondary lymphoid tissues, necessary for T_{regs} development.[24–26] However, in non-lymphoid organs such as skin or gastrointestinal tract, ILCs have recently been identified as other main IL-2

producers[8, 27] and murine studies showed that IL-2 produced by ILC3s and not T cells is necessary to maintain intestinal T_{regs}.[8] Further IL-2 producers include, among others, dendritic cells, contributing to the maintenance of T_{regs} in certain organs[24, 26] and aiding in T cell activation upon antigen presentation,[28] B cells,[26, 29] and mast cells.[30]

How can one cytokine induce both immunosuppression and -activation? The most apparent explanation is that T_{regs} constitutively express high levels of IL-2Rα (CD25), crucial to form the high-affinity IL-2R (described below), while resting effector T cells (T_{effs}) usually express the intermediate-affinity IL-2R and only transiently induce expression of IL-2Rα upon activation. Expression of the high-affinity IL-2R increases IL-2 affinity 100-fold compared to the intermediate-affinity IL-2R lacking IL-2Rα, leading to a higher IL-2 sensitivity of T_{regs} compared to effector cells (Figure 1).[31, 32] Besides increased activation, the high-affinity IL-2R enables T_{regs} to capture and compete for IL-2 as part of their inhibitory function, which limits bioavailability of the cytokine for – and, with that, activation of – effector cells.[5, 33] However, differential IL-2R expression does not seem to be the sole reason for dual functions of IL-2 as T cell blasts with high IL-2Rα expression still show lower IL-2 sensitivity compared to T_{regs}.[32] Rather, cell-specific differences in signaling cascades add to the differential function of IL-2 in T_{regs} and effector cells.

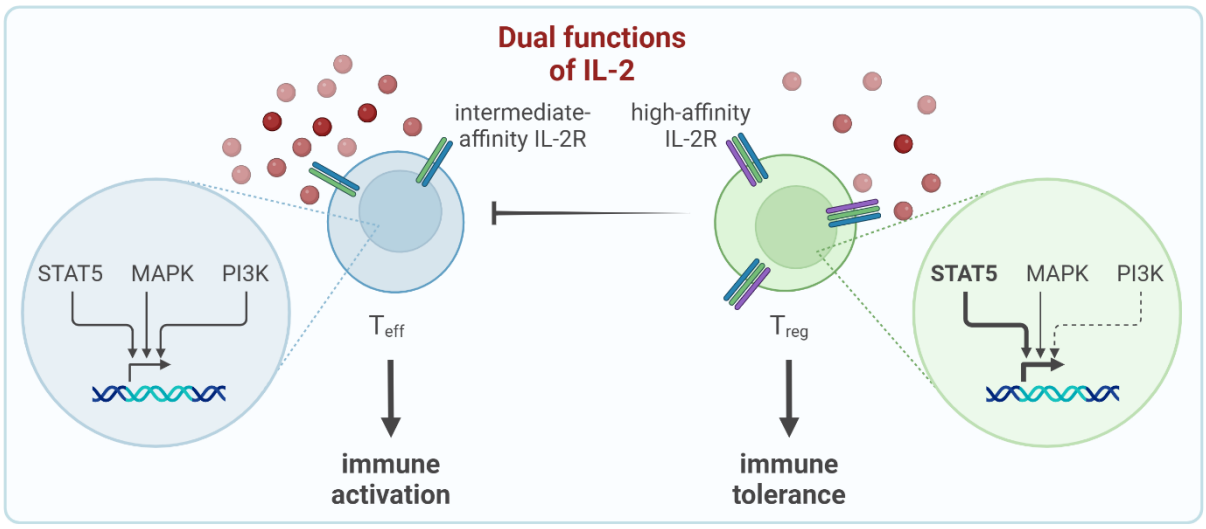

Figure 1: Extracellular and intracellular differences determine the dual functions of IL-2 in immunity. High expression of trimeric high-affinity IL-2R on regulatory T cells (T_{regs}) enables binding of IL-2 even at low concentrations while resting effector T cells (T_{effs}), which usually express the dimeric intermediate-affinity IL-2R, are activated at higher IL-2 levels. Upon IL-2 binding, the signal transducer and activator of transcription 5 (STAT5) signaling pathway seems to be favored in T_{regs}. Overall, expression of IL-2-dependent genes is 100-fold higher in T_{regs} compared to effector cells. PI3K: class I phosphatidylinositol 3-kinase; MAPK: mitogen-activated protein kinase. Created with BioRender.com.

The binding of IL-2 can induce a variety of signaling cascades including activation of Janus family kinases (JAKs), leading to phosphorylation of signal transducer and activator of transcription (STAT), mitogen-activated protein (MAP) kinase cascade, and class I phosphatidylinositol 3-kinase (PI3K) signaling.[34] In T_{regs}, STAT5 signaling may be favored due to increased expression of phosphatase and tensin homolog (PTEN), inhibiting PI3K signaling.[35–37] This could partly account for increased phosphorylation of STAT5 (pSTAT5) at concentrations 10 times lower than T_{effs} or NK cells.[32] Furthermore, T_{regs} have been shown to harbor increased activity of protein phosphatase 2A (PP2A)[32]

which boosts IL-2 signaling by removing inhibitory phosphorylations from IL-2R, leading to increased pSTAT5 activation.[38] Overall, T_{regs} were shown to induce IL-2-dependent genes at IL-2 concentrations 100-fold lower compared to $CD4^+$ memory T cells.[32] Together, differences in intracellular signaling in addition to differential receptor expression shape the dual functions of IL-2 in immunity (Figure 1).

1.1.1 Deficiencies in IL-2R subunits disturb the balance of the immune system

The IL-2R complex is composed of up to three subunits, IL-2Rα, IL-2Rβ (CD122), and IL-2Rγ (CD132). All three subunits form the high-affinity IL-2Rαβγ which can bind IL-2 at picomolar concentrations (Kd~10 pM) and is specific for IL-2.[39] Within the IL-2R complex, IL-2Rβ and IL-2Rγ represent the signaling subunits,[40] forming the intermediate-affinity dimeric IL-2Rβγ (Kd~1 nM) which is shared by IL-15.[41] IL-2Rγ is also termed the common γ chain (γc) as it is further shared by IL-4, IL-7, IL-9, and IL-21.[42–45] Furthermore, IL-2Rα alone forms the low-affinity IL-2R which does not harbor intracellular signaling capacity (Kd~10 nM).[46]

The binding of IL-2 to the IL-2R complex occurs in a stepwise process. Initially, IL-2 binds to IL-2Rα, leading to a conformational change in IL-2 which increases the binding affinity of the cytokine to IL-2Rβ.[31, 47] Further association with IL-2Rγ initiates intracellular signaling.[31, 47, 48] Besides activation of signaling cascades, IL-2 binding also induces internalization of receptor subunits. While IL-2Rβ and IL-2Rγ are degraded in endosomes, IL-2Rα and bound IL-2 can be recycled which leads to an extracellular IL-2 reservoir and can further increase the durability of IL-2 signaling (Figure 2).[49, 50]

The expression of IL-2R subunits is cell-specifically regulated and further influenced by the activation status of cells. NK cells are the cells with the highest IL-2Rβ expression over $CD4^+$ and $CD8^+$ T cells.[26, 32] Resting T_{effs} and NK cells, expressing the dimeric IL-2Rβγ, can therefore be activated at high IL-2 concentrations (Figure 2).[16, 32, 51] Furthermore, besides T_{regs}, ILCs and especially ILC2s express IL-2Rα, which can thus be activated at low IL-2 concentrations (Figure 2).[21, 26, 32, 52] Resting αβ and γδ T cells and NK cells, as part of ILC1s, express low if any IL-2Rα but can induce expression upon activation by antigen or high IL-2 doses, increasing IL-2 sensitivity.[13, 16, 51, 53, 54] Furthermore, dendritic cells have been shown to express the low-affinity monomeric IL-2Rα in the absence of IL-2Rβ expression which enables $IL\text{-}2R\alpha^+$ dendritic cells to sequester IL-2 from T_{effs}.[26, 55]

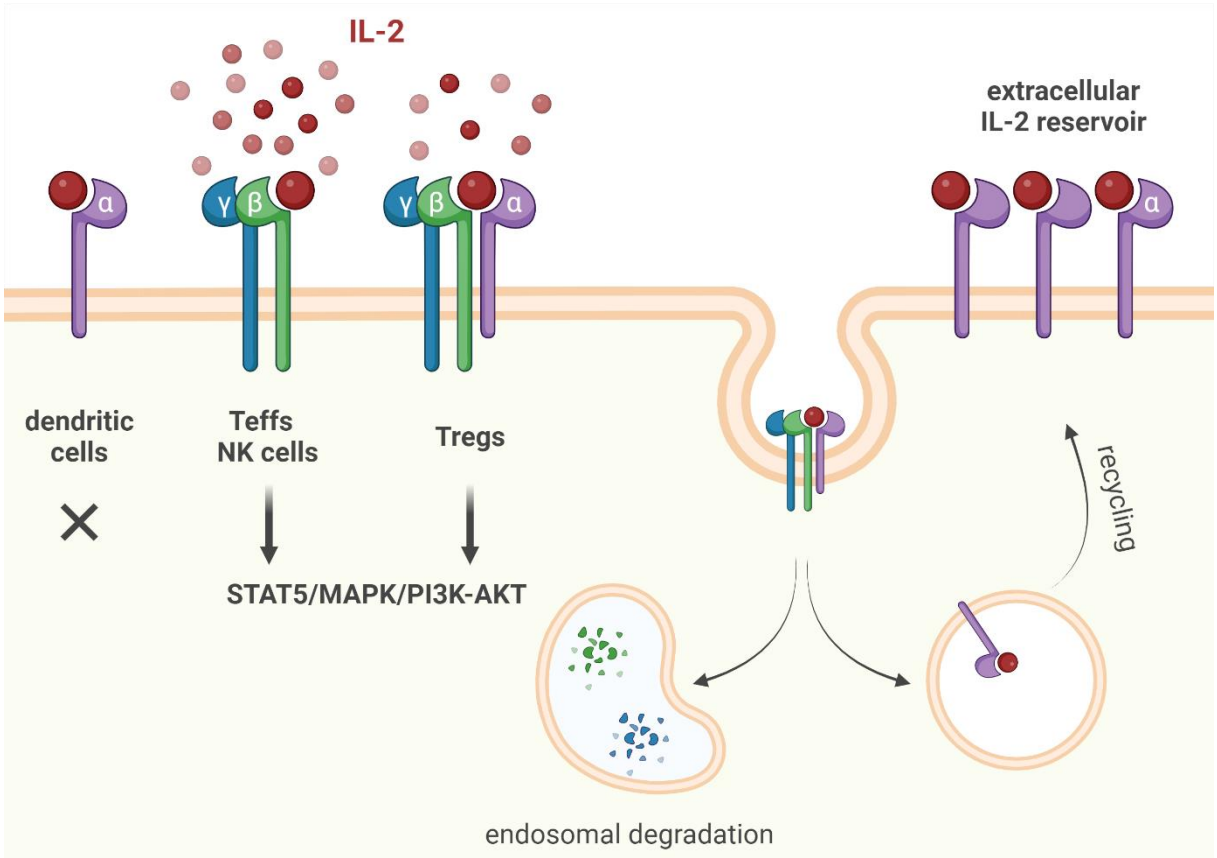

Figure 2: IL-2R subunits are differentially regulated upon IL-2 binding. Of the three IL-2R subunits, IL-2Rβ and IL-2Rγ are needed for intracellular signaling induced by IL-2. Cells expressing the intermediate-affinity dimeric IL-2Rβγ can be activated at high IL-2 concentrations and additional IL-2Rα expression increases sensitivity towards low IL-2 concentrations. Following IL-2 binding, the IL-2-IL-2R complexes are internalized, leading to endosomal degradation of IL-2Rβ and IL-2Rγ. On the other hand, IL-2Rα, together with bound IL-2, is recycled to the cell surface, forming an extracellular IL-2 reservoir on the respective cell. Adapted from Roser et al. (2024), in production.[56] Created with BioRender.com.

Mutations in IL-2R subunits lead to a mix of inflammatory phenotypes and infections, further highlighting the dual role of IL-2R signaling in immunity. *IL2RG* deficiencies lead to X-linked severe combined immunodeficiency (X-SCID), most frequently associated with a drastic decrease or absence of T and NK cells, while B cell numbers usually are unaltered ($T^{-}B^{+}NK^{-}$ phenotype).[57–59] Patients suffering from X-SCID present with severe bacterial, viral, or fungal opportunistic infections.[59, 60]

On the other hand, mutations in *IL2RA* or *IL2RB* manifest in immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX)-like syndrome, characterized by inflammatory organ manifestations, lymphoproliferation, memory phenotypes of T cell subsets, and chronic infections which are dominated by cytomegalovirus and other herpesvirus infections.[61–64] Patients with *IL2RA* mutations show inflammatory infiltrates in several organs, leading to autoimmune-like phenotypes such as dermatitis or enteropathy.[61, 62] Furthermore, multiple cytokines such as IL-2, IL-4, IL-6, TNF-α, and IFN-γ are increased in these patients.[61, 62] While IL-2 responsiveness is decreased, overall T_{reg} numbers seem to be comparable to healthy subjects.[61, 62, 65] Autoimmune manifestations likely are induced by decreased IL-2 consumption of IL-2Rα-deficient T_{regs}, leading to increased IL-2 levels which might favor activation of effector cells.[62] This notion is supported by transfer experiments of IL-2Rα-expressing T_{regs} into *Il2rα*$^{-/-}$ mice, drastically reducing inflammatory phenotypes including IL-2 levels and imbalanced $CD4^{+}$:$CD8^{+}$ ratios.[66]

While various studies investigate mutations in IL-2Rα, only two reports are published characterizing *IL2RB* mutations in depth. Similar to *IL2RA* mutations, *IL2RB* mutations present with multi-organ inflammation in the skin and gut, among other organs.[63, 64] Patients with hypomorphic *IL2RB* mutations show increased levels of IL-2 and IL-15, however, due to the lack of IL-2Rβ surface expression, T cells do not respond to either of the cytokines *in vitro*. In contrast to IL-2Rα mutations, T_{regs} are basically absent in patients with defective IL-2Rβ, likely inducing autoimmune manifestations.[63, 64] While some phenotypes are reflected in *Il2rb*$^{-/-}$ mouse models, including reduction of T_{regs} and systemic inflammation, differences occur in organ manifestations compared to patients with *IL2RB* mutations as no skin abnormalities or colitis were observed in mice.[67, 68]

Overall, IL-2R signaling is crucial to mounting effective immune responses on the one side while maintaining immune tolerance on the other side. This dualism of IL-2 positions the cytokine at several critical points in the immune system which is advantageous to boost or inhibit immune responses to alleviate immune-mediated diseases.

1.1.2 Recombinant IL-2: An immunotherapy with adverse effects

The T cell-activating properties of IL-2 made it an interesting treatment option to boost effector immune responses for anti-tumor therapy. In the 1990s, aldesleukin, a recombinant IL-2 (recIL-2) marketed as Proleukin®, was among the pioneering immunotherapies approved by the FDA for the treatment of metastatic melanoma and metastatic renal cell carcinoma. Aldesleukin is commonly applied in high doses (600,000-720,000 IU/kg) every 8 h for a maximum of 14-15 doses in two cycles[69] to activate T_{effs} and NK cells for anti-tumor activity. However, although survival rates are promising in patients who respond to the treatment, objective response rates only are around 15%.[70, 71] One reason for this is the low half-life of recIL-2 of about 15 min due to renal clearance which limits optimal, continuous activation of effector cells and, thus, anti-tumor responses.[72] Furthermore, along with T_{effs} and NK cells, T_{regs} are activated and expanded during high-dose aldesleukin therapy which can inhibit anti-tumor responses, therefore limiting therapeutic effects.[73–75] However, increasing recIL-2 doses to achieve preferential activation of effector cells is prevented by dose-limiting toxicity, leading to severe systemic adverse effects (Figure 3).

Common adverse effects during high-dose aldesleukin therapy include more general symptoms such as fever, nausea, vomiting, or diarrhea.[76] Furthermore, vascular-leak-syndrome frequently is induced, characterized by increased permeability of endothelial cells and most likely resulting from the myriad of cytokines secreted during high-dose recIL-2 therapy.[69] Additionally, organ-specific immunotoxicity occurs presenting as hepato- or cardiac toxicity, colitis, neurotoxicity, or cutaneous toxicity manifested as skin rashes.[69] These skin rashes might correlate with peripheral eosinophilia induced during aldesleukin therapy,[22, 77] which could therefore resemble cutaneous drug reactions such as drug reaction with eosinophilia and systemic symptoms (DRESS) syndrome.[78] However, pathophysiological mechanisms of DRESS, and specifically of recIL-2-induced adverse effects are still

barely understood and while numerous studies have investigated adverse events such as vascular-leak-syndrome, associated with failure of multiple organs,[79–85] studies clarifying key mechanisms of recIL-2-induced skin rashes are scarce.

Although toxicity and poor efficacy initially led to a drawback of recIL-2 therapy, nowadays over 40 IL-2-derived products are being investigated in preclinical and clinical trials,[86] highlighting the potential of the cytokine for immunotherapy. Besides anti-cancer therapy, the dual roles of IL-2, either boosting or diminishing inflammatory responses, expand the therapeutic application to treat inflammatory diseases such as autoimmunity. In these therapies, low-dose recIL-2 aims to specifically expand and activate T_{regs}, expressing the high-affinity IL-2R.[87] The resulting anti-inflammatory response is favorable in inflammatory conditions such as chronic graft-versus-host disease, type 1 diabetes, and systemic lupus erythematosus, among others.[87–91] However, the therapeutic window for low-dose immunosuppressing recIL-2 therapy is small due to the risk of activating effector cells, especially NK cells, which might diminish therapeutic efficacy (Figure 3).[89, 91–93]

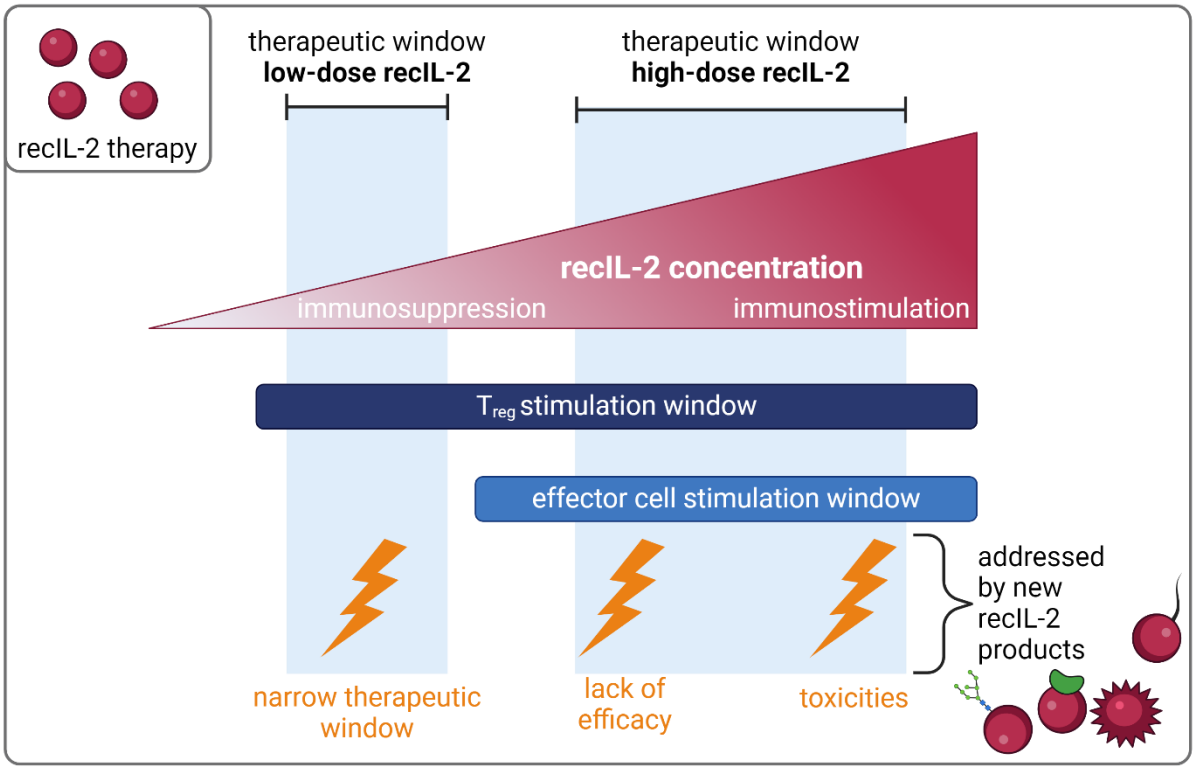

Figure 3: Limitations of low- and high-dose recIL-2 therapies. The immunosuppressive capacity of low-dose recIL-2 therapy is limited due to the risk of activating effector cells such as $CD8^+$ T or NK cells, leading to a narrow therapeutic window. High doses of recIL-2 for anti-cancer therapy, however, bear the potential of being ineffective due to simultaneous activation of T_{regs} while inducing immunotoxicity at increasing doses. New IL-2-derived products aim to tackle these caveats by increasing specificity and half-life, leading to higher efficacy while preventing adverse effects. Adapted from Roser et al. (2024), in production.[56] Created with BioRender.com.

To overcome narrow therapeutic windows, toxicities, and lack of efficacy, new recIL-2 molecules are being developed, aiming to increase half-life *in vivo* and to activate target cells more specifically (Figure 3). Strategies include modifying the IL-2 molecule to favor binding to one IL-2R complex, e.g. by increasing binding specificity to IL-2Rβ while reducing affinity to IL-2Rα-expressing cells, thus reducing undesired activation of T_{regs} while concomitantly increasing activation of effector

lymphocytes for tumor therapy.[94, 95] Furthermore, to increase stability, recIL-2 molecules might be coupled to structures such as polyethylene glycol (PEG), allowing slow release of active recIL-2, therefore gradually increasing bioavailability over time.[96, 97]

One prominent product optimized for anti-tumor therapy using PEGylation is bempegaldesleukin (NKTR-214), for which PEG chains were introduced at the IL-2Rα binding side of recIL-2 (aldesleukin), thus reducing binding to IL-2Rαβγ-expressing cells such as T_{regs}.[96, 98] This favors binding to and activation of IL-2Rβγ$^+$ effector cells which induces anti-tumor efficacy in mice and humans while simultaneously increasing *in vivo* stability over unmodified aldesleukin.[98, 99] However, although phase 1/2 studies with NKTR-214 as a combinational treatment with a checkpoint inhibitor initially seemed promising,[100] phase 3 studies and all further clinical development has been stopped due to insufficient efficacy, possibly due to induction of endogenous IL-2 leading to T_{reg} activation.[101, 102] Furthermore, while adverse effects such as hypotension, indicative for vascular-leak-syndrome, are significantly reduced compared to unmodified aldesleukin, several drug-related adverse events occur in NKTR-214 mono- and combinational therapy and, of those, skin rash was one of the most commonly reported toxicities (54% and 39% upon monotherapy and combinational therapy, respectively).[99, 100] While usually not life-threatening, skin rashes and other cutaneous adverse drug reactions (CADRs) including pruritus represent a substantial factor to negatively impact overall well-being of patients which might hamper therapeutic efficacy due to termination of treatment.[69] This highlights the need to understand mechanisms leading to the development of recIL-2-induced skin rashes to minimize or even prevent onset of this adverse effect in future IL-2-based therapies.

