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Review

Interleukin-2: Biology, Design and Application

Natalia Arenas-Ramirez,[1,2] Janine Woytschak,[1,2] and Onur Boyman[1,*]

Interleukin-2 (IL-2) exerts crucial functions during immune homeostasis via its effects on regulatory T (Treg) cells, and the optimizing and fine-tuning of effector lymphocyte responses. Thus, somewhat paradoxically, low doses of recombinant IL-2 have been used for Treg cell-based immunosuppressive strategies against immune pathologies, while high-dose IL-2 has shown some success in stimulating anti-tumor immune responses. Recent studies of the functional, biophysical and structural characteristics of IL-2 have led to the generation of IL-2 formulations, including IL-2/mAb complexes and IL-2 variants (muteins) that selectively enhance IL-2's immune stimulatory versus inhibitory properties. Here, we review these findings, placing new mechanistic insights into improved next-generation IL-2 formulations within the broader context of IL-2 biology. We conclude by integrating these findings into a framework for understanding IL-2-mediated selective immune modulation.

Introduction

Discovered, isolated, and cloned between 1976 and 1983, IL-2 was the first immunotherapy demonstrating clinical efficacy in metastatic cancer [1–5]. Despite these promising data, IL-2 immunotherapy has not been widely adopted for various reasons, including its difficult administration due to its short *in vivo* half-life ($T_{½}$), its toxic adverse effects when administered at high doses (as needed for antitumor immunotherapy), and its ability to stimulate both cytotoxic effector T cells and regulatory T (Treg) cells [6]. While activation of Treg cells is an unwanted effect in anticancer IL-2 immunotherapy, as Treg cells can dampen effector T cell responses against tumor antigens, the property of IL-2 – even at low doses – to stimulate Treg cells could be harnessed for the treatment of Treg cell-deficient autoimmune and chronic inflammatory disorders [7].

The past 10 years rekindled an interest in IL-2 immunotherapy, which came from structural and biophysical insights into the complex formed by IL-2 with its IL-2 receptor (IL-2R) subunits [8] and the finding that IL-2 could be modified to selectively stimulate either cytotoxic effector T cells or Treg cells [9]; these studies have led to the generation of IL-2 formulations with improved and selective immune stimulatory capacities [10]. Furthermore, clinical trials using low-dose IL-2 have demonstrated IL-2's potential in expanding Treg cells and modulating immune pathologies [11,12]. Four recent publications exemplify this development and have motivated this review, in that they provide structural insight into the selective IL-2-mediated modulation of immune responses using IL-2/monoclonal antibody (mAb) complexes [13], highlight the possibility of using IL-2 muteins to antagonize, rather than stimulate, IL-2R-induced signals [14], and report novel data on the clinical use of low-dose IL-2 therapy in organ-specific and systemic autoimmune disease [15,16].

To place these findings in context, we begin by providing an introduction into the biology of IL-2 and its receptors, followed by a discussion of IL-2/mAb complexes and IL-2 muteins, and end by

Trends

Interleukin-2 (IL-2) exerts immunosuppressive and immunostimulatory effects by activating regulatory T (Treg) versus cytotoxic effector cells.

These IL-2 effects hinge on different IL-2 receptor (IL-2R) expression patterns: $CD8^+$ T and natural killer cells carry high levels of dimeric IL-2Rs comprising IL-2Rβ (CD122) and IL-2Rγ (γ_c); Treg cells express high IL-2Rα (CD25) levels along with intermediate levels of CD122 and γ_c.

Selective IL-2 formulations, such as IL-2 complexes and IL-2 muteins, preferentially stimulate cells expressing high CD25 versus high CD122 levels, and recent studies extend these concepts to also include muteins that inhibit IL-2R-induced responses.

These data converge into a framework of IL-2-mediated selective immune modulation where CD25-biased IL-2 formulations primarily expand Treg cells and CD122-directed IL-2 formulations stimulate cytotoxic effector cells.

[1]Department of Immunology, University Hospital Zurich, University of Zurich, CH-8091 Zurich, Switzerland
[2]These authors contributed equally.

*Correspondence: onur.boyman@uzh.ch (O. Boyman).

summarizing the key concepts arising from these studies as well as indicating gaps in our current understanding.

The Biology of IL-2

IL-2 is a 15.5–16-kDa, four-α-helix-bundle cytokine (Figure 1) that exerts its actions via binding to various IL-2Rs, notably monomeric, dimeric, and trimeric IL-2Rs [6,17,18]. Monomeric IL-2Rs, comprising IL-2Rα (CD25), are usually cell membrane associated but also exist in soluble form and bind IL-2 with a low K_d of $\sim 10^{-8}$ M. Interaction of IL-2 with CD25 alone does not induce a signal [19]; hence, isolated membrane-bound or soluble CD25 molecules might serve as scavenger or decoy receptors for IL-2 [6,20]. Conversely, both dimeric and trimeric IL-2Rs lead to a downstream signal on binding to IL-2. Dimeric IL-2Rs comprise IL-2Rβ (CD122) and IL-2Rγ [better known as common γ-chain (γ_c) or CD132], whereas trimeric IL-2Rs comprise CD25, CD122, and γ_c (Figure 2). Of note, CD122 is also part of IL-15R, whereas γ_c is shared by IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 [18]. Considering only IL-2Rs with signaling capacity, dimeric IL-2Rs can be referred to as low-affinity (K_d $\sim 10^{-9}$ M) and trimeric IL-2Rs as high-affinity (K_d $\sim 10^{-11}$ M) IL-2Rs [19]. On a molecular level, a single trimeric IL-2R binds IL-2 with roughly 10–100-fold higher affinity than a single dimeric IL-2R, illustrating that one function of CD25 is to improve IL-2 binding to the dimeric IL-2R. Association of IL-2 with IL-2R causes internalization of the quaternary complex, on which IL-2, CD122, and γ_c become degraded in vesicles, whereas CD25 is recycled via endosomes to the cell surface [21]. Notably, internalization of IL-2 has been suggested to depend on the cytoplasmic part of γ_c, suggesting that γ_c-mediated signaling is involved [22].

On triggering of IL-2R, signal transduction occurs via three major pathways, involving: (i) Janus kinase (JAK)–signal transducer and activator of transcription (STAT); (ii) phosphoinositide 3-kinase (PI3K)–AKT; and (iii) mitogen-activated protein kinase (MAPK) [6,18] (see Figure 2 for more details). Moreover, IL-2 signaling activates the transcription factor B lymphocyte-induced maturation protein 1 (Blimp1), encoded by *Prdm1*, which serves as a negative feedback loop by repressing IL-2 production.

Several immune cells have been shown to secrete IL-2 when activated, including T cell receptor (TCR) $\alpha\beta^+$ and TCR$\gamma\delta^+$ T cells, natural killer (NK) cells, NKT cells, dendritic cells (DCs), and mast cells [6]. At resting conditions, $CD4^+$ helper T (Th) cells are the