1.2 RecIL-2-induced skin rash – an adverse effect with unknown mechanisms

In the initial years of high-dose aldesleukin therapy, skin rashes frequently developed a few days after the onset of therapy, affecting up to 72% of patients.[103] Among the most frequent cutaneous reactions described for aldesleukin therapy are erythematous rashes and diffuse macular erythemas, often accompanied by pruritus.[104–107] Initially localized on proximal extremities, head, or back of patients, these skin rashes might spread and can become more generalized.[104–106, 108–110] Usually, rashes resolve after stopping recIL-2 therapy but might rapidly reoccur once treatment is continued.[103, 106, 108, 111]

Histological analyses of biopsies from patients suffering from skin rashes during high-dose aldesleukin therapy show thickening of the epidermis, necrosis of keratinocytes, and prominent cell infiltrates in perivascular location.[104, 106] These infiltrates predominantly are characterized as mononuclear cells, which mostly seem to be composed of CD3$^+$ T cells, while NK cells, B cells, and macrophages are less frequent.[104, 106, 112] CD4$^+$ T cells predominate over CD8$^+$ T cells and some of these CD4$^+$ T cells are positive for IL-2Rα,[103, 104, 106, 113] suggesting activated T_{effs} or T_{regs}. The occurrence of granulocytes, namely eosinophils, and neutrophils, further characterizes the mixed inflammatory infiltrates in skin rashes during aldesleukin therapy.[77, 105–108] Another hallmark is the increased expression of adhesion molecules including endothelial-leukocyte adhesion molecule-1 (ELAM-1),

vascular cell adhesion protein-1 (VCAM-1), and especially intercellular adhesion molecule-1 (ICAM-1), expressed on keratinocytes and stromal, endothelial, and mononuclear cells.[103, 104, 114, 115]

Although immune cells seem to play a central role in inducing skin damage and, consequently, skin rashes during aldesleukin therapy, molecular and cellular key mechanisms leading to the adverse effect are still unknown. This might in part be due to the lack of more recent reports, phenotyping biopsies from patients in depth. The following sections focus on two mechanisms that might contribute to inducing skin rashes during recIL-2 therapies. However, due to the pleiotropic effects of IL-2 and because mechanisms of recIL-2-induced skin rashes have only scarcely been investigated in current literature, other mechanisms such as sensitization reactions or activation of further cells might be involved in inducing the adverse effect during recIL-2 therapies.

1.2.1 RecIL-2-induced skin rashes: A type 2-driven adverse effect?

Given clinical reports identifying mixed inflammatory infiltrates in biopsies of patients affected by skin rashes, it stands to reason that these infiltrates are crucial to induce dermatologic complications. Accumulation of mononuclear cells in the skin during high-dose aldesleukin therapy might partly be mediated by the local proliferation of lymphocytes in response to aldesleukin. In this regard, T cells and ILC2s from the skin proliferate in response to recIL-2 without antigen stimulation.[23, 116] Subsequent to IL-2 activation, ILC2s are known to secrete type 2 cytokines IL-5 and IL-13 which could induce local inflammation in the skin.[21–23] In line with this, in the skin of $Rag^{-/-}$ mice, lacking T and B cells, ILC2s were specifically activated upon injection of an antibody-coupled recIL-2 construct, leading to dermatitis in half of the mice.[21] Furthermore, IL-5-producing ILC2s are reported to be crucial for recIL-2-induced peripheral eosinophilia as another common adverse effect during recIL-2 therapy,[22] thus underlining the relevance of ILC2s in recIL-2-induced adverse effects. On the other hand, antigen-independent activation of T cells leading to e.g. cytokine secretion or other effector functions is only reported in response to cytokine mixtures[117, 118] but not towards sole IL-2 stimulation and, thus, the impact of T cells on recIL-2-induced skin rash is unclear.

Besides lymphoid cells, mast cells are reported to be increased in skin rashes of aldesleukin-treated patients[104] and, similarly, mast cells appeared to be activated in dermatitis sections of recIL-2-treated $Rag^{-/-}$ mice.[21] Mast cells and ILC2s specifically interact in the skin and activation of mast cells possibly is induced through direct interaction with recIL-2-activated ILC2s such as proposed for atopic dermatitis,[21, 119] further suggesting type 2-driven immunity underlying recIL-2-induced skin rashes. Possible mast cell activation might initiate a positive feedback loop through the secretion of leukotrienes or prostaglandins, which could further enhance the activation of ILC2s (Figure 4).[120–122]

Following activation of immune cells in the skin, especially ILC2s and mast cells, recruitment of cells from the circulation might be induced, leading to the typical mixed infiltrates seen in biopsies of patients suffering from recIL-2-induced skin rashes. Specifically, IL-5 and IL-13 secreted by recIL-2-

activated ILC2s, and tryptase and chymase possibly secreted by mast cells might induce infiltration of eosinophils and neutrophils into the skin of recIL-2-treated patients.[123–125] Furthermore, possible histamine secreted by mast cells can induce IL-8 secretion by keratinocytes which might support neutrophil infiltration (Figure 4).[126]

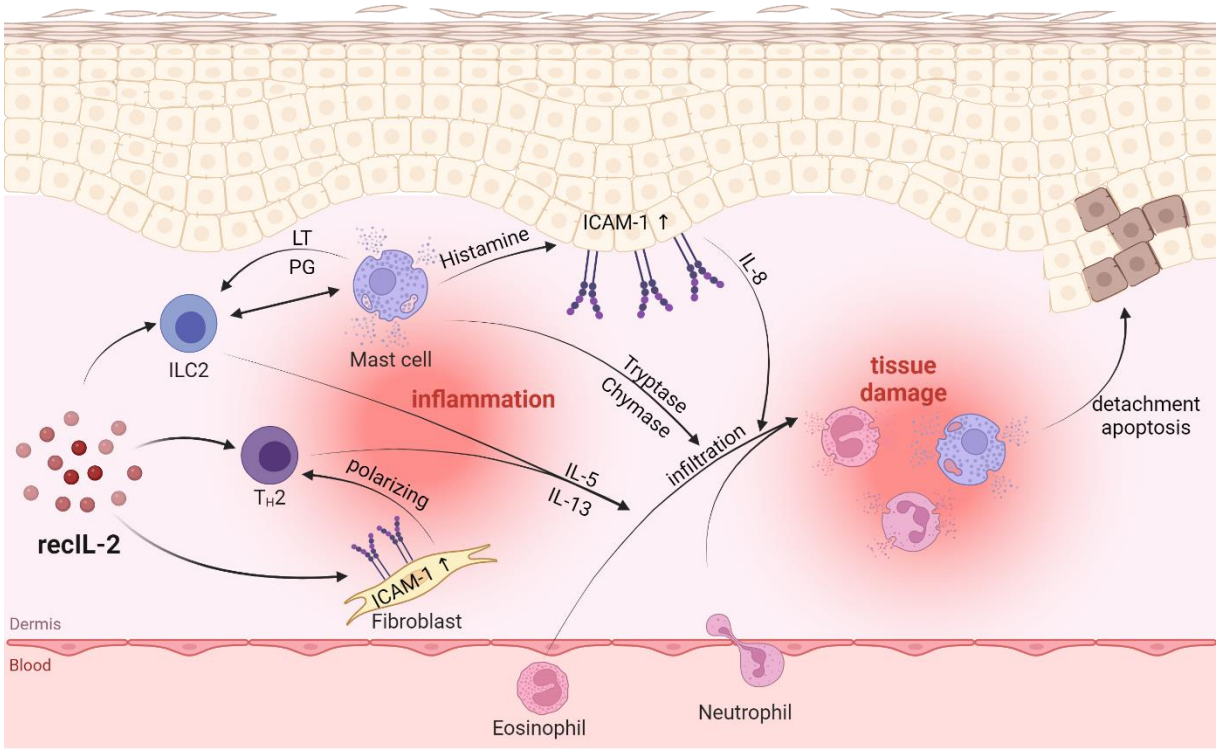

Figure 4: Proposed key cells inducing skin rashes during recIL-2-therapy. Upon recIL-2 therapy, type 2 immune cells including group 2 innate lymphoid cells (ILC2s) might be activated. Secondary activation of mast cells could induce a positive-feedback loop due to the secretion of leukotrienes (LT) and prostaglandins (PG). Secretion of type 2 cytokines and mast cell mediators might induce infiltration of granulocytes, further facilitated by increased ICAM-1 expression. Activation of resident and recruited cells could finally induce tissue damage, manifested as skin rashes in the clinics. Adapted from Sommer et al., under revision in Journal of Immunotoxicology. Created with BioRender.com.

Increased expression of ICAM-1 which was invariably reported in clinical studies could further support the accumulation of immune cells in the skin and might be induced on keratinocytes by possible secretion of mast cell histamine[126] or on fibroblasts directly through recIL-2.[127] RecIL-2 stimulation of fibroblasts might further strengthen type 2 immune responses through secretion of monocyte chemoattractant protein-1 (MCP-1), associated with polarization of T cells towards T_H2 cells.[127–129] Ultimately, activation of skin-resident and recruited cells could lead to a massive secretion of tissue-damaging proteins and enzymes, including secretion of eosinophilic major basic protein found in the proximity of eosinophils in biopsies affected by skin rashes.[77, 105] Overall, this might facilitate tissue damage, resulting in skin rashes during recIL-2 therapy (Figure 4). These mechanisms might resemble pathologies such as those described for atopic dermatitis or DRESS, as one known form of drug-induced cutaneous adverse effects.[78, 130] However, although current literature suggests type 2-driven immune responses during recIL-2-induced skin rashes with ILC2s as key cells initiating the cascade, most of the results are based on mice models lacking T and B cells which makes translation to a fully immunocompetent organism challenging. Thus, further research is needed to investigate immunological cascades leading to recIL-2-induced skin rash.

1.2.2 Potential relevance of IL-2R subunits in recIL-2-induced adverse effects

ILC2s seem to be central to inducing adverse effects such as skin rashes during recIL-2 therapy – cells which are known to express IL-2Rα even under steady-state conditions.[21, 131, 132] Therefore, it stands to reason that the IL-2R subunit is a key molecule in recIL-2-induced adverse effects including skin rashes. In line with this, $Rag^{-/-}$ mice treated with recIL-2 and an IL-2Rα-specific antibody, JES6-1, developed spontaneous dermatitis,[21] supporting the notion that activation of IL-2Rα$^+$ cells is central to inducing skin rashes during recIL-2 application. Expression of the high-affinity IL-2Rαβγ increases the affinity of the receptor towards IL-2 up to 100-fold compared to the dimeric IL-2Rβγ, lacking IL-2Rα.[31] Thus, IL-2-induced activation is increased in cells expressing the trimeric IL-2R such as T_{regs}, ILC2s, or activated lymphocytes. As IL-2 itself (including recIL-2 therapy) induces upregulation of IL-2Rα on lymphocytes such as T cells or ILCs including NK cells,[16, 21, 53, 75, 133, 134] these cells become more sensitive towards further IL-2 stimulation. During continuous recIL-2 therapy, this could tip the balance of inhibitory T_{reg} and inflammatory effector cell responses towards the inflammatory side which might ultimately induce toxicity (Figure 5A). In favor of this hypothesis is the observation that the occurrence of adverse effects during high-dose recIL-2 therapy is associated with improved anti-tumor response rates, which rely on activation of effector lymphocytes.[135] Still, the direct role of IL-2Rα during recIL-2-induced skin inflammation is hypothetical and needs further investigation.

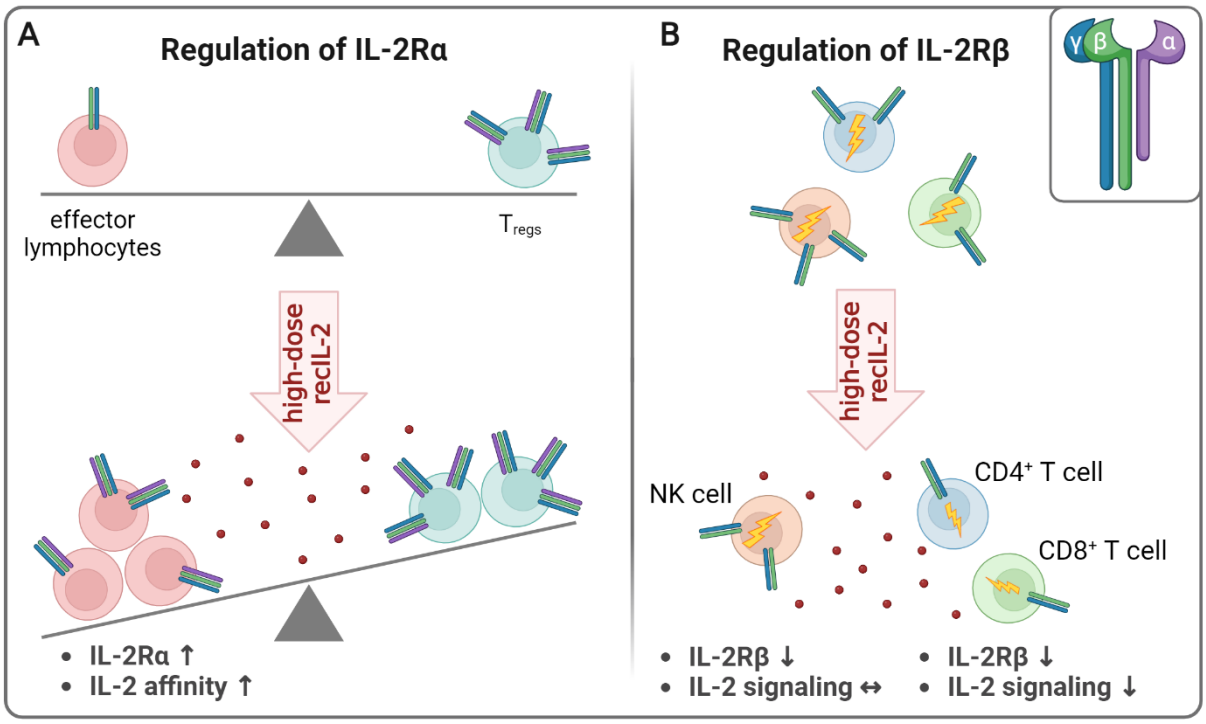

Figure 5: Possible role of IL-2R subunits inducing adverse effects during high-dose recIL-2 therapy. A During IL-2 stimulation, IL-2Rα expression is increased in effector lymphocytes, in turn enhancing IL-2 affinity. This might tip the balance from immune homeostasis towards toxic effector functions which could ultimately contribute to adverse effects during recIL-2 therapy. **B** Possibly, continuous high-dose IL-2 stimulation leads to decreased IL-2Rβ surface expression especially on T cells due to intracellular degradation of IL-2Rβ upon IL-2 binding, and internalization. In turn, IL-2R signaling might be differentially impacted which could favor the onset of adverse effects during high-dose recIL-2 therapy. Created with BioRender.com.

While IL-2Rα expression is increased in IL-2-stimulated lymphocytes, expression of IL-2Rβ might be differentially regulated. In contrast to IL-2Rα, IL-2Rβ is intracellularly degraded upon IL-2 binding and internalization.[49] Continuous IL-2 stimulation such as during recIL-2 therapy might therefore exhaust IL-2Rβ storages due to permanent internalization and degradation of the receptor which could induce a lack of IL-2Rβ on the cell surface of stimulated cells. On that note, upon *IL2RB* mutation, dermatitis and colitis are described[63, 64] – similar to patients suffering from recIL-2-induced adverse effects, possibly suggesting similar pathophysiological mechanisms leading to organ manifestations. Furthermore, patients with *IL2RB* mutations show increased IL-2 plasma concentrations, resembling increased IL-2 bioavailability during recIL-2 therapy. In patients suffering from hypomorphic *IL2RB* mutations, T cells basically lack IL-2Rβ surface expression, leading to a reduction in IL-2Rβ signaling.[63, 64] In contrast, NK cells, which express significantly more IL-2Rβ compared to T cells,[32] show reduced IL-2Rβ surface expression but the majority of cells remains IL-2Rβ^{+}, thus not impacting IL-2R signaling in NK cells.[63, 64] Likewise, possible reduction of IL-2Rβ surface expression during recIL-2 therapy might especially affect T over NK cells, resulting in reduced IL-2 signaling in T cells which could have implications in the induction of skin rashes (Figure 5B). However, regulation of IL-2Rβ during recIL-2 therapy is currently poorly described and further research is needed to investigate the impact of continuous recIL-2 stimulation on IL-2R signaling capacity.

2 AIMS

Skin rashes are frequent adverse effects during therapy with unmodified recIL-2 (aldesleukin) and new recIL-2 alternatives with currently unresolved pathophysiological mechanisms. To ensure the safety of current and future IL-2-based and other therapies, identifying possible key molecules and cells inducing the adverse effect is crucial. Therefore, the major aim of this thesis was to investigate possible mechanisms leading to recIL-2-induced adverse effects with a focus on skin rashes.

Current literature suggests a central role of IL-2Rα and ILC2s, inducing type 2-driven immune responses in recIL-2-induced dermatitis. Using a novel skin-specific genetic deletion approach, the first aim therefore was to investigate the significance of IL-2Rα-expressing cutaneous cells during recIL-2-induced skin inflammation and the type of immunity induced by recIL-2 therapy. We hypothesized that IL-2Rα-expressing cells in the skin induce dermatitis during recIL-2 application and that skin inflammation predominantly is characterized by type 2 immune responses, driven by ILC2s (**research paper 1**).

Furthermore, IL-2 stimulation induces receptor internalization and degradation of IL-2Rβ which might induce similar cellular responses as in patients suffering from hypomorphic *IL2RB* mutations, manifested in autoimmunity including skin rashes. Thus, the second aim was to investigate the regulation of IL-2R subunits and signaling upon continuous stimulation of human lymphocytes with recIL-2. We hypothesized that high-dose recIL-2 stimulation decreases IL-2Rβ surface expression and IL-2R signaling capacity, especially in T cells over NK cells (**research paper 2**).

3 PUBLICATIONS

3.1 Interleukin-2-induced skin inflammation

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Charline Sommer[1*], Jarish N. Cohen[2,3*], Susann Dehmel[1], Vanessa Neuhaus[1], Dirk Schaudien[1], Armin Braun[1,4], Katherina Sewald[1#], Micheal D. Rosenblum[2#]

[1] 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)

[2] Department of Dermatology, University of California, San Francisco, San Francisco, CA (USA)

[3] Department of Pathology, University of California, San Francisco, San Francisco, CA (USA)

[4] Institute of Immunology, Hannover Medical School, Hannover (Germany)

[*] Shared first authorship

[#] Shared last authorship

Corresponding author:

Michael D. Rosenblum, Department of Dermatology, University of California, San Francisco, 505 Parnassus Ave, San Francisco, CA 94143, USA, Michael.Rosenblum@ucsf.edu

Author contribution statement:

Charline Sommer substantially contributed to conception and design of the study and to the manuscript with selection of endpoints and controls; was mainly involved in *in vivo* treatment of mice and performed all tissue harvesting; performed all flow cytometric measurements and respective data analysis and performed the statistical analysis for all data; interpreted all data and drafted and wrote the manuscript and implemented all required revisions.

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European Journal of Immunology Clinical-Basic-Translational

Allergy and inflammation

Research Article

Interleukin-2-induced skin inflammation

Charline Sommer[1], *Jarish N. Cohen*[2,3], *Susann Dehmel*[1], *Vanessa Neuhaus*[1], *Dirk Schaudien*[1], *Armin Braun*[1,4], *Katherina Sewald*[1] and *Michael D. Rosenblum*[2]

[1] 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
[2] Department of Dermatology, University of California, San Francisco, California, USA
[3] Department of Pathology, University of California, San Francisco, California, USA
[4] Institute of Immunology, Hannover Medical School, Hannover, Germany

Recombinant human IL-2 has been used to treat inflammatory diseases and cancer; however, side effects like skin rashes limit the use of this therapeutic. To identify key molecules and cells inducing this side effect, we characterized IL-2-induced cutaneous immune reactions and investigated the relevance of CD25 (IL-2 receptor α) in the process. We injected IL-2 intradermally into WT mice and observed increases in immune cell subsets in the skin with preferential increases in frequencies of IL-4- and IL-13-producing group 2 innate lymphoid cells and IL-17-producing dermal γδ T cells. This overall led to a shift toward type 2/type 17 immune responses. In addition, using a novel topical genetic deletion approach, we reduced CD25 on skin, specifically on all cutaneous cells, and found that IL-2-dependent effects were reduced, hinting that CD25 — at least partly — induces this skin inflammation. Reduction of CD25 specifically on skin Tregs further augmented IL-2-induced immune cell infiltration, hinting that CD25 on skin Tregs is crucial to restrain IL-2-induced inflammation. Overall, our data support that innate lymphoid immune cells are key cells inducing side effects during IL-2 therapy and underline the significance of CD25 in this process.

Keywords: Drug-induced skin inflammation · IL-2 therapy · Immunotherapy · Innate lymphoid cells · γδ T cells

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

As one of the first immunomodulatory cancer therapies to be approved by the FDA, recombinant IL-2 (aldesleukin), aimed to induce therapeutic effects by activating T- and NK cells. However, high-dose application necessary for antitumor activity led to severe systemic side effects, including skin rashes in up to 72% of the patients [1]. Currently, modifications of IL-2 have been undertaken to increase the specificity of treatment for both cancer and autoimmune diseases [2, 3]. However, a better under-

Correspondence: Dr. Michael D. Rosenblum
e-mail: Michael.Rosenblum@ucsf.edu

Charline Sommer and Jarish N. Cohen shared first authorship.
Katherina Sewald and Michael D. Rosenblum shared last authorship.

standing of the molecular mechanisms of IL-2-induced dermatitis is needed.

Affinities to IL-2 are determined by differential expression of IL-2 receptor (IL-2R) complexes on the respective cell. The dimeric intermediate-affinity IL-2Rβγ (CD122 and CD132) is shared by IL-15 [4] and expressed by resting T and NK cells, among others, which can in turn be activated at high IL-2 concentrations [5–7]. Additional expression of the IL-2Rα subunit (CD25), which is specific for IL-2, can induce the formation of the high-affinity trimeric IL-2Rαβγ which can bind IL-2 at picomolar concentrations [8]. Regulatory T cells (Tregs) which have high CD25 expression [5, 9] are able to scavenge free IL-2 through their high-affinity IL-2R, further promoting immune homeostasis [10, 11]. In addition to Tregs, innate lymphoid cells (ILCs) including activated NK cells as members of group 1 ILCs (ILC1s) [12] as well as activated αβ and γδ T cells might express the high-affinity IL-2R [6, 7, 13–15].

Similar to patients treated with high-dose IL-2, dermatitis occurred in $Rag^{-/-}$ mice treated with IL-2 and a CD25-specific antibody, [13] suggesting a central role of CD25 expression in IL-2-induced skin reactions. Histologically, skin reactions showed infiltrates of eosinophils and neutrophils in both mice and humans [13, 16, 17]. In addition, the expansion of group 2 ILCs (ILC2s), which are the innate counterpart of type 2 T helper cells, was observed with increased production of IL-5 and IL-13 upon IL-2 treatment [13, 18]. Together, these data led us to hypothesize that skin rashes during IL-2 therapies are CD25-dependent and characterized by type 2 immunity.

To test these hypotheses, we applied recombinant human IL-2 (aldesleukin) intradermally in mice and characterized the resulting immune response. Second, to evaluate the role of CD25 in IL-2-induced dermatitis, we deleted CD25 locally on skin cells and measured the impact of CD25 reduction on IL-2-induced skin reactions. Lastly, because CD25-expressing Tregs have a central role in maintaining immune homeostasis, we analyzed the effects of CD25 deletion on skin Tregs upon IL-2 injections.

Results

IL-2-induced skin inflammation is characterized by increases in innate immune cells

To characterize cutaneous immune reactions elicited by IL-2, WT mice were injected intradermally with recombinant human IL-2. For dose-finding experiments, IL-2 was injected once or twice daily for 7 consecutive days (Fig. 1A). The IL-2 dose applied here roughly corresponds to one high-dose intravenous injection given in the clinics [19]. However, the intermediate IL-2Rβγ in mice has a lower affinity to human IL-2 than human IL-2Rβγ [20, 21] and, thus, the chosen dosing regimen aimed to induce local over systemic effects in mice.

Histological analysis of skin at the injection site revealed mild inflammatory cell infiltration in control-treated skin (Fig. 1B). In contrast, injections of IL-2 once per day induced prominent inflammatory cell infiltrates consisting of lymphocytes, granulocytes, and macrophages (Fig. 1C). Numbers of these cells were highly increased in skin of mice injected twice daily (Supporting information Fig. S2), leading to cell infiltrates within large subdermal areas. To ensure only local immune reactions in skin without inducing systemic effects, IL-2 injections were performed once daily in all further experiments.

Flow cytometric analysis of immune cell populations in skin and skin-draining lymph nodes (SDLN) supported histological findings with significant increases in numbers of $CD45^+$ cells in the side of skin treated with IL-2 as compared with control sides (Fig. 2B). These increases in skin immune cell numbers were observed for $CD4^+$ and $CD8^+$ T-cell subsets (5.4- and 4.1-fold, respectively), dermal γδ T cells (8.6-fold), ILCs (9.0-fold), and CD3-$NK1.1^+$ "NK-like" group 1 ILCs (further referred to as ILC1s; 27.2-fold), but not for dendritic epidermal T cells (DETCs; Fig. 2C) and were accompanied by induction of the proliferation marker Ki-67 in T-cell subsets and ILCs (Supporting information Fig. S3A). In addition to increases in lymphocytes, IL-2 injections induced increases in neutrophil (13.6-fold) and eosinophil numbers (7.3-fold) in the skin (Fig. 2D). Notably, frequencies of immune cell subsets were only significantly increased for dermal γδ T cells (mean, 4.4–7.3%), ILCs (mean, 4.9–8.0%), and ILC1s (mean, 2.1–10.5%; Fig. 2E), but not for $CD4^+$ or $CD8^+$ T-cell subsets or granulocytes (Supporting information Fig. S3B).

CD25 as the subunit crucial to form the high-affinity trimeric IL-2R could be central in inducing IL-2-dependent side effects. Upon IL-2 injections, slight increases in frequencies of $CD25^+$ cells were observed for $CD4^+$ Teffs and $CD8^+$ T cells (Fig. 2F) in addition to increases in MFI of CD25 on $CD4^+$ Teffs (Supporting information Fig. S3C). Strikingly, CD25 expression of dermal γδ T cells, which only showed low if any CD25 expression under control conditions, was significantly increased upon IL-2 treatment, both regarding $CD25^+$ frequencies (mean 2.0% vs. 13.6% upon IL-2 injection) as well as MFI (Fig. 2F; Supporting information Fig. S3C).

Skin-specific reduction of CD25 reduces IL-2-induced accumulation of cells in the skin

To clarify the role of CD25-expressing cells in IL-2-induced skin inflammation, we aimed to delete CD25 specifically on skin cells by applying a newly developed method that allows inducible skin-specific, nonsystemic deletion of target genes [22]. $Rosa26^{CreERT2}xCD25^{fl/fl}$ mice were generated and 4-hydroxytamoxifen (4-OHT) was applied topically to induce pancellular CD25 deletion on one side of the back skin of these mice (Supporting information Fig. S4A). Acetone served as a vehicle control on the contralateral back skin, enabling us to investigate changes within the same mouse. Upon 4-OHT application without IL-2 injections, CD25 expression of skin Teffs, ILCs, and type 1 conventional DCs was almost completely abolished with frequencies of $CD25^+$ cells <3% (Supporting information Fig. S4B), which was reflected by reduced MFIs on the respective cells (Sup-

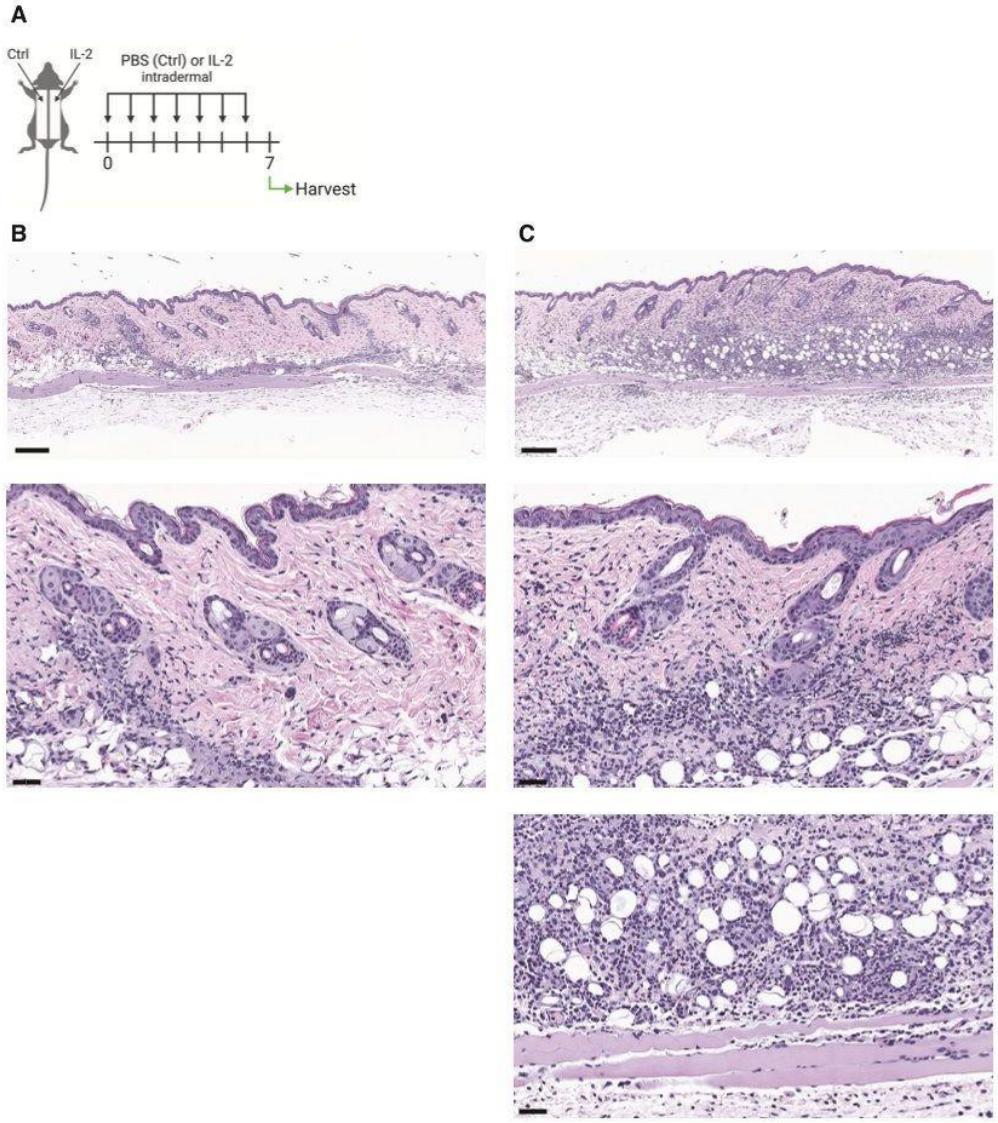

Figure 1. Intradermal IL-2 injections induce skin inflammation characterized by considerable immune cell infiltrates. (A) WT mice were intradermally injected with IL-2 (aldesleukin) daily for 7 consecutive days before analysis of skin. Created with BioRender.com. Representative histological images of injection sides from control (B) and IL-2-treated skin (C). Overview pictures (upper images; scale bars = 200 μm) and close-ups (middle and lower images; scale bars = 40 μm) are shown.

porting information Fig. S4C). In addition, CD25 expression on skin Tregs was significantly reduced with respect to $CD25^+$ cell frequencies (mean 11.9%) and total MFI. No effects on $CD25^+$ cell frequencies were observed in SDLN, providing evidence that deletion of the receptor was restricted to skin (Supporting information Fig. S4D).

To investigate the effects of skin-specific CD25 deletion on IL-2-induced skin inflammation, 4-OHT was applied to induce deletion prior to the initiation of IL-2 injections. Application of 4-OHT was continued during IL-2 treatment to ensure continuous CD25 deletion (Fig. 3A). The resulting reduction of CD25 in skin of IL-2-treated mice was partial but nonetheless significant for Tregs, DETCs, ILCs including ILC1s, and type 1 conventional DCs as shown by frequencies of $CD25^+$ cells and MFIs (Fig. 3B, C). Dermal γδ T cells showed slight decreases in MFI of CD25. Lower deletion efficacy upon IL-2 injection might be due to preferential proliferation of cells that remain $CD25^+$. Furthermore, 4-OHT was found to mostly penetrate upper layers of the skin including epidermis and dermis (unpublished observations). Therefore, IL-2-induced cell infiltrates in lower sections such as the subcutis will be less affected by 4-OHT, resulting in incomplete deletion. In contrast to the skin, frequencies of

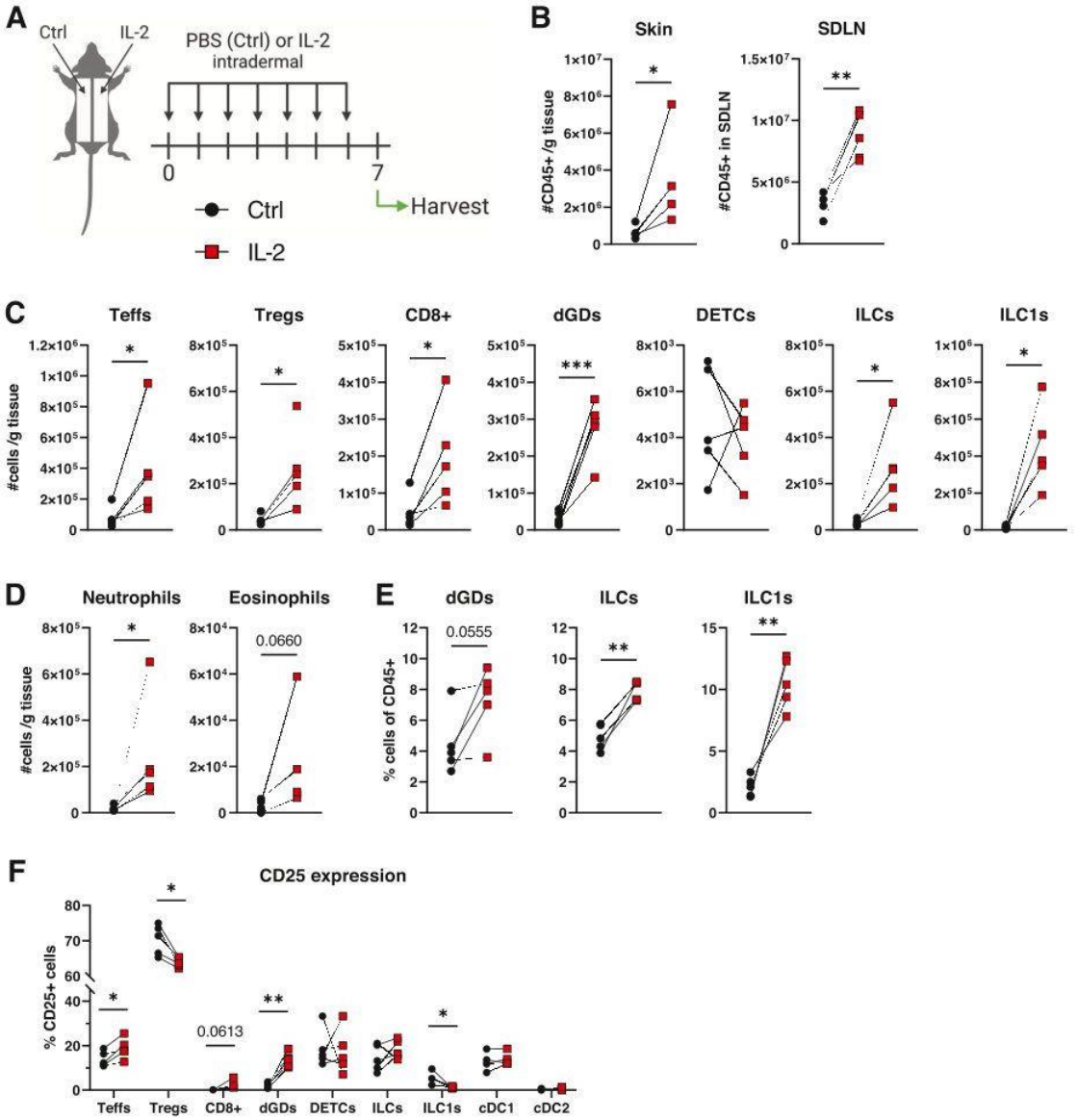

Figure 2. IL-2-induced skin inflammation is characterized by increases in frequencies of innate immune cells. (A) WT mice were intradermally injected with IL-2 (aldesleukin) daily for 7 consecutive days before analysis of skin and skin-draining lymph nodes (SDLN). Created with BioRender.com. (B) Absolute CD45$^+$ immune cell numbers in skin and SDLN. (C) Absolute numbers of T-cell subsets (Teffs: CD4$^+$ T effector cells; dGDs: dermal γδ T cells; DETCs: dendritic epidermal T cells), innate lymphoid cells (ILCs), and CD3-NK1.1$^+$ group 1 ILCs (ILC1s). (D) Absolute numbers of neutrophils and eosinophils in skin. (E) Frequencies of innate immune cells upon IL-2 injection. (F) CD25 expression as shown by CD25$^+$ frequencies of the respective cells. cDC, conventional dendritic cell. n = 5 mice from two independent experiments; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as determined by paired t-tests. All other conditions n.s.

CD25$^+$ immune cells in corresponding SDLN were largely similar between control and 4-OHT-treated sides (Supporting information Fig. S5).

Upon reduction of CD25 expression on the 4-OHT-treated side, total numbers of immune cells were significantly decreased in skin (Fig. 3D), with reduced Teffs, Tregs, dermal γδ T cells, and ILCs including ILC1s (Fig. 3E) as well as eosinophils but not neutrophils (Fig. 3F). Numbers of CD8$^+$ T cells, on the other hand, were unaltered – comparable to CD25 expression on CD8$^+$ T cells on the 4-OHT-treated vs. control side (Fig. 3B, D). Proportions of T-bet$^+$ type 1 and GATA-3$^+$ type 2 T-helper cells and ILCs were unaltered upon CD25 reduction (Fig. 3G). Together, these data suggest that CD25 expression on skin immune cells is required for their accumulation following intradermal IL-2 injection.

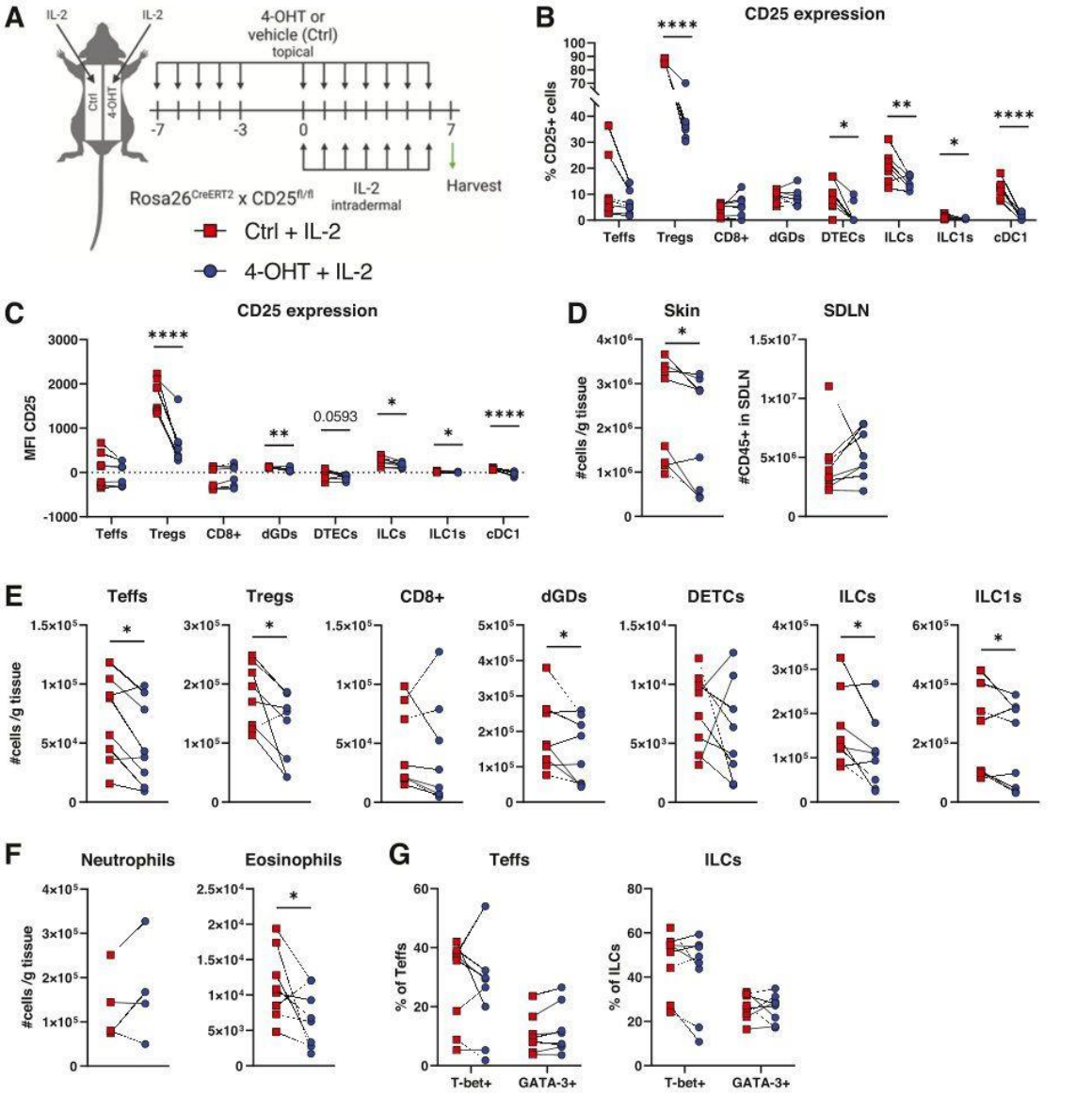

Figure 3. Reduction of CD25 expression on skin cells reduces IL-2-induced increases in skin immune cell numbers. (A) Cre recombinase of Rosa26^{CreERT2}xCD25$^{fl/fl}$ mice was induced by topical 4-hydroxytamoxifen (4-OHT) application prior to IL-2 injections on both sides to decrease CD25 on all skin cells. Acetone on the contralateral side of the back of the same mouse served as vehicle control. Created with BioRender.com. (B) CD25 expression as shown by CD25$^+$ cell frequencies of the respective cell subsets. Teffs: CD4$^+$ T-effector cells; dGDs: dermal γδ T cells; DETCs: dendritic epidermal T cells; ILCs: innate lymphoid cells; ILC1s: CD3-NK1.1$^+$ group 1 ILCs; cDC1: type 1 conventional dendritic cell. (C) MFI of CD25 on respective cells. (D) Immune cell numbers on control and 4-OHT-treated sides in skin and skin-draining lymph nodes (SDLN). (E) Absolute cell numbers of lymphocyte immune cell subsets in skin tissue. (F) Absolute numbers of neutrophils and eosinophils in the skin. (G) Expression of T-bet and GATA-3 transcription factors in Teffs and ILCs. n = 4–8 mice from three independent experiments; $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ as determined by paired t-tests. All other conditions n.s.

CD25 reduction on Tregs increases IL-2-induced accumulation of cells in skin

Tregs are known to exert part of their immunoregulatory role through expression of CD25 [23, 24]. To investigate the impact of CD25 expression on Tregs in controlling IL-2-induced inflammation in the skin, we aimed to specifically delete CD25 on skin Tregs using FoxP3^{CreERT2}xCD25$^{f/fl}$ mice and the topical 4-OHT treatment approach (Fig. 4A). Effects of skin-specific CD25 deletion on FoxP3$^+$ Tregs without IL-2 injections were extensively described elsewhere [22]. Intradermal IL-2 injections and continuous 4-OHT treatment (Fig. 4A) led to significantly reduced frequencies

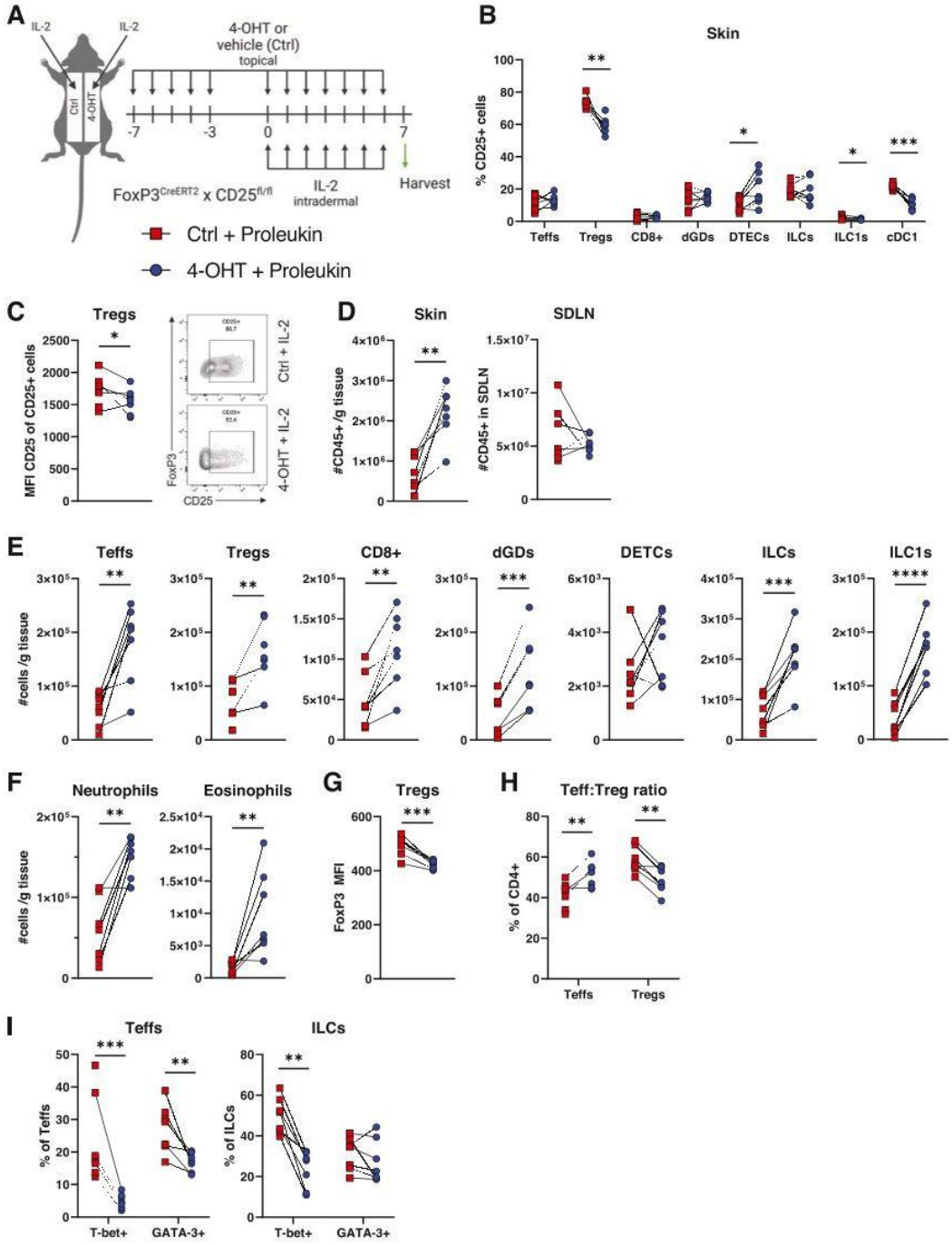

Figure 4. Reduction of CD25 on Tregs leads to significant increases in IL-2-induced skin inflammation. (A) Cre recombinase of FoxP3^{CreERT2}xCD25$^{fl/fl}$ mice was induced by topical 4-hydroxytamoxifen (4-OHT) application to delete CD25 on skin Tregs prior to IL-2 injections on both sides. Acetone on the contralateral side of the back of the same mouse served as vehicle control. Created with BioRender.com. (B) CD25 expression as shown by CD25$^+$ frequencies of the respective cells. Teffs, CD4$^+$ T effector cells; dGDs, dermal γδ T cells; DETCs, dendritic epidermal T cells; ILCs, innate lymphoid cells; ILC1s, CD3-NK1.1$^+$ group 1 ILCs; cDC1, type 1 conventional DC. (C) CD25 MFI of CD25$^+$ Tregs (left) and representative CD25 gating on Tregs (right). (D) Immune cell numbers on control and 4-OHT-treated sides in skin and skin-draining lymph nodes (SDLN). (E) Absolute cell numbers of immune cell subsets in skin tissue. (F) Absolute numbers of neutrophils and eosinophils in skin. (G) MFI of FoxP3 in Tregs in skin. (H) Frequencies of skin Teffs and Tregs of CD4$^+$ cells. (I) Expression of T-bet and GATA-3 transcription factors in Teffs and ILCs. $n = 7$ mice; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as determined by paired t-tests. All other conditions n.s.

of CD25$^+$ Tregs to an average of 58.7% (Fig. 4B). Remaining CD25$^+$ Tregs displayed reduced CD25 expression (Fig. 4C). On the other hand, while CD25 expression was not affected on most cells, expression on ILC1s and type 1 conventional DCs was diminished 1.5-fold and 2.2.-fold, respectively (Fig. 4B). Frequencies of CD25$^+$ cells in SDLN were found to be slightly decreased on Tregs, ILC1s, and type 1 conventional DCs (Supporting information Fig. S6).

Strikingly, numbers of immune cells significantly increased (three-fold) in 4-OHT-treated skin upon IL-2 injections whereas numbers in corresponding lymph nodes were similar for both control and 4-OHT-treated sides (Fig. 4D). Similarly, absolute numbers of all T-cell subsets — except DETCs — ILCs including ILC1s, and granulocytes were highly increased in 4-OHT-treated skin (Fig. 4E, F). In addition, FoxP3 expression in Tregs was slightly reduced in skin treated with 4-OHT (Fig. 4G), and a shift of the Teff:Treg ratio was observed toward Teffs (Fig. 4H). Significant decreases of type 1 and 2 T-helper cell (mean 23.4% vs. 4.9% and 27.4% vs. 17.0%, respectively) as well as ILC1 frequencies (mean 50.1% vs. 23.6%) were observed upon CD25 reduction on Tregs (Fig. 3I). Proportions of ILC2s were unaltered (Fig. 3I). Taken together, these data indicate that CD25 expression on skin Tregs restrains accumulation of cutaneous immune cells resulting in a higher Teff:Treg ratio following intradermal IL-2 injections.

Dermal γδ T cells and ILC2s induce mixed-type 2/type 17 immunity in skin of IL-2-injected mice

Intradermal IL-2 injections induced increases in various immune cell numbers at the injection sides with specific increases in γδ T cells, and ILCs, including ILC1s. To further characterize the quality of the immune response induced by IL-2 treatment, intracellular cytokine expression was investigated in IL-2-injected WT mice using flow cytometry (Fig. 5A). While prevalence of IFN-γ$^+$ immune cells was significantly decreased (9.6% vs. 6.6% of CD45$^+$ cells), frequencies of IL-17$^+$ CD45$^+$ cells were markedly enhanced in response to IL-2 injections (5.2% vs. 9.1%; Fig. 5B). Frequencies of TNF-α-, IL-4-, and IL-13-producing CD45$^+$ immune cells were largely unaltered. Overall, IL-2 injections led to a shift toward type 2/type 17 immune responses compared with control conditions, whereas type 1 immune responses were unaltered or slightly decreased (Fig. 5C).

To determine cells responsible for shifts in immunity, intracellular cytokine production was further analyzed in immune cell subpopulations. While no significant changes in IFN-γ-producing Teffs and CD8$^+$ T cells were observed, prevalence of IFN-γ$^+$ ILC1s was significantly decreased (Fig. 5D) suggesting that ILC1s may become dysfunctional due to overstimulation from daily IL-2 application, similar to tumor-infiltrating NK cells in mice and humans [25, 26]. Significant increases in type 2 cytokine-producing ILCs, namely group 2 ILCs, were found upon IL-2 treatment (1.0% vs. 3.0% for IL-4-producing cells; 4.7% vs. 11.3% for IL-13-producing cells). The most striking changes in cytokine production were observed for dermal γδ T cells which showed significant and prominent increases in frequencies of IL-17-producing cells from an average of 29.2% to 56.1% (Fig. 5D). In conclusion, we observed a mixed type 2/type 17 immunity in response to IL-2 injections with increases in frequencies of IL-4- and IL-13-producing ILC2s and IL-17-producing γδ T cells.

Discussion

High-dose IL-2 therapy has been a promising treatment option for malignant cancers but was limited due to systemic toxicity. Still, pathophysiological mechanisms of IL-2-induced side effects such as skin rashes are obscure. Here, we provide new molecular and cellular insights into IL-2-induced skin inflammation and the relevance of CD25 using different mouse models.

Intradermal IL-2 injections in WT mice led to striking increases in several lymphoid immune cell numbers around the injection site. This might be due to infiltration of cells toward IL-2 or other inflammatory stimuli such as described for T cells [27, 28] or in inflamed tissue for ILC2s and γδ T cells [29, 30]. Besides recruitment of cells, IL-2 can directly induce proliferation of cutaneous T cells and ILC2s in mice and humans [13, 31, 32]. Therefore, increasing Ki-67 expression in cells of IL-2-treated skin could hint toward local proliferation of tissue-resident as well as migrated cells. We further observed infiltrates of neutrophils and eosinophils in response to IL-2 injections. Similar observations of lymphoid and granulocytic infiltrates were made after subcutaneously injecting IL-2 into rats [33] and in early clinical reports of high-dose IL-2-treated patients suffering from skin rash [16, 17, 34, 35]. In our current study, we observed increases in the representation of ILCs including ILC1s and dermal γδ T cells, accompanied by significant changes in cytokine production in all three cell populations which hints toward a more specific effect of IL-2 on these innate cell subsets.

Frequencies of IL-4- and IL-13-producing ILCs were increased upon IL-2 injections — signature cytokines for group 2 ILCs [12]. Both human as well as murine ILC2s are well described to be activated in response to IL-2, leading to secretion of type 2 cytokines [13, 18, 32]. In this context, ILC2s seem to play a central role in IL-2-induced eosinophilia [18] and skin rashes in mice treated with the antibody JES6-1 and IL-2 [13]. Our data further support the central role of ILC2s in IL-2-induced side effects as ILC2s seem to be responsible for the shift toward type 2 immunity observed in response to IL-2 injections. Furthermore, we found that IL-2 injections highly increased prevalence of IL-17$^+$ dermal γδ T cells. In mice, IL-17 production of γδ T cells is induced by IL-2 alone without further stimuli [36], hinting that IL-2 injections might directly induce IL-17 production in our study. Furthermore, Shibata et al. [37] reported that CD25$^+$ γδ T cells produce IL-17 and that CD25 expression was dependent on IL-2. Therefore, upregulation of CD25 on γδ T cells might be crucial to induce IL-17 production observed in our study, suggesting a central role of CD25 expression in inducing the shift toward type 17 immune reac-

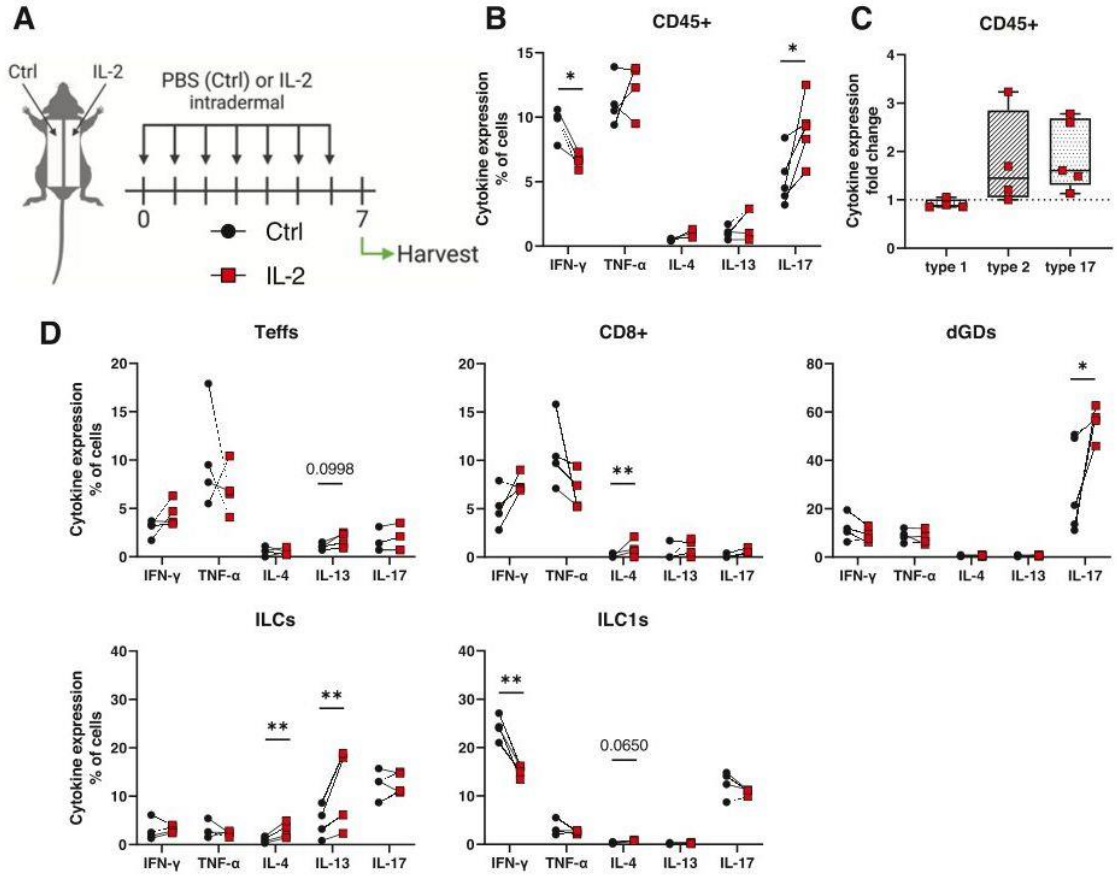

Figure 5. IL-2 injections induce a mixed type 2/type 17 immune reaction in skin, associated with dermal γδ T cells and ILCs. (A) Experimental set-up for injection of WT mice with IL-2. Created with BioRender.com. (B) Expression of cytokines in CD45$^+$ cells upon IL-2 injection. (C) Relative cytokine expression in immune cells in IL-2-treated vs. Ctrl skin. Frequencies of cytokine expression in CD45$^+$ cells (type 1: IFN-γ + TNF-α; type 2: IL-4 + IL-13; type 17: IL-17) upon IL-2 injection was normalized to control conditions to yield fold changes. (D) Cytokine expression in different lymphocyte populations. Teffs: CD4$^+$ T effector cells; dGDs: dermal γδ T cells; ILCs: innate lymphoid cells; ILC1s: CD3-NK1.1$^+$ group 1 ILCs. n = 4–5 mice representative for two independent experiments; *$p < 0.05$, **$p < 0.01$ as determined by paired t-tests. All other conditions n.s.

tions in response to IL-2 injections. Besides direct IL-2-induced effects, a variety of cytokines are secreted during IL-2 therapy in mice and humans [18, 35, 38], which might further impact cutaneous cells indirectly. For instance, cell plasticity in the skin of IL-2-treated mice could be affected by the complex inflammatory milieu induced by IL-2 injections. Changes in plasticity have been reported for ILCs during the onset of psoriasis [39] or γδ T cells stimulated with IL-23, IL-1β, and IL-2 [40]. Similarly, possible secretion of cytokines such as IL-33 in response to tissue damage could further support IL-2-induced type 2 responses by directly activating type 2 T helper cells or ILC2s, leading to synergistic effects of IL-33 and IL-2 on type 2 cytokine secretion [41–43]. The overall shift toward a mixed type 2/type 17 immunity along with mixed neutrophilic and eosinophilic infiltrates into IL-2-treated skin might resemble pathologies such as atopic dermatitis or psoriasis [44, 45].

To further investigate the relevance of CD25 expression during IL-2-induced skin inflammation, we aimed to specifically delete CD25 on all skin cells. Although we achieved moderate partial rather than full deletion, we found that immune cell numbers were reduced upon IL-2 injections. In previous studies, macroscopic skin rashes in mice were observed when combining IL-2 treatment with the CD25-specific antibody JES6-1 [13]. Furthermore, modifying IL-2 therapy to reduce or prevent binding to CD25$^+$ cells reduces the occurrence of IL-2-induced side effects such as systemic inflammation or eosinophilia in preclinical models [46, 47]. Together with our data, these observations highlight the role of the IL-2Rα subunit in inducing side effects during IL-2 therapy.

In contrast to CD25 reduction on all skin cells, skin-specific reduction of CD25 on FoxP3$^+$ Tregs drastically increased inflammatory infiltrates upon IL-2 injections. These observations were accompanied by an imbalance in the Teff:Treg ratio in favor of CD4$^+$ Teffs, highlighting the role of CD25 on Tregs to maintain immune tolerance [23, 24]. Furthermore, FoxP3 expression was slightly decreased upon reduction of CD25 on Tregs and IL-2 treat-

ment, hinting toward a reduction of regulatory function [48, 49]. In a previous study, we found that skin-specific CD25 deletion in FoxP3$^+$ Tregs did not affect FoxP3 expression in skin Tregs under steady-state conditions [22]. Therefore, decreased FoxP3 expression in the current study seems to be due to the additional IL-2 stimulation and could be an indirect effect due to IL-2-induced inflammation in the skin of mice [48, 49]. Furthermore, decreases in type 1 and 2 T-helper cells as well as ILC1s hint toward a higher prevalence of RORγt-expressing cells, suggesting a shift to type 17 immunity upon CD25 reduction on Tregs. Overall, our data highlight the crucial role of CD25 in Tregs on restraining IL-2-induced inflammation in the skin.

Together, we show here that IL-2 injections induce cutaneous immune reactions characterized by a shift toward type 2/type 17 immunity which largely seems to be induced by ILC2s and dermal γδ T cells. In addition, while CD25-expressing Tregs suppress these inflammatory reactions, reduction of CD25 on all skin cells reduced IL-2-induced inflammation, showing that the receptor is responsible for mediating at least part of these inflammatory reactions. Our study gives new molecular and cellular insight into IL-2-dependent skin reactions, identifying a central role of innate immune cells in inducing the side effects. The results presented here might therefore help to increase the safety of current or future IL-2-based therapeutics.

Data limitations and perspectives

In this study, we demonstrate that IL-2 induces skin inflammation as evidenced by increases in immune cell numbers and a shift toward type 2/type 17 immunity. To differentiate effects induced by IL-2 directly from indirect effects induced by produced cytokines, mediators such as IL-13, IL-17, or IL-33 might be reduced by blocking antibodies or in knockout mouse models. Similarly, deleting ILCs or γδ T cells — cells that seem to be responsible for the shift in immunity — could help to further understand the role of these unconventional immune cells in inducing side effects during IL-2 therapy.

Methods

Mice

All animals used for experiments were held in specific pathogen-free cages in the animal facility of the University of California, San Francisco. Mice of both genders were included, and littermate controls were used. Genetic strains of mice included WT C57BL/6 (B6), Rosa26-^{CreERT2}, FoxP3-^{CreERT2}, and CD25$^{fl/fl}$ (Il2ra$_{tm1c(EUCOMM)Wtsi}$) (all on B6 background). Animal experiments were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee and Laboratory Animal Resource Center of the University of California, San Francisco.

Administration of 4-OHT and IL-2

Dorsal back hair of mice was shaved along both flanks leaving a stripe of hair down the middle. To induce Cre-mediated recombination, 100 μL of 4-OHT (50 μg/mL, Sigma-Aldrich; diluted in acetone, Sigma-Aldrich) was applied dropwise to the surface of one side of shaved back for 5 consecutive days starting at day 7 before IL-2 injections [22]. As a control, 100 μL acetone was applied to contralateral shaved back skin. For IL-2 injections, human recombinant IL-2 (aldesleukin) was diluted in PBS and 30 μg/kg/injection in 100 μL was intradermally injected (approx. 10,000 IU/injection), either to both sides of shaved back skin or unilaterally in experiments using WT B6 mice. In experiments using B6 mice, 100 μL PBS was injected intradermally into contralateral back skin as a control. IL-2 was intradermally injected for 7 consecutive days and 4-OHT or acetone control was topically applied during IL-2 injections daily for 7 days.

Tissue processing

After euthanasia of mice, skin and SDLN (axillary, brachial, and inguinal) were processed as described previously [22] before staining for flow cytometry.

Flow cytometry

Single-cell suspensions were initially Fc-blocked using anti-CD16/anti-CD32 (Bio X Cell) and surface-stained in addition to the fixable viability Ghost Dye Violet 510 (Tonbo Biosciences) for 30 min at 4°C in PBS containing 2% FBS. Intracellular staining was performed in perm/wash buffer at 4°C overnight after permeabilization of cells using the Cytofix/Cytoperm kit (BD Biosciences). For intracellular cytokine staining, isolated cells were incubated in media containing 1× cell stimulation cocktail (Tonbo) at 37°C, 5% CO_2, and transferred to 4°C after 6 h. Surface and intracellular staining was performed as described on the next day. To determine absolute cell numbers, CountBright Absolute Counting Beads (Invitrogen) were added before measurement. Antibodies used for flow cytometry are summarized in Supporting information Table S1.

Measurement of stained cells was performed on a BD Fortessa flow cytometer using FACS Diva software (BD Biosciences). FlowJo version 10.8.1 was used to analyze cell populations as depicted in Supporting information Fig. S1. As no markers to distinguish NK cells from ILC1s were included, CD3-NK1.1$^+$ cells are defined as “NK-like” ILC1s and further referred to as ILC1s.

Histology

Skin strips (approx. 4–5 mm width, 10–20 mm length) were fixed in 10% neutral-buffered formalin and paraffin-embedded for histological analysis. Hematoxylin and eosin (H&E) staining of tissue

was performed by the University of California, San Francisco Dermatopathology Service and visualized with an Aperio AT2 scanner (Leica Biosystems) at 40× resolution using a 20×0.75NA Plan Apo objective with a 2× optical magnification changer. Image resolution was 40×: 0.25 μm/pixel. Aperio ImageScope software (version 12.4.0.5043, Leica Biosystems) was used to digitally view slides. The pathological evaluation was performed by a board-certified veterinary pathologist.

Statistics

Statistical analysis was performed using GraphPad Prism (version 9) and paired *t*-tests were applied to determine *p*-values as indicated in the figure legends. In case of Rosa26^{CreERT2}xCD25$^{fl/fl}$ mice, animals with low reduction of CD25 (<30% reduction on CD45^{+} cells after 4-OHT application and IL-2 injection as compared with control side) were excluded from the analysis. For all other experiments, no animals were excluded.

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Conflict of interest: The authors declare no commercial or financial conflict of interest.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval statement: Animal experiments were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee and Laboratory Animal Resource Center of the University of California, San Francisco.

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Abbreviations: **4-OHT**: 4-hydroxytamoxifen · **B6**: C57BL/6 mice · **DETCs**: dendritic epidermal T cells · **IL-2R**: IL-2 receptor · **ILC1**: group 1 innate lymphoid cell · **ILC2**: group 2 innate lymphoid cell · **NK**: natural killer · **SDLN**: skin-draining lymph nodes · **Teffs**: T-effector cells · **Tregs**: regulatory T cells

Full correspondence: Dr. Katherina Sewald, 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, 30625 Hannover, Germany
e-mail: Katherina.Sewald@item.fraunhofer.de

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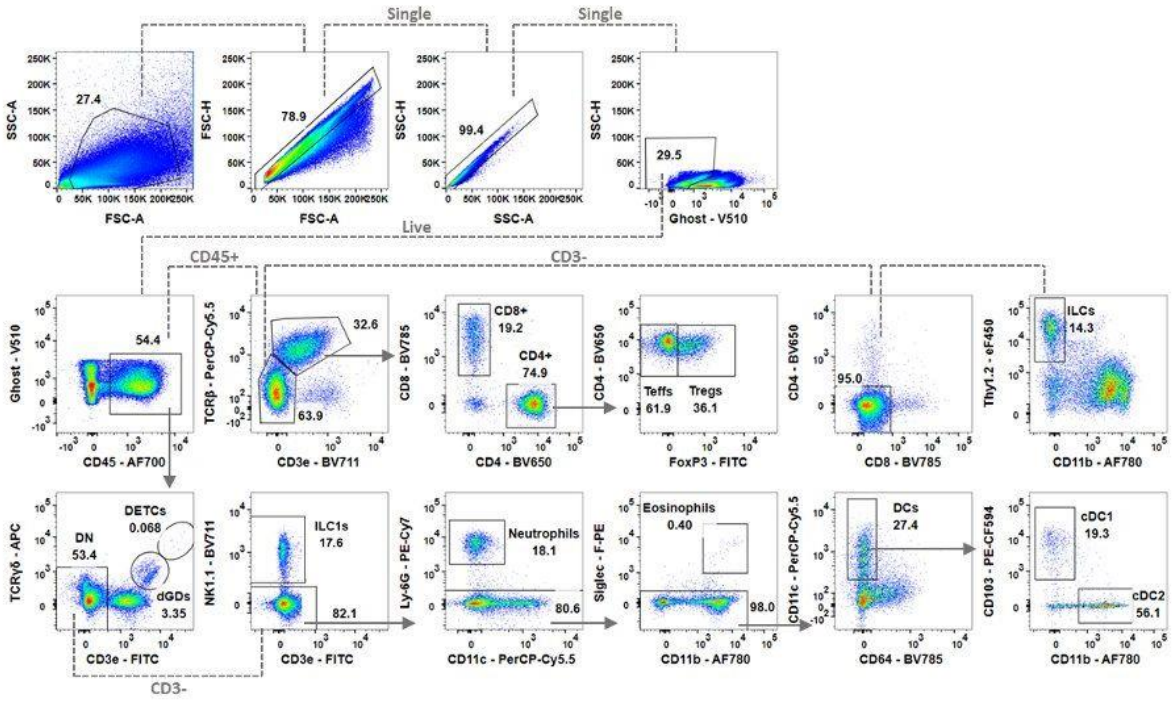
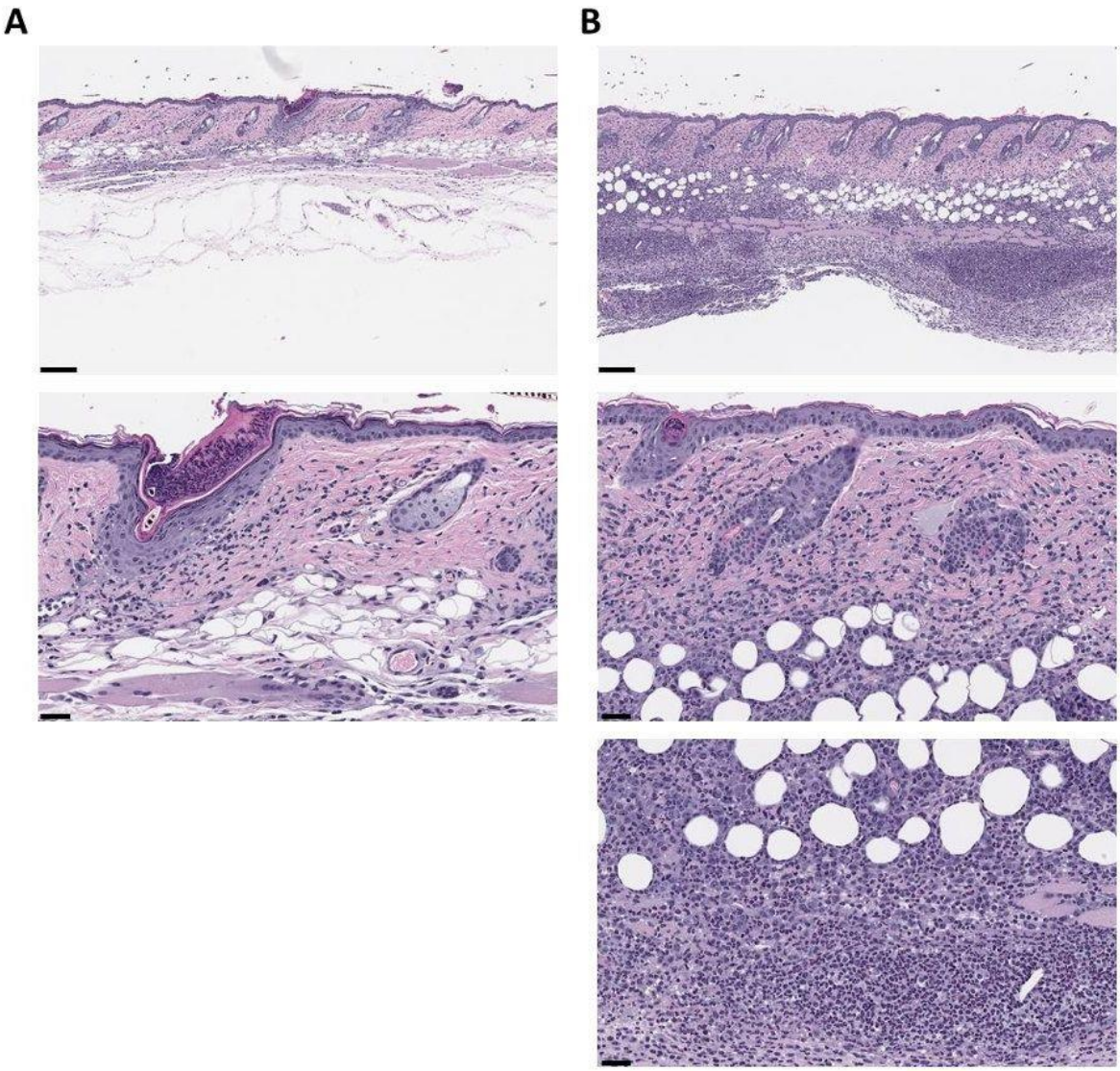
Interleukin-2-induced skin inflammation

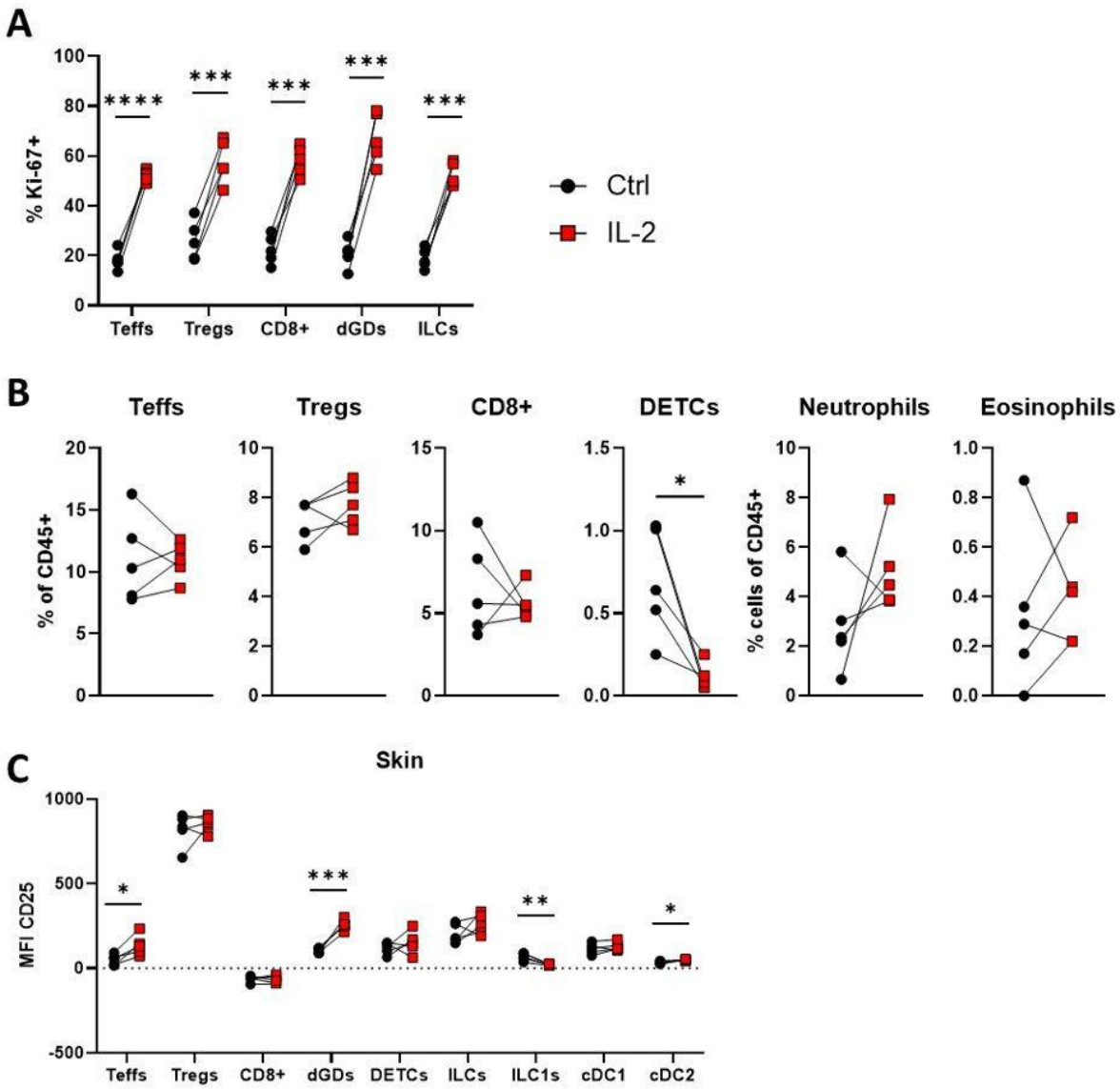
- Supplementary Material -



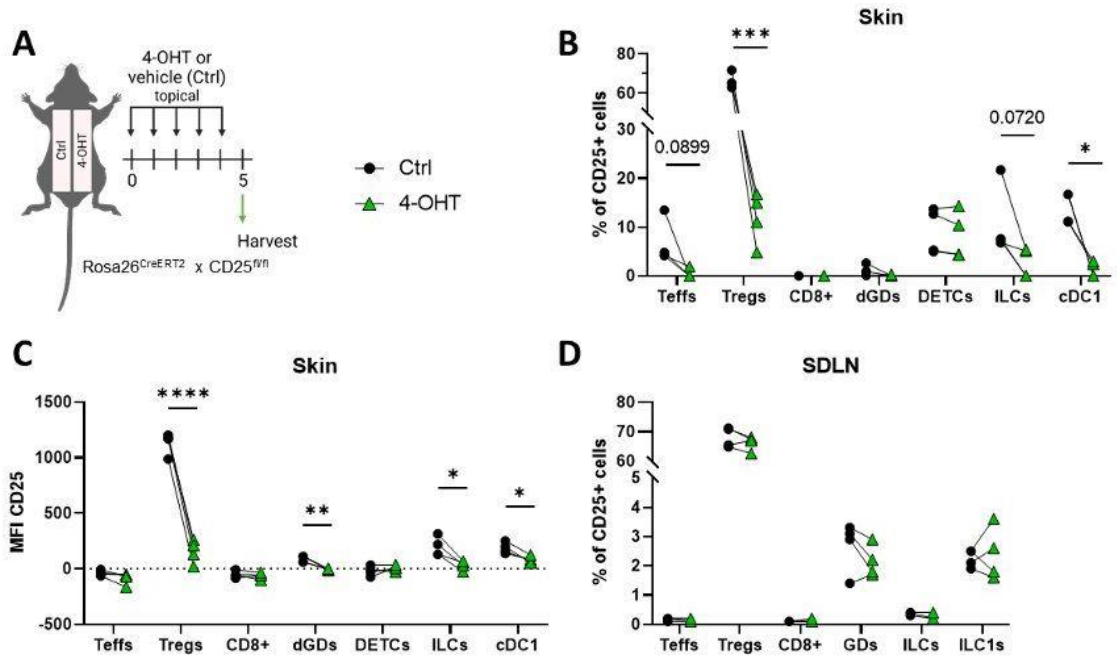
Supplementary Figure S1: Representative gating strategy used to identify immune cell subsets in skin and skin-draining lymph nodes. Teffs: CD4+ FoxP3- T effector cells; Tregs: CD4+ FoxP3+ regulatory T cells, ILCs: innate lymphoid cells; dGDs: dermal γδ T cells; DETCs: dendritic epidermal T cells; ILC1s: CD3-NK1.1+ group 1 ILCs; cDC: conventional dendritic cell.



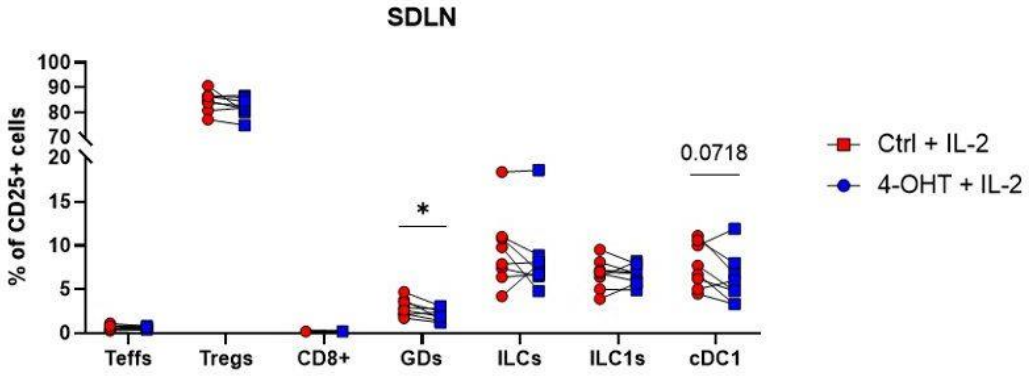
Supplementary Figure S2: Histological images of skin injected twice daily with IL-2. Wild type mice were intradermally injected with IL-2 (aldesleukin) twice daily for 7 consecutive days before analysis of skin. Representative histological images of injections sides for control (**A**) and IL-2 (**B**). Overview pictures (upper images; scale bars 200 µm) and close ups (middle and lower images; scale bares 40 µm) are shown.



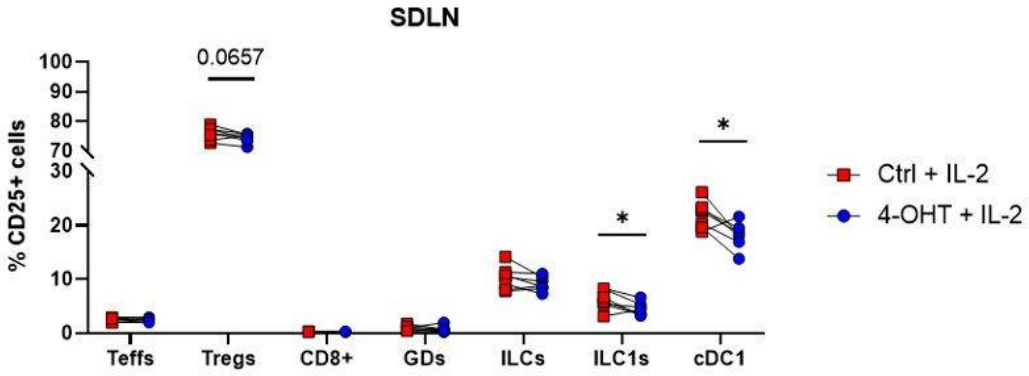
Supplementary Figure S3: Intradermal IL-2 injections induce proliferation of lymphocytes. Wild type mice were intradermally injected with IL-2 (aldesleukin) or PBS (Ctrl) daily for 7 consecutive days before analysis of skin. **A** Proliferation of CD4+ Teffs and Tregs, CD8+, dermal γδ T cells (dGDs), and innate lymphoid cells (ILCs) as shown by Ki-67+ cells. **B** Frequencies of T cell and granulocyte subsets in skin of control and IL-2-treated sides. DETCs: dendritic epidermal T cells. **C** Mean fluorescence intensity (MFI) of CD25 on immune cell subsets in skin of control and IL-2-treated mice. ILC1s: CD3-NK1.1+ group 1 ILCs; cDC: conventional dendritic cell. n=5 mice; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ as determined by paired t-tests. All other conditions n.s.



Supplementary Figure S4: Skin-specific deletion of CD25 on all cells. **A** Experimental set-up to delete CD25 on all skin cells: Cre recombinase of Rosa26^{CreERT2} x CD25$^{fl/fl}$ mice was induced by 4-Hydroxytamoxifen (4-OHT), applied topically daily for 5 consecutive days. Acetone served as vehicle control. Created with Biorender.com. **B** CD25 expression in the skin as shown by CD25+ cell frequencies of the respective cell subsets. Teffs: CD4+ T effector cells; dGDs: dermal γδ T cells; DETCs: dendritic epidermal T cells; ILCs: innate lymphoid cells; cDC1: type 1 conventional dendritic cell. **C** Mean fluorescence intensity (MFI) of CD25 on respective cells in the skin of control and 4-OHT-treated side. cDC1: type 1 conventional dendritic cell. **D** CD25 expression in skin-draining lymph nodes (SDLN) as shown by CD25+ cell frequencies of the respective cell subsets. ILC1s: CD3-NK1.1+ group 1 ILCs. Due to very low frequencies, ILC1s were excluded from CD25 analysis of the skin and cDC1 were excluded for SDLN. n=4 mice; * p<0.05, ** p<0.01, *** p<0.001, *** p<0.0001 as determined by paired t-tests. All other conditions n.s.



Supplementary Figure S5: CD25 expression is similar in lymph nodes of control and 4-OHT-treated sides after IL-2 injections. CD25 deletion of Rosa26^{CreERT2} x CD25$^{fl/fl}$ mice was induced by topical 4-Hydroxytamoxifen (4-OHT) application prior to IL-2 injections on both sides. Acetone served as vehicle control. CD25 expression in skin-draining lymph nodes (SDLN) as shown by CD25+ cell frequencies of the respective cells subsets is shown. Teffs: CD4+ T effector cells; GDs: γδ T cells; ILCs: innate lymphoid cells; ILC1s: CD3-NK1.1+ group 1 ILCs; cDC1: type 1 conventional DC. n=8 mice; * $p<0.05$ as determined by paired t-tests. All other conditions n.s.



Supplementary Figure S6: CD25 expression decreases on some immune cells in lymph nodes of control and 4-OHT-treated sides after IL-2 injections. CD25 deletion of FoxP3^{CreERT2} x CD25$^{fl/fl}$ mice was induced by topical 4-Hydroxytamoxifen (4-OHT) application prior to IL-2 injections on both sides. Acetone served as vehicle control. CD25 expression in skin-draining lymph nodes (SDLN) as shown by CD25+ cell frequencies of the respective cell subsets is shown. Teffs: CD4+ T effector cells; GDs: γδ T cells; ILCs: innate lymphoid cells; ILC1s: CD3-NK1.1+ group 1 ILCs; cDC1: type 1 conventional DC. n=7 mice; * p<0.05 as determined by paired t-tests. All other conditions n.s.

Supplementary Table S1: List of anti-mouse antibodies used for flow cytometry.

Antibody	Clone	Company
CD45-AF700	30-F11	eBioscience
CD45-BV605	30-F11	BD
CD3e-BV711	145-2C11	BD
CD3e-FITC	500A29	BD
TCRβ-PerCP-Cy5.5	H57-597	Tonbo
TCRβ-APC	H57-597	eBioscience
CD8-BV785	53-6.7	Biolegend
CD4-BV650	RM4-5	BD
CD4-BC605	RM4-5	Biolegend
TCRγδ-APC	eBioGL3	eBioscience
NK1.1-APC-eFluor780	PK126	eBioscience
NK1.1-BV711	PK126	Biolegend
Thy1.2-eFluor450	53-2.1	Invitrogen
Ly-6G-PE-Cy7	1A8	Invitrogen
Siglec-F-PE	E80-2440	BD
CD11b-AF780	M1/70	eBioscience
CD11c-PerCP-Cy5.5	N418	Biolegend
CD103-PE-CF594	M290	BD
CD64-BV785	X54-5/7.1	BD
CD25-BV650	PC61	Biolegend
CD25-APC	PC61	eBioscience
TNFα-PerCP-eFluor710	MP6-X122	eBioscience
IFNy-AF488	XMG1.2	eBioscience
IL-17A-PE-Cy7	TC11-18H10.1	Biolegend
IL-4-PE-CF594	11B11	BD
IL-13-PE	eBio13A	eBioscience
FoxP3-AF700	FJK-16s	eBioscience
FoxP3-FITC	FJK-16s	eBioscience
Ki-67-BV650	B56	BD
T-bet-PE-Cy7	4B10	Invitrogen
GATA-3-PE	TWAJ	eBioscience

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

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Charline Sommer[1], Sophie Jacob[1], Tonia Bargmann[1], Muhammad Shoaib[2], Basel Alshaikhdeeb[2], Venkata P. Satagopam[2], Susann Dehmel[1], Vanessa Neuhaus[1], Armin Braun[1,3#], Katherina Sewald[1#]

[1] 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)

[2] Luxembourg Centre for Systems Biomedicine, University of Luxembourg (Luxembourg)

[3] Institute of Immunology, Hannover Medical School, Hannover (Germany)

[#] shared last authorship

Corresponding author:

Katherina Sewald, Fraunhofer ITEM, Preclinical Pharmacology and In-Vitro Toxicology, Nikolai-Fuchs-Str. 1, 30625 Hannover, Germany, Katherina.Sewald@item.fraunhofer.de

Author contribution statement:

Charline Sommer substantially contributed to conception and design of the study and to the manuscript with selection of endpoints and controls; was mainly involved in isolation and culture of peripheral blood mononuclear cells (PBMCs) and performed flow cytometric measurements; analyzed all flow cytometric data and performed the statistical analysis for all data; interpreted all data and drafted and wrote the manuscript and implemented all required revisions.

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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).

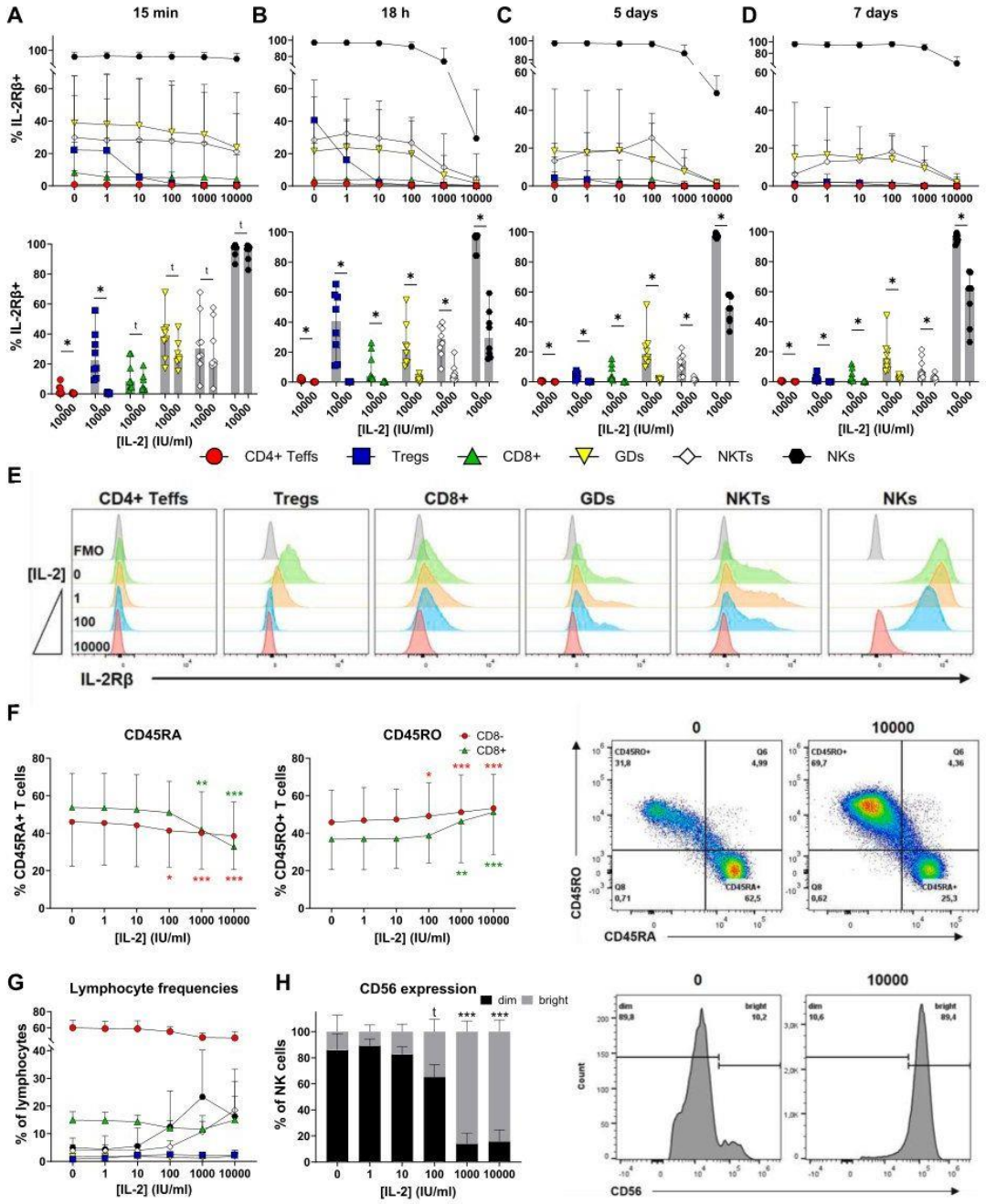

Fig. 1. Continuous hdIL-2 stimulation reduces IL-2Rβ surface expression on lymphocytes. Human PBMCs were stimulated with increasing IL-2 doses for up to 7 days prior to analysis of CD4+ Teffs, CD4+ Tregs, CD8+ T cells, γδ T cells (GDs), NKT cells, and NK cells. Mean frequencies of IL-2Rβ + cells after 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.

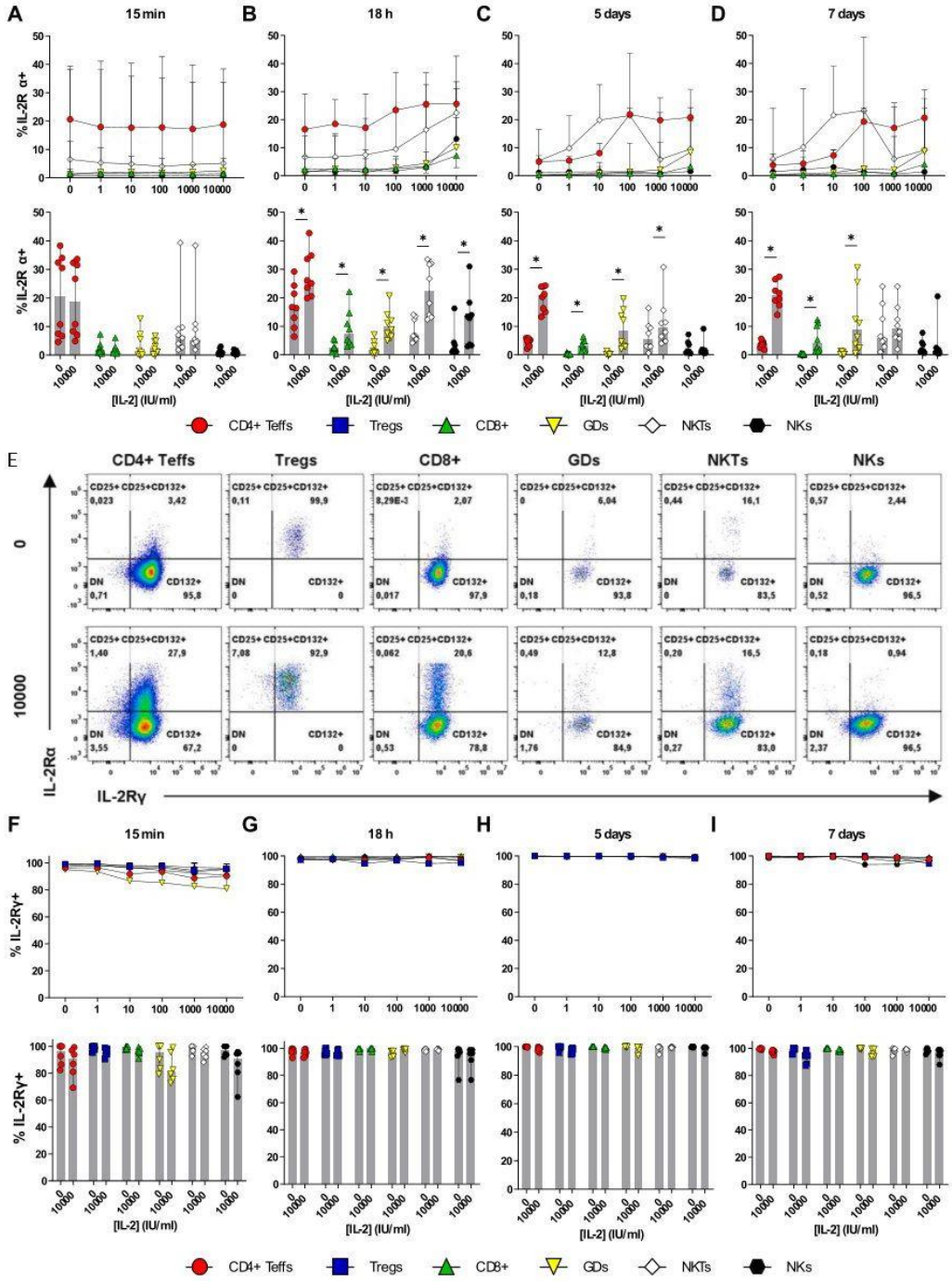

Fig. 2. Differential regulation of IL-2Rα and IL-2Rγ surface expression upon continuous IL-2 stimulation. Human PBMCs were stimulated with increasing IL-2 doses for up to 7 days prior to analysis. A-D Mean frequencies of CD4+ Teffs, CD8+ T cells, γδ T cells (GDs), NKT cells, and NK cells positive for 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).

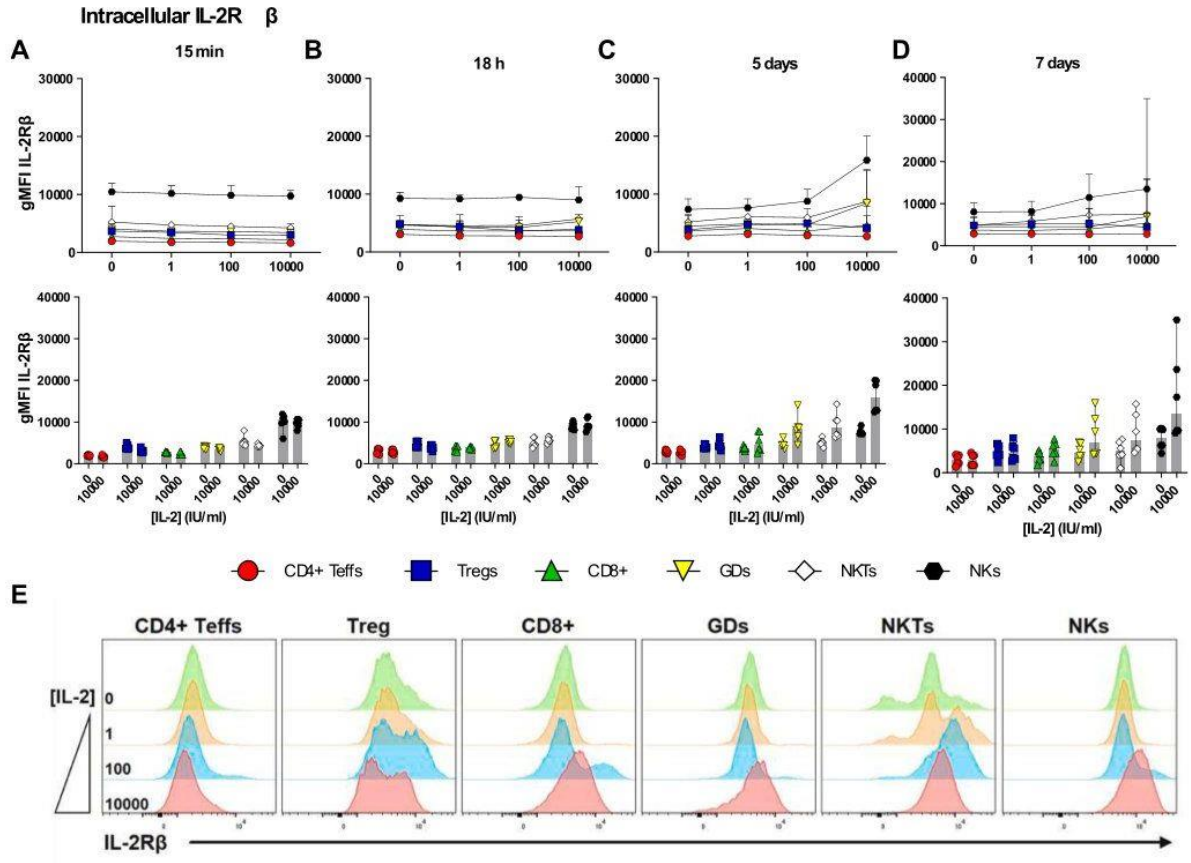

Fig. 3. Intracellular IL-2Rβ abundance is cell-specifically regulated upon IL-2 stimulation. Human PBMCs were stimulated with increasing IL-2 doses for up to 7 days prior to analysis. A-E Intracellular IL-2Rβ abundance was analyzed in CD4+ Teffs, CD4+ Tregs, CD8+ T cells, γδ T cells (GDs), NKT cells, 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

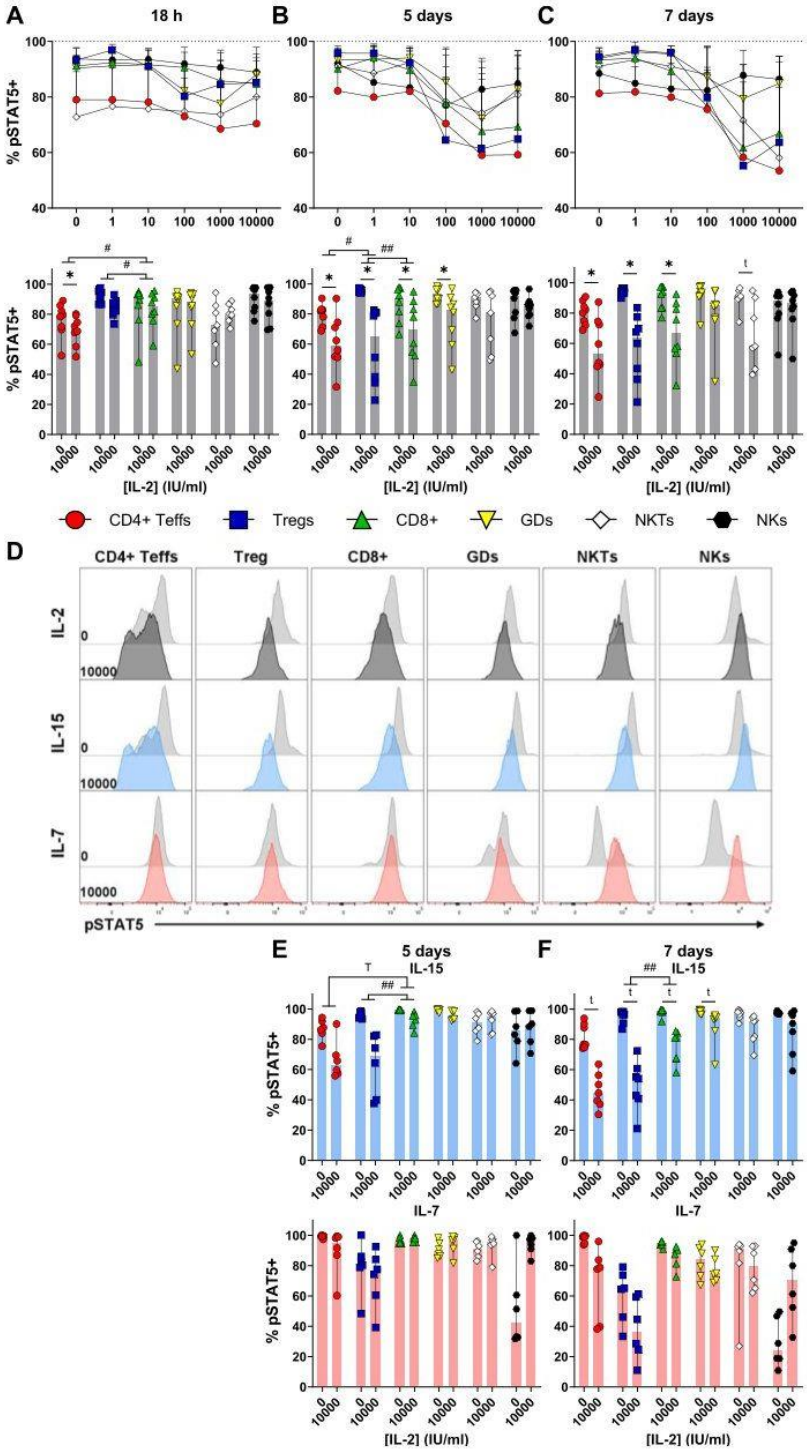

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Fig. 4. IL-2R signaling capacity is reduced in cells with previous high-dose IL-2 stimulation. Human PBMCs were stimulated with increasing IL-2 doses. After the indicated time points, cells were re-stimulated with high-dose IL-2 (10,000 IU/mL), IL-15, 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:**

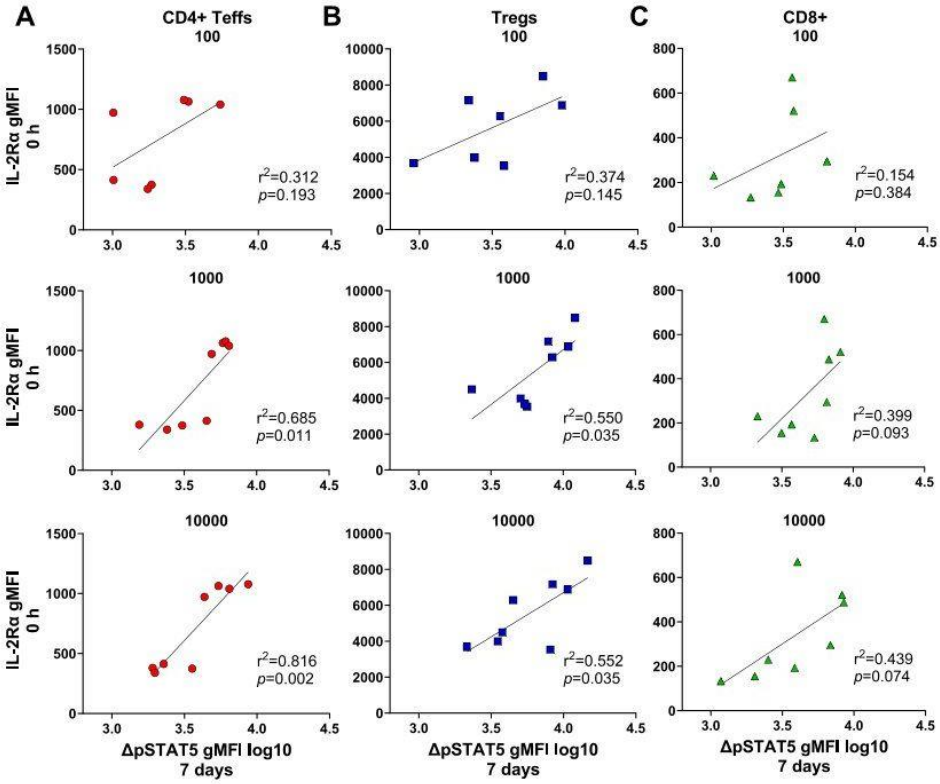

Fig. 5. IL-2Rα expression correlates with decreases in IL-2 signaling capacity of high-dose IL-2-stimulated T cells. Donor-dependent correlation of baseline IL-2Rα gMFI on CD4+ Teffs (A), CD4+ Tregs (B), and CD8+ T cells (C) without IL-2 stimulation with differences in pSTAT5 signal on day 7. For pSTAT5 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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2024.110288.

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Supplements

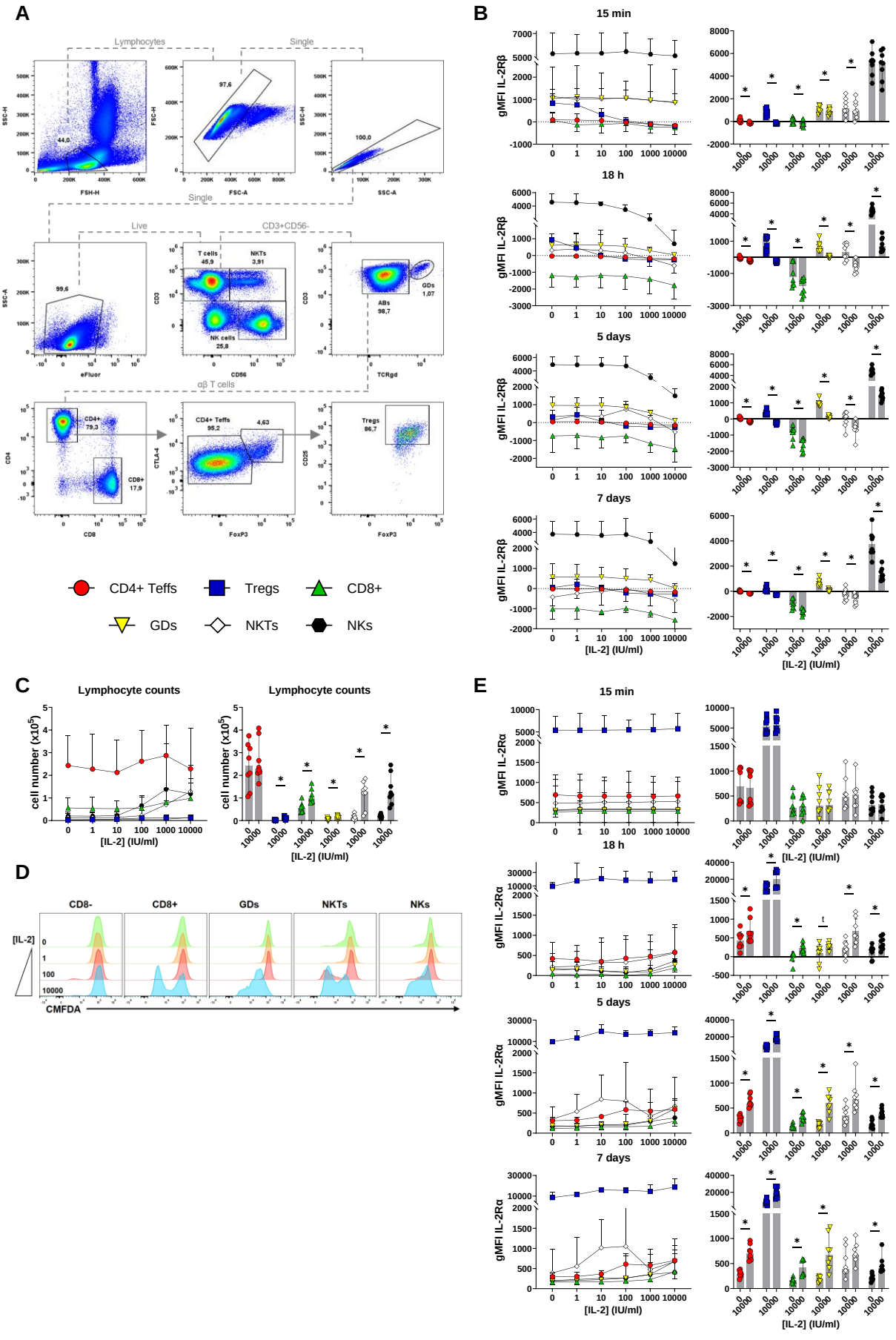

Figure S1: High-dose IL-2 stimulation increases numbers of lymphocyte subsets. Human PBMCs were stimulated with increasing IL-2 doses for 7 days prior to analysis of CD4+ Teffs, CD4+ Tregs, CD8+ T cells, γδ T cells (GDs), NKT cells, and NK cells. **A** Representative gating strategy to characterize lymphocyte subsets (ABs: αβ T cells). **B** gMFI of extracellular IL-2Rβ of cells after 15 min, 18 h, 5 days, or 7 days of IL-2 stimulation in different concentrations (left panels). Bar graphs (right panels) show IL-2Rβ gMFIs on lymphocytes as shown on the left without IL-2 (0 IU/mL) or upon high-dose IL-2 stimulation (10,000 IU/mL). **C** Cell counts after IL-2 stimulation for 7 days. Dose-dependent changes in cell numbers (left) and cell numbers without (0 IU/mL) and with high-dose IL-2 (10,000 IU/mL) comparatively are shown. **D** Representative histograms of CMFDA dilution after 7 days of IL-2 stimulation (CD8–: CD8– αβ T cells). **E** gMFI of extracellular IL-2Rα of cells after 15 min, 18 h, 5 days, or 7 days of IL-2 stimulation in different concentrations (left panels). Bar graphs (right panels) show IL-2Rα gMFIs on lymphocytes as shown on the left without IL-2 (0 IU/mL) or upon high-dose IL-2 stimulation (10,000 IU/mL). n=8 donors, two independent experiments (n=4 donors each), median ± range. * $p<0.05$, other conditions non-significant, analyzed by multiple Wilcoxon tests with Holm-Šídák method.

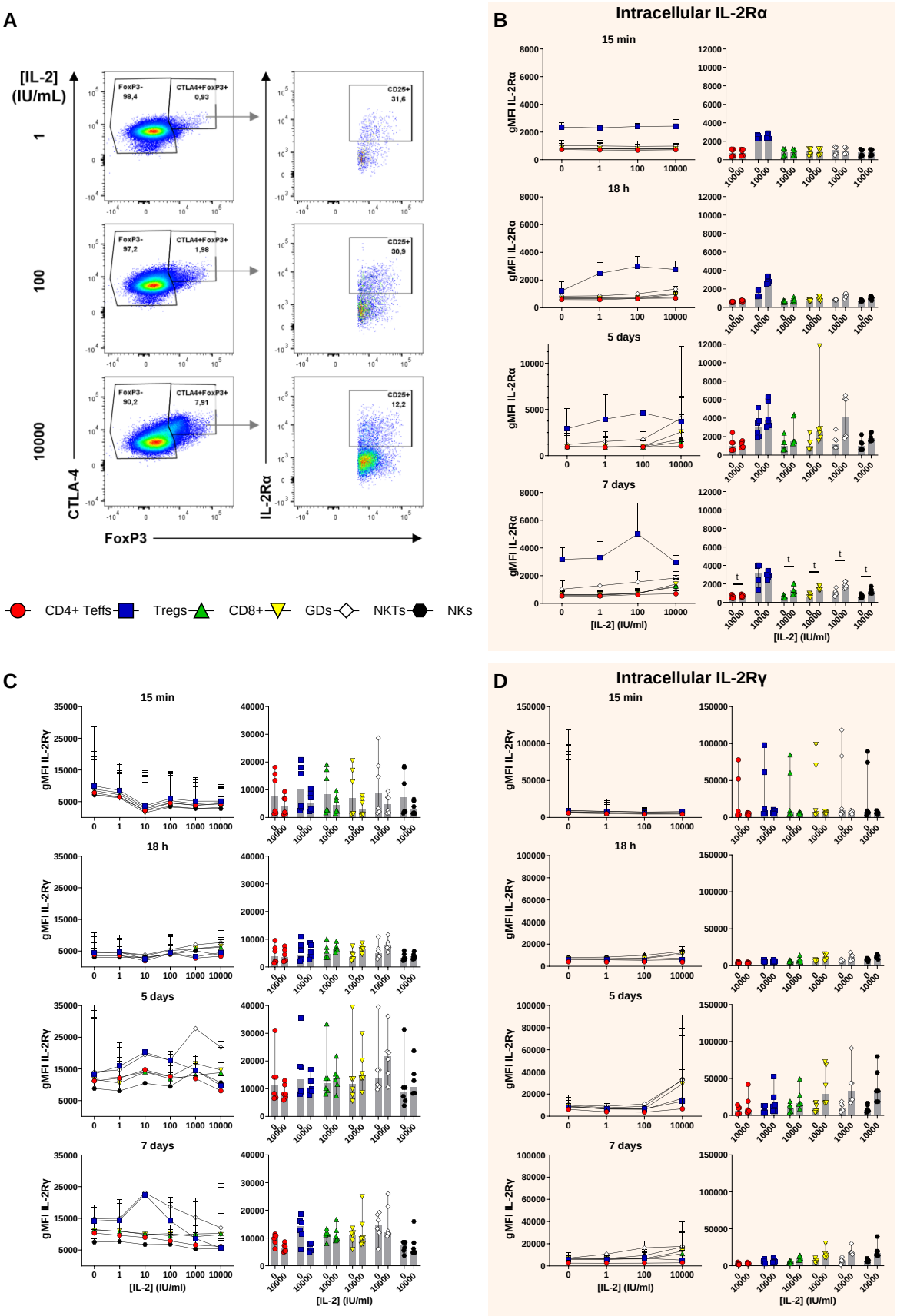

Figure S2: Intracellular IL-2Rα and IL-2Rγ abundance is cell-specifically increased on IL-2-stimulated cells. A Expression of CTLA-4 and FoxP3 in high-dose IL-2-stimulated CD4+ Teffs. CD4+ Teffs were isolated from PBMCs and cultured for 7 days with increasing IL-2 concentrations prior to

analysis. Gating representative for 3 donors. **B-D** 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. **B** gMFI of intracellular IL-2Rα of cells after 15 min, 18 h, 5 days, or 7 days of IL-2 stimulation in different concentrations (left panels). Bar graphs (right panels) show IL-2Rα gMFI on lymphocytes as shown on the left without IL-2 (0 IU/mL) or upon high-dose IL-2 stimulation (10,000 IU/mL). **C+D** gMFI of extracellular (**C**) or intracellular (**D**) IL-2Rγ of cells after 15 min, 18 h, 5 days, or 7 days of IL-2 stimulation in different concentrations (left panels). Bar graphs (right panels) show IL-2Rγ gMFI on lymphocytes as shown on the left without IL-2 (0 IU/mL) or upon high-dose IL-2 stimulation (10,000 IU/mL). n=3-6 donors, two independent experiments (n=1-3 donors each), median ± range. No significant differences between 0 and 10,000 IU/mL (t $p<0.1$), analyzed by multiple Wilcoxon tests with Holm-Šídák method.

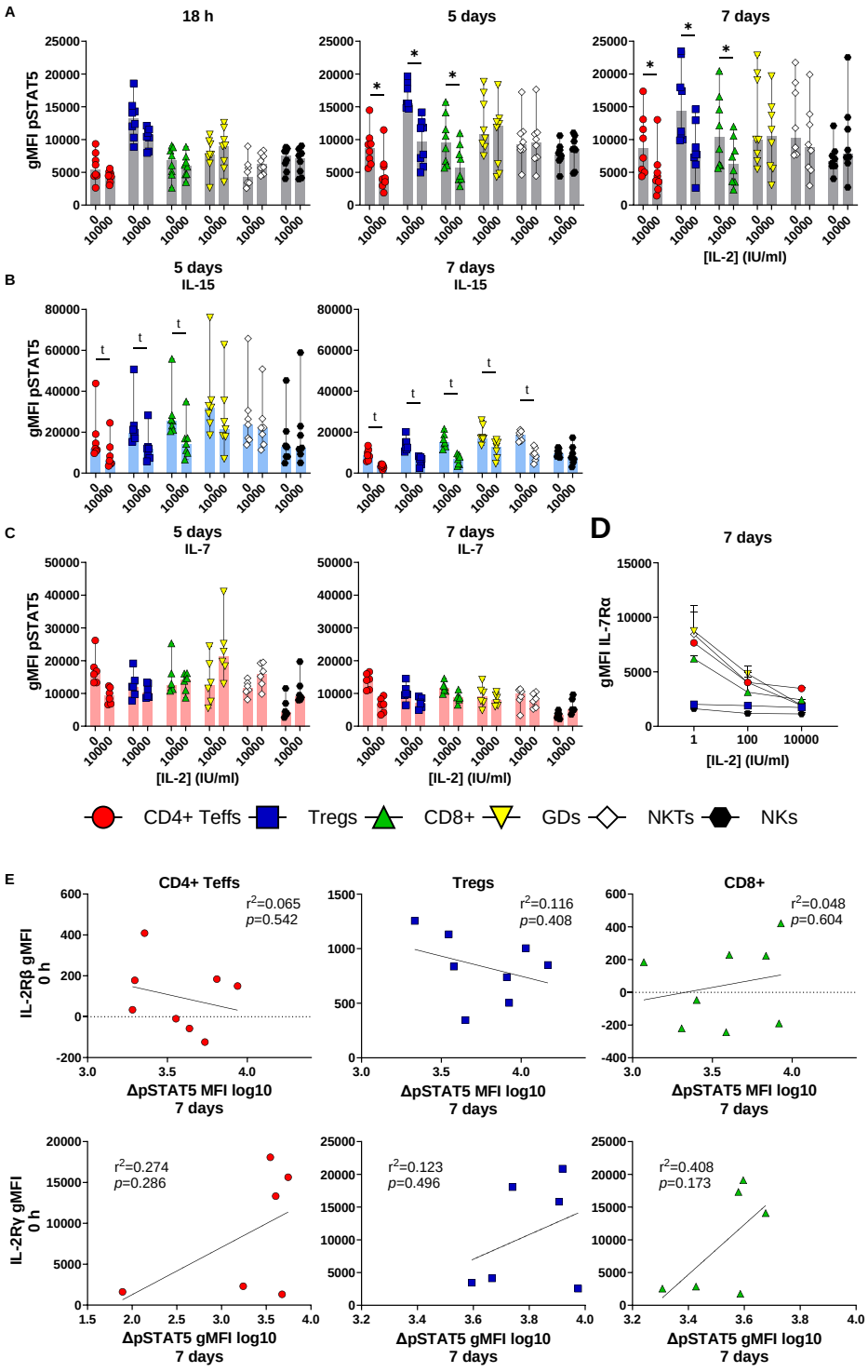

Figure S3: pSTAT5 signal after re-stimulation of high-dose IL-2-stimulated cells. Human PBMCs were stimulated with increasing IL-2 doses for up to 7 days prior to analysis of CD4+ Teffs, CD4+ Tregs, CD8+ T cells, γδ T cells (GDs), NKT cells, and NK cells. **A-C** After stimulation with (10,000 IU/mL) or without IL-2 (0 IU/mL) for up to 7 days, cells were re-stimulated with high-dose IL-2 (10,000 IU/mL) (**A**), IL-15 (**B**), or IL-7 (**C**) and pSTAT5 signal was measured as shown in gMFI. n=6-8 donors, two independent experiments (n=3-4 donors each), median ± range. * $p<0.05$, all other conditions non-significant (t $p<0.1$), analyzed by multiple Wilcoxon tests with Holm-Šídák method.

D Surface expression of IL-7Rα after 7 days of IL-2 stimulation on respective cell subsets as shown by gMFI. Tregs are gated as CD3+CD4+ FoxP3+ CD127low/–CD25+. n=3 donors. **E** Donor-dependent correlation of baseline IL-2Rβ (upper panels) and IL-2Rγ (lower panels) gMFI of αβ T cells without IL-2 stimulation with differences in pSTAT5 signal on day 7. For pSTAT5 signal, cells were stimulated with high-dose IL-2 (10,000 IU/mL) or left unstimulated for 7 days and re-stimulated with high-dose IL-2 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 (10,000 IU/mL)). The common logarithm (log10) of ΔpSTAT5 is shown. n=6-8 donors, two independent experiments (n=3-4 donors each). r^2 and *p*-values determined using simple linear regression.

4 DISCUSSION

Systemic toxicity hindered the use of high-dose recIL-2 therapy, a once-promising treatment option for malignant cancers. Although adverse effects initially led to a drawback of the therapeutic, the promise it holds for the treatment of both cancer and inflammatory diseases initiated a revival of recIL-2 therapies, leading to currently more than 40 different IL-2-based molecules in clinical trials.[86] However, while adverse effects such as vascular-leak-syndrome as one of the most severe toxicities during recIL-2 therapies are reduced in current studies, recIL-2-induced skin rashes still frequently occur. Although usually not life-threatening, these CADRs significantly impair patient well-being and might pose a reason to stop treatment.[69] However, pathophysiological mechanisms underlying recIL-2-induced skin rashes remain unclear. The current thesis sheds light on IL-2R expression dynamic during recIL-2 stimulation and key cells and molecules that might be involved in the induction of recIL-2-induced immunotoxicities such as skin rashes using human- and murine-based model systems.

4.1 IL-2Rα$^+$ skin effector cells induce skin inflammation upon recIL-2 application

Skin rashes represent one of the most frequent adverse effects during recIL-2 therapy – both using unmodified aldesleukin as well as during treatment with new recIL-2 products.[99, 100, 103] To characterize recIL-2-induced dermatitis and the relevance of IL-2Rα in the process, different mouse models were used, highlighting the role of innate lymphoid immune subsets and IL-2Rα in recIL-2-induced skin inflammation (research paper 1).[136]

Similar to early clinical reports, the application of recIL-2 (aldesleukin) in wild-type mice resulted in considerable mixed cell infiltrates of lymphocytes in addition to neutrophils and eosinophils in the skin.[77, 104–106] While numbers of all lymphocyte subsets investigated (except dendritic epidermal T cells [DETCs]) increased upon dermal recIL-2 injections, frequencies of αβ T cell subsets were similar in control and recIL-2-treated skin. On the other hand, proportions of dermal γδ T cells and ILCs including ILC1s were increased, suggesting a more specific effect of recIL-2 on these innate immune cell subsets. In line with this, cytokine expression in all three cell subsets was significantly impacted upon recIL-2 injections, inducing an overall shift towards type 2 and type 17 immunity.

While frequencies of IFN-γ-producing ILC1s decreased, possibly due to overstimulation such as seen for NK cells – which are part of ILC1s – in tumors of mice and humans,[137, 138] proportion of IL-4- and IL-13-producing ILC2s significantly increased upon recIL-2 application. In line with our study, ILC2s and type 2 cytokine production were shown to be specifically increased in dermatitis sections of Rag$^{-/-}$ mice receiving a recIL-2-antibody complex.[21] Furthermore, *in vivo* reduction of ILC2s verified that the cells are the main IL-5 producers resulting in peripheral eosinophilia as another common adverse effect during recIL-2 therapy.[22] Our current study further underlines the central role of ILC2s inducing

adverse effects during recIL-2 application as these seem to induce the overall shift towards type 2 immunity upon dermal recIL-2 injections. Activation of ILC2s might result from direct recIL-2 stimulation as IL-2 is well-known to induce type 2 cytokine secretion in ILC2s.[21–23] Furthermore, recIL-2 induces secretion of various other cytokines in both mice and humans,[22, 106, 139] suggesting that other inflammatory stimuli besides recIL-2 might support the activation of ILC2s. For instance, alarmins such as IL-33 might be secreted upon progressive tissue damage in recIL-2-treated skin which could induce synergistic effects in ILC2 activation[139–142] and might implement a vicious cycle of boosting type 2 responses, leading to progressive tissue damage and IL-33 secretion in the clinics. Furthermore, besides type 2 responses, recIL-2 injections significantly increased the frequency of IL-17$^+$ dermal γδ T cells. In mice, IL-2Rα$^+$ γδ T cells are reported to produce IL-17 and IL-17 production might be induced upon IL-2 stimulation.[143, 144] IL-2Rα expression on γδ T cells further was shown to depend on IL-2,[144] suggesting that recIL-2-induced upregulation of the receptor subunit might be crucial to induce the shift towards type 17 immunity in our study. Overall, dermal recIL-2 injections induced a mixed type 2/type 17 immune response which seems to be mediated by ILC2s and dermal γδ T cells (Figure 6). Thus, recIL-2-induced skin inflammation might resemble pathologies such as atopic dermatitis or psoriasis in which the frequency of ILC2s or γδ T cells is significantly increased compared to healthy skin in humans, respectively.[130, 145]

One limitation of this study is that aldesleukin does not induce macroscopic skin rashes in wild-type mice or other rodents,[146] contrary to the clinics. However, skin rashes were observed in Rag$^{-/-}$ mice treated with an IL-2Rα-specific antibody-coupled recIL-2.[21] These studies suggest that either the lack of T and B cells in Rag$^{-/-}$ animals is crucial to induce the adverse effect in mice or that specific activation of IL-2Rα$^+$ cells – such as ILC2s – is key. Thus, by applying an IL-2Rα-specific recIL-2 complex, type 2 immune responses might preferentially be mediated with minimal activation of γδ T cells. The lack of type 17 immunity to counterbalance immune reactions could consequently induce macroscopic skin rashes in mice. In human skin, γδ T cells are reported to rarely produce IL-17 and IL-2 stimulation rather induces TNF-α- and IFN-γ-producing human γδ T cells *in vitro*.[17, 147] This indicates that in patients suffering from recIL-2-induced skin rashes, type 2 immune responses might predominate, manifesting in skin rashes. However, this hypothesis would need further investigation such as by using γδ T cell knockout mice models or human skin-derived samples.

To examine the significance of IL-2Rα expression in recIL-2-induced skin inflammation in more detail, we aimed to specifically delete IL-2Rα in all skin cells. Although moderate partial reduction rather than full deletion was observed, recIL-2-induced accumulation of immune cells was significantly decreased. To increase deletion efficacy, knockout might be induced systemically "the classical way", by tamoxifen injection. However, the tremendous advantage of establishing the 4-Hydroxytamoxifen (4-OHT)-based system is that it allows local over systemic effects so that deletion is restricted to skin sections to which 4-OHT was applied. This is especially of relevance as systemic IL-2Rα deletion results in systemic inflammation, leading to autoimmunity[66, 148, 149] which would complicate interpretation of

results upon recIL-2 application. The 4-OHT approach additionally reduces animal numbers used as the system allows each animal to represent its own internal control, harboring both wild-type and knockout skin. Overall, our results of skin-specific reduction of IL-2Rα further underline the central role of the receptor subunit leading to recIL-2-induced immunotoxicity as shown by skin inflammation (Figure 6). These observations are therefore in line with clinical studies, reporting reduced systemic inflammation using recIL-2 constructs with reduced IL-2Rα binding affinity.[95, 97]

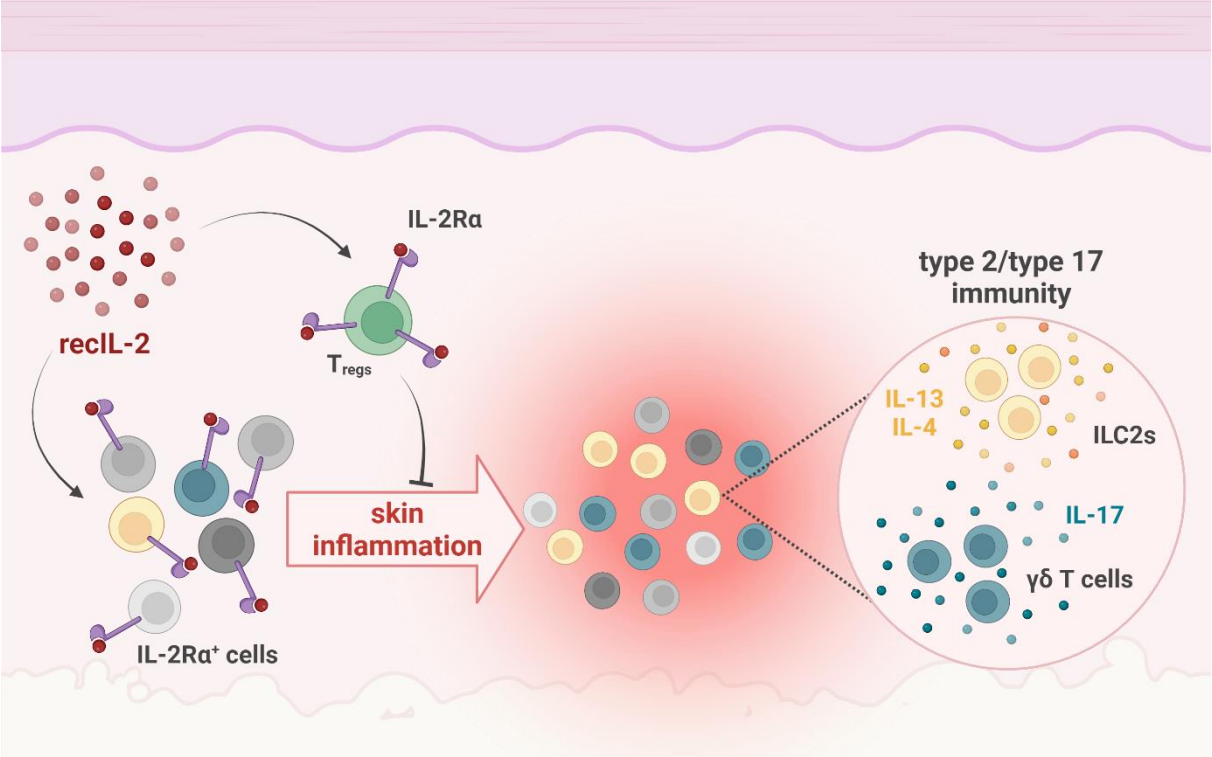

Figure 6: RecIL-2 induces mixed type 2/type 17 immunity in an IL-2Rα-dependent manner. In the skin of mice, group 2 innate lymphoid cells (ILC2s) and dermal γδ T cells induce a shift towards type 2- and type 17-driven inflammatory responses. While IL-2Rα-expressing T_{regs} inhibit this inflammation, IL-2Rα$^+$ effector cells seem to drive these responses. Adapted from Sommer et al. (2024).[136] Created with BioRender.com.

In contrast to the reduction of IL-2Rα on all cells, skin-specific reduction on FoxP3$^+$ T_{regs} drastically increased inflammatory infiltrates upon recIL-2 injections. This finding was accompanied by an imbalance of the CD4$^+$ T_{eff}:T_{reg} ratio in favor of CD4$^+$ T_{effs} and slight decreases in FoxP3 expression, suggesting loss of regulatory function.[150, 151] Moreover, decreased frequencies of ILC1s and T_H1 and T_H2 cells point to an increased frequency of RORγt-expressing cells, indicating a shift towards type 17 immunity following IL-2Rα reduction on T_{regs}. Overall, our results highlight the role of IL-2Rα expression on T_{regs} to maintain immune balance.[152, 153] Reduction of IL-2Rα could result in two major mechanisms, destabilizing immunity: First, T_{regs} are known to exert part of their regulatory function as "IL-2 sinks", consuming IL-2 through their high-affinity IL-2R which limits IL-2 for effector cells.[5, 33] Second, IL-2 can directly induce the inhibitory function of T_{regs}, irrespective of their T cell receptor.[5] Thus, increased bioavailability of recIL-2 could directly support activation of effector cells while the suppressive function of T_{regs} might concurrently be decreased due to reduced IL-2 signaling upon reduction of IL-2Rα. Overall, our findings underline the critical function of IL-2Rα on T_{regs}, limiting inflammatory immune responses such as recIL-2-induced skin inflammation (Figure 6).

Together, research paper 1 demonstrates that cutaneous immune reactions induced by recIL-2 injections are marked by a shift towards type 2/type 17 immunity, which appears to be primarily induced by innate lymphoid immune cells, namely ILC2s and dermal γδ T cells in mice. In addition, while IL-2Rα-expressing T_{regs} suppress these inflammatory reactions, reduction of IL-2Rα on all skin cells decreased recIL-2-induced skin inflammation, indicating that the receptor on effector cells is responsible for mediating at least part of these inflammatory reactions. These results highlight the relevance of innate lymphoid immune cells and IL-2Rα in the induction of adverse effects during recIL-2 therapy.

4.2 IL-2R signaling is especially impaired in high-dose recIL-2-stimulated T_{regs}

IL-2Rα is well-known to be upregulated upon recIL-2 application in mice and humans.[16, 75, 133, 134, 136] However, regulation of other subunits of the IL-2R complex – IL-2Rβ and IL-2Rγ – is poorly described. To characterize the regulation of IL-2R subunits and impact on IL-2R signaling capacity during recIL-2 stimulation, human PBMCs were stimulated with increasing recIL-2 (aldesleukin) doses, inducing vast changes in IL-2Rβ surface expression and corresponding reduction in IL-2R signaling capacity especially in T over NK cells (research paper 2) (Figure 7).[154] These findings are comparable to cellular responses of patients with hypomorphic *IL2RB* mutations which present with multi-organ autoimmunity such as skin rashes.[63, 64]

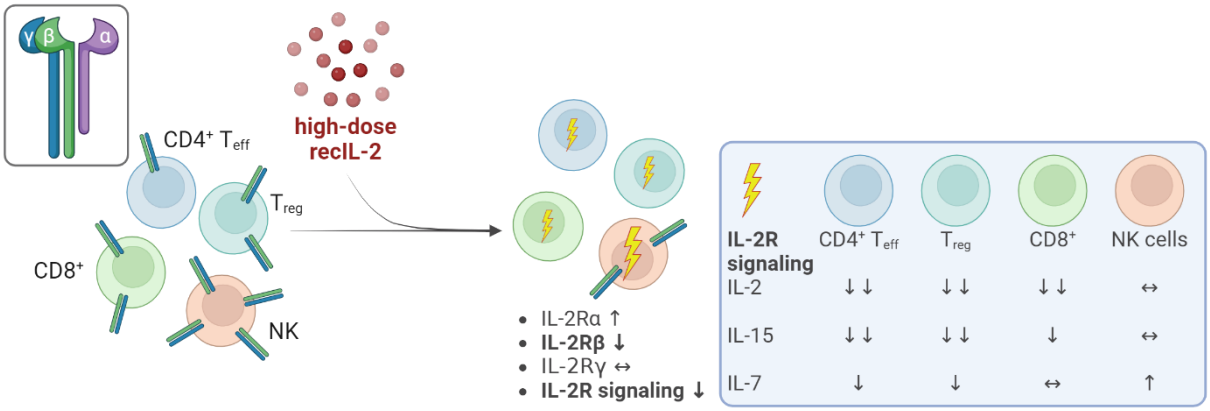

Figure 7: IL-2R subunits are differentially regulated, resulting in reduced IL-2R signaling in high-dose recIL-2-stimulated T cells. While surface expression of IL-2Rα is increased and IL-2Rγ abundance is largely unaltered, extracellular IL-2Rβ expression is highly decreased, especially on T cell subsets. In turn, the signaling of cytokines binding to IL-2R subunits is decreased in T cells, particularly in $CD4^+$ T cells. Adapted from Sommer et al. (2024).[154] Created with BioRender.com.

Even though stimulation with high recIL-2 concentrations (>100 IU/mL) led to significant decreases in IL-2Rβ surface expression on all lymphocyte subsets investigated, these were especially prominent for T cell subsets as IL-2Rβ was low (<7%) or basically absent (<1%) on γδ T and NKT cells or αβ T cells after 18 h of stimulation, respectively. While expression remained low on these cell subsets throughout the culture period, IL-2Rβ expression on NK cells – which was similarly reduced 18 h after high-dose IL-2 stimulation – increased over time so that after 7 days, more than 50% of NK cells remained IL-2Rβ$^+$. These results correlate with observations in patients with hypomorphic *IL2RB* mutations, leading to reduced IL-2Rβ signaling in T cells as shown by decreased STAT5 phosphorylation by IL-2 or IL-15, both signaling through the IL-2Rβγ complex.[63, 64] Similarly, in our

study, we showed that IL-2 and IL-15 signaling in high-dose recIL-2-stimulated T cells is impaired, suggesting that decreases in IL-2Rβ surface expression by recIL-2 stimulation impact T cell function. Decreased IL-2 and IL-15 signaling was especially prominent in $CD4^+$ T cells, particularly T_{regs}. On the other hand, signaling in NK cells, maintaining high IL-2Rβ surface expression, in both our study and in patients with deficient IL-2Rβ is not affected.[63, 64] In contrast to patients deficient in IL-2Rβ, IL-7 signaling was slightly reduced in high-dose recIL-2-stimulated $CD4^+$ T cells which might be due to reduced IL-2Rγ expression or lower expression of IL-7Rα (CD127) on stimulated T cells observed by us and others,[14, 155] or both. IL-2R-induced activation of STAT5 was shown to be crucial to induce regulatory activity of T_{regs}, preventing systemic inflammation in mice *in vivo*.[5] Thus, reduced IL-2Rβ surface expression leading to lower activation of STAT5 in high-dose recIL-2-stimulated T_{regs} could impair suppressive function of T_{regs}, possibly disturbing immune balances in recIL-2-treated patients.

While previous studies report reduced IL-2Rβ surface expression on recIL-2-stimulated T and NK cells *in vitro*,[156–158] our study is the first determining functional consequences of decreased IL-2Rβ expression upon high-dose recIL-2 stimulation. On the other hand, IL-2Rβ regulation in the clinics is barely described. A clinical report investigating recIL-2 therapy for treatment of multiple melanoma and lymphoma found increased IL-2Rβ expression on lymphocytes 24 h after the last recIL-2 dose was applied.[159] However, in this study, recIL-2 was applied less frequently and in lower concentrations compared to conventional anti-cancer high-dose recIL-2 therapy ($1.8x10^6$ IU/day daily vs. 600,000-720,000 IU/kg/dose every 8 h in high-dose IL-2 therapy) and induced only minimal adverse effects. The differential dosing regime and time point chosen to measure IL-2Rβ expression might explain differences compared to our study, showing a dynamic, dose-dependent regulation of IL-2Rβ.

Cell-specific differences upon continuous recIL-2 stimulation might in part explain specific increases in NK cell frequencies observed during recIL-2 therapy in the blood of high-dose aldesleukin-treated cancer patients.[73] In line with this, NK cells are typically increased along with T_{regs} upon low-dose recIL-2 therapy[89, 91–93] and $CD56^+$ cell frequencies (namely NKT and NK cells) were dose-dependently increased upon recIL-2 stimulation of PBMCs (research paper 2). Within the NK cell compartment, we observed increases in $CD56^{bright}$ NK cells upon recIL-2 stimulation *in vitro* which seems to be due to a preferential proliferation of $CD56^{bright}$ NK cells.[19, 160] Similarly, $CD56^{bright}$ NK cells expanded during recIL-2 therapy as well as in patients with defective IL-2Rβ.[63, 64, 73, 92, 160] Classically, $CD56^{bright}$ NK cells are associated with cytokine production while $CD56^{dim}$ NK cells rather show cytotoxic function[161] which might suggest increased cytokine secretion rather than cytotoxicity during high-dose recIL-2 stimulation. However, NK cells have been associated with inducing vascular-leak-syndrome during high-dose recIL-2 anti-cancer therapy in mice, and thus, the role of NK cells in the induction of adverse effects such as skin rashes during recIL-2 therapy needs to be further evaluated in future studies. Similarly, the relevance of NKT and γδ T cells in recIL-2-induced immune dysregulation, possibly contributing to adverse effects, will need to be investigated in more detail in the future. While our results showed that IL-2Rβ expression was significantly reduced on high-dose recIL-2-stimulated

γδ T and NKT cells, a small proportion seems to remain IL-2Rβ$^+$ and IL-15 signaling was impaired to a lesser extent compared to adaptive αβ T cells. Possibly, these differential responses towards high-dose recIL-2 are due to higher intracellular IL-2Rβ abundance induced by recIL-2 compared to αβ T cells which could in turn cell-specifically impact cellular function.

Within the cell populations analyzed, CD4$^+$ T cell subsets and especially T_{regs} seem to be more broadly affected by increasing recIL-2 concentrations as evidenced by significant decreases in IL-2Rβ surface expression as early as 15 min after onset of stimulation, slight decreases in IL-2 signaling capacity after only 18 h of recIL-2 stimulation, more prominent decreases in IL-15 signaling, and decreases in IL-7 signaling in some donors. These observations suggest differential responses of CD4$^+$ T cells towards IL-2 compared to other lymphocytes such as CD8$^+$ T cells. In line with this, previous studies report that lineage-specific mechanisms control IL-2 responsiveness of CD4$^+$ and CD8$^+$ T cells.[5, 162–164] Specifically, IL-2 lowers the threshold for T cell receptor signaling in CD8$^+$ but not CD4$^+$ T cells[162] and IL-2 consumption by T_{regs} is required to suppress CD8$^+$ T cells only.[5] These differences might partly be mediated by higher intracellular IL-2Rβ storage in CD8$^+$ over CD4$^+$ T_{effs}, mirroring differential IL-2Rβ surface expression and inducing biphasic IL-2 signaling in murine CD4$^+$ T cell blasts but constant signaling in CD8$^+$ T cell blasts.[163] In line with these observations, we found that intracellular IL-2Rβ abundance is higher in unstimulated human CD8$^+$ T cells compared to CD4$^+$ T_{effs}. Smith et al. hypothesized that these differences might account for the preferential proliferation of CD8$^+$ compared to CD4$^+$ T_{effs} during *in vitro* IL-2 stimulation or recIL-2 therapy,[163, 164] also found in our study. Similarly, intracellular IL-2R abundance might also account for differences in CD4$^+$ and CD8$^+$ T_{effs} in our study. However, differential IL-2Rβ storage might only explain some cell-specific differences towards continuous recIL-2 stimulation, but not all. On that note, intracellular IL-2Rβ in CD4$^+$ T_{regs}, which showed similar extensive changes of IL-2R signaling as CD4$^+$ T_{effs} upon continuous recIL-2 stimulation, was comparable to murine CD8$^+$ T cell blasts and even higher than in human CD8$^+$ T cells.[163] Rather, recIL-2 stimulation might induce cell-specific gene expression as intracellular IL-2Rβ and IL-2Rγ significantly and dose-dependently increased with recIL-2 stimulation in CD8$^+$ T cells (along with γδ T, NKT, and NK cells) while recIL-2 did not enhance intracellular IL-2Rβ or IL-2Rγ abundance in CD4$^+$ T cell subsets. In line with this hypothesis, expression of *IL2RB* and *IL2RG* was shown to increase upon recIL-2 stimulation of human CD8$^+$ T cells and NK cells dose-dependently but expression was largely unaltered in differentiated T_H1 and T_H2 cells.[158]

Besides differential gene expression of IL-2R subunits, cell-specific expression of IL-2Rα could additionally account for more pronounced changes in recIL-2-stimulated CD4$^+$ T cells. Compared to CD8$^+$ T cells, CD4$^+$ T cells and especially T_{regs} show a higher abundance of IL-2Rα surface expression,[32] thus equipping CD4$^+$ T cells with a higher IL-2 sensitivity.[13, 14, 26, 32] Formation of the high-affinity IL-2Rαβγ in turn increases receptor turnover and, with that, relatively more IL-2Rβ might be degraded which could further impair IL-2R signaling. In line with this, we found that IL-2Rα expression on CD4$^+$ T_{effs} and T_{regs} significantly correlated with decreased IL-2 signaling in high-dose recIL-2-

stimulated compared to unstimulated cells. A similar trend was observed for $CD8^+$ T cells, but not for baseline expression of IL-2Rβ or IL-2Rγ. These results further strengthen the role of IL-2Rα in immune dysregulation upon recIL-2 stimulation. Potentially, in $CD4^+$ T cells with relatively high IL-2Rα and lower IL-2Rβ expression, IL-2Rβ might be the IL-2R subunit limiting effective IL-2 signaling during continuous recIL-2 stimulation while in high IL-2Rβ producers such as NK cells, IL-2Rα could be limiting. As IL-2Rβ is the crucial subunit to induce IL-2 signaling, differential relative expression of IL-2Rα and IL-2Rβ could thus be a central factor for cell-specific immune dysregulation upon continuous high-dose recIL-2 stimulation.

Ultimately, similarities in cellular responses of high-dose recIL-2-stimulated immune cells and cells from patients with hypomorphic *IL2RB* mutations might induce comparable autoimmune-like organ manifestations in both settings. Specifically, upon mutation of IL-2Rβ, organs such as skin or gut are affected – similar to patients treated with high-dose recIL-2.[63, 64, 69, 105, 106, 165] Upon *IL2RB* mutation, T_{regs} are significantly reduced which might be induced by the permanent reduction or absence of IL-2Rβ, preventing IL-2 and IL-15 signaling.[63, 64, 166] This contrasts with reduction of IL-2Rβ surface expression upon continuous recIL-2 stimulation which is induced and likely transient. Although abundance of circulating T_{regs} is increased in patients treated with recIL-2,[73–75, 90, 91] decreased IL-2, IL-15, and – for $CD4^+$ T cells – IL-7 signaling capacity observed upon high-dose recIL-2 stimulation of T cells might impact maintenance and function of these immune cells in organs. All three cytokines are well-known factors for T cell survival, proliferation, and homeostasis and especially IL-15 and IL-7 are central in maintaining tissue memory T cells in organs including the skin.[167–170] Thus, reduced sensitivity towards IL-15 and IL-7 in $CD4^+$ T cells, especially T_{regs}, observed early upon high-dose IL-2 stimulation might impair peripheral function and maintenance, potentially disturbing tissue homeostasis. As IL-2R signaling is impaired at later time points in $CD8^+$ T cells and γδ T and NKT cells and unaffected in NK cells, this immune disbalance might provide a window for increased activation of effector cells. A recent study has shown that even partial skin-specific reduction of T_{regs} in mice leads to inflammation through autoreactive cells in the skin, highlighting that the skin is especially sensitive to T_{reg} disbalances.[27] This might be due to higher T_{reg} frequencies in human and murine skin (mean 20% and 30% of $CD4^+$ T cells, respectively) over other organs such as the gut, lung, liver, or the circulation (<10% of $CD4^+$ T cells in humans).[171–175] In turn, even a slight reduction in T_{reg} numbers and function in the skin of high-dose recIL-2-treated patients due to reduced maintenance could allow activation of autoreactive and/or innate immune cells such as ILCs including NK cells, facilitating autoimmune-like skin rashes in the clinics. Alternative to autoreactivity, effector responses might be directed towards skin commensals in response to disrupted T_{reg} balances such as reported for mice treated with checkpoint inhibitors,[176] which – like recIL-2 – are frequently associated with skin rashes in the clinics.[177] However, these hypotheses are highly speculative and will need further testing using clinical samples or skin-resident cells.

Overall, continuous high-dose recIL-2 stimulation cell-specifically reduces IL-2Rβ surface expression on human lymphocytes, leading to decreased IL-2R signaling capacity in T cells which correlates with high IL-2Rα baseline expression. $CD4^+$ T cells and especially T_{regs} seem to be more broadly affected compared to $CD8^+$ T cells and innate immune cells including γδ T, NKT, and NK cells which might be due to differential extracellular IL-2Rα and intracellular IL-2Rβ abundance in addition to lineage-specific intracellular mechanisms upon recIL-2 stimulation. Correlating *in vitro* findings with the clinical situation will help to decipher if reduced IL-2Rβ expression together with impaired function might be involved in recIL-2-induced adverse effects such as skin rashes.

4.3 Conclusion: The role of IL-2Rα during recIL-2-induced adverse effects

In both studies of the present thesis, IL-2Rα was identified as a central molecule correlating with skin inflammation and reduced IL-2R signaling capacity. Possibly, IL-2Rα might impact recIL-2-induced adverse effects such as skin rashes through different mechanisms: Firstly, $IL\text{-}2R\alpha^+$ cells in the skin were shown to contribute to skin inflammation in mice (research paper 1) which might be due to activation of effector cells expressing IL-2Rα under baseline conditions (such as ILC2s) or upregulating IL-2Rα due to recIL-2 stimulation, tipping the balance towards inflammatory and tissue-damaging effector responses (Figure 8A). Moreover, increased IL-2Rα expression correlated with decreased IL-2R signaling capacity through IL-2Rβ reduction on high-dose recIL-2-stimulated T cells, especially in $CD4^+$ T cell subsets (research paper 2). Possible reduced maintenance and function of these cells – particularly T_{regs} – might disturb the delicate immune balance in tissues such as the skin which could favor autoimmune-like skin rashes (Figure 8B). As tissue-resident immune cells differ vastly from their peripheral counterparts, such as observed for skin T_{regs} in mice,[27, 173] it would be crucial to investigate IL-2R regulation and function upon continuous recIL-2 stimulation of cutaneous immune cells and the impact on homeostasis in the skin. Due to the lower affinity of murine IL-2Rβγ towards human IL-2 (as opposed to murine IL-2Rα which shows similar affinity as the human receptor),[178, 179] human-based model systems would be favorable in this context.

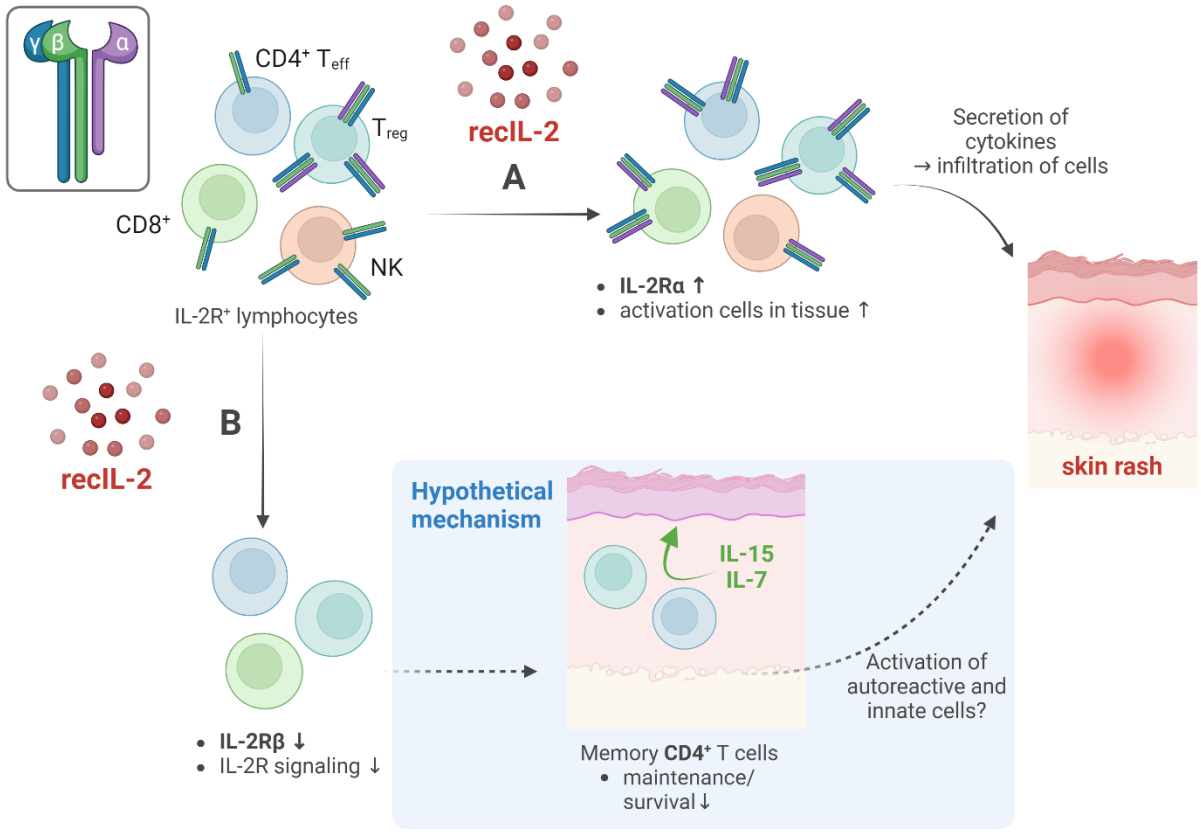

Figure 8: Potential IL-2Rα-related mechanisms contributing to recIL-2-induced skin rashes. A RecIL-2 stimulation induces upregulation of IL-2Rα on effector lymphocytes in the skin, leading to increased IL-2 sensitivity and facilitating effector functions such as cytokine secretion which seems to contribute to further cell infiltration and, ultimately, skin rashes. **B** High IL-2Rα expression furthermore was shown to correlate with the reduction in IL-2R signaling capacity upon high-dose recIL-2 stimulation which therefore might contribute to adverse effects indirectly by reducing sensitivity towards survival and maintenance factors, especially in $CD4^+$ T cells. This might facilitate the activation of tissue-resident autoreactive or innate cells and could favor autoimmune-like skin rashes. Created with BioRender.com.

Together, this thesis suggests a key role of IL-2R subunits in the induction of adverse effects during recIL-2 therapy. Specifically, IL-2Rα$^+$ effector cells drive recIL-2-induced skin inflammation which might be biased towards type 2 immunity, inducing skin rashes in the clinics. While IL-2Rα is upregulated on immune cell subsets by recIL-2 stimulation, IL-2Rβ surface expression is significantly and dynamically reduced which leads to impaired IL-2R signaling in T but not NK cells. $CD4^+$ T cells and especially T_{regs} seem to be more broadly affected compared to other (T) cell subsets, possibly driving immune disbalances resulting in autoimmune-like skin rashes. The differential impact of recIL-2 might partly be driven by high IL-2Rα expression on $CD4^+$ T_{regs} in addition to distinct gene expression induced by recIL-2. Thus, IL-2Rα might be an additional prognostic factor for immune dysregulation during recIL-2 therapy. These new molecular and cellular insights may contribute to improving the safety of recIL-2-based or other therapies.

5 OUTLOOK

The current thesis postulates that cell-specific and dynamic expression of IL-2R subunits is central in recIL-2-induced adverse effects such as skin rashes. To transfer results generated in murine models to humans, cells isolated from human skin and human skin explant cultures will be used in future studies, helping to clarify the role of dermal ILC2s and γδ T cells in human skin. Similarly, IL-2Rβ regulation upon recIL-2 stimulation should be investigated using skin-derived cells which might differ in function and phenotype from their circulating counterpart to correlate the potential impact on skin homeostasis. The usage of skin cells further allows analyzing the interaction and activation of tissue-resident immune cell subsets such as T cells and ILCs in the context of recIL-2-induced adverse effects.

Furthermore, the impact of IL-2Rβ reduction on cell functionality besides IL-2R signaling should be analyzed. Specifically, as STAT5 activation is known to directly impact regulatory function of T_{regs}, it would be interesting to investigate suppressive capacity upon continuous recIL-2 stimulation of $CD4^+$ T_{regs}. Besides *in vitro* stimulation assays, investigating IL-2R regulation and IL-2R signaling capacity in a clinical, therapeutic setting is of high importance, especially regarding high-dose recIL-2 treatment or using new IL-2-based approaches to verify the clinical relevance of the findings presented here. Specifically, analyzing clinical samples will help to verify if baseline expression of IL-2Rα correlates with onset and severity of skin rashes and if dynamic regulation of IL-2Rβ surface expression is a prognostic factor for the onset of skin rashes during recIL-2 therapy. Possibly, differential expression and regulation of IL-2R subunits might serve as biomarkers for patient-specific adverse effects, leading to skin rashes in a subset of patients only. Based on the mechanism of recIL-2-induced skin rash proposed herein, patient-specific differences might further result from varying T_{reg} proportions in human skin (ranging from ~15-35% of $CD4^+$ T cells)[171] which could render certain patients more sensitive towards disturbances in T_{reg} function compared to others. Furthermore, receptor polymorphisms might impact IL-2 sensitivity and in turn function of cells, such as seen in patients with Crohn's Disease[180] which could further add to the differential development of adverse effects upon recIL-2 therapy. Correlating additional factors such as skin T_{reg} frequency and possible IL-2R polymorphisms with recIL-2-induced skin rashes might help to further unravel mechanisms inducing the adverse effect which will help to increase the safety of IL-2-based therapies.

APPENDIX

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List of Abbreviations

4-OHT	4-Hydroxytamoxifen
CADR	cutaneous adverse drug reaction
DETC	dendritic epidermal T cell
DRESS	drug reaction with eosinophilia and systemic symptoms
ELAM-1	endothelial-leukocyte adhesion molecule-1
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
IL	interleukin
IL-2R	interleukin-2 receptor
ILC	innate lymphoid cell
ILC1	group 1 innate lymphoid cell
ILC2	group 2 innate lymphoid cell
ILC3	group 3 innate lymphoid cell
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
JAK	Janus family kinase
MAP	mitogen-activated protein
MCP-1	monocyte chemoattractant protein-1
NK	natural killer
NKT	natural killer T
PBMCs	peripheral blood mononuclear cells
PEG	polyethylene glycol
PI3K	class I phosphatidylinositol 3-kinase
PP2A	protein phosphatase 2A
pSTAT	phosphorylated signal transducer and activator of transcription
PTEN	phosphatase and tensin homologue
recIL-2	recombinant interleukin-2
STAT	signal transducer and activator of transcription
T_{eff}	effector T cell
T_H	T helper

TNF	tumor necrosis factor
T_{reg}	regulatory T cell
VCAM-1	vascular cell adhesion protein-1
X-SCID	X-linked severe combined immunodeficiency

List of Figures

List of Publications

Published

Sommer, C., Jacob, S., Bargmann, T., Shoaib, M., Alshaikhdeeb, B., Satagopam, V.P., Dehmel, S., Neuhaus, V., Braun, A.#, and Sewald, K.# (2024). Bridging therapy-induced phenotypes and genetic immune dysregulation to study interleukin-2-induced immunotoxicology. Clinical Immunology, 110288. 10.1016/j.clim.2024.110288.
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List of Conferences Associated with This Thesis

- German Pharm-Tox Summit: *Interleukin-2 induces type 2 and 17 skin inflammation in a CD25-dependent manner*. **Oral Presentation**. March 2024, Munich, Germany.
- German Pharm-Tox Summit: *IL-2 receptor signaling capacity is cell-specifically reduced in high-dose IL-2-stimulated PBMCs.* March 2024, Munich, Germany.
- Society of Toxicology: *High-dose IL-2 reduces IL-2R signaling capacity in T cells.* March 2024, South Lake City, USA.
- International Congress of Immunology (IUIS): *High-dose IL-2 stimulation reduces IL-2 receptor signaling capacity in T cells but not NK cells*. November-December 2023, Cape Town, South Africa.
- International Congress of Immunology (IUIS): *Natural killer cells are donor-dependently regulated in interleukin-2-stimulated human lung slices.* September-December 2023, Cape Town, South Africa.
- imSAVAR Consortium Meeting: *High-dose IL-2 stimulation reduces IL-2 receptor signaling capacity in T cells but not NK cells.* October 2023, Hannover, Germany.
- Models of Lung Disease Workshop: *Interleukin-2 induces donor-dependent immunological changes in human precision-cut lung slices.* June 2023, Hannover, Germany.
- Alternatives to Animal Testing Workshop: *Interleukin-2 induces donor-dependent immunological changes in human precision-cut lung slices.* June 2023, Hannover, Germany.
- Annual Meeting, German Center for Lung Research (DZL): *Natural killer cells are donor-dependently regulated in interleukin-2-stimulated human lung slices.* June 2023, Fürstenfeldbruck, Germany.
- Spring School, Deutsche Gesellschaft für Immunologie (DGfI): *Interleukin-2 stimulation of human precision-cut lung slices induces donor-dependent immune responses.* March 2023, Ettal, Germany.
- Symposium of the Young European Federation of Immunological Societies (yEFIS): *Interleukin-2 induces donor-dependent immunological changes in human precision-cut lung slices.* November 2022, Berlin, Germany.
- European Congress of Immunology: *Drafting an evidence-based immune-related adverse outcome pathway of interleukin-2-induced adverse effects on human lung tissue*. **Oral Presentation**. September 2021, online.
- World Immune Regulation Meeting: *Type 2 innate lymphoid cells and mast cells possess potential key roles in skin rash during IL-2 therapy*. July 2021, online.
- Autumn School, Deutsche Gesellschaft für Immunologie (DGfI): *Secondary activation of mast cells might contribute to lung toxicity during interleukin-2 therapy.* October 2020, Merseburg, Germany.

Declaration

Herewith, I confirm that I have written the present PhD thesis myself and independently, in compliance with “the policy of Hannover Medical School on the safeguarding of good scientific practice and procedural rules for dealing with scientific misconduct” and that I have not submitted it at any other university worldwide. Herewith, I agree that MHH can check my thesis by plagiarism detection software as well as randomly check the primary data. I am aware that in case of suspicion, ombudsman proceedings according to § 9 of MHH ’Guidelines of Hannover Medical School to guarantee good scientific practice and dealing with scientific fraud’ will be initiated. During such proceedings, the PhD process is paused.

Hannover, April 2024 Charline Sommer

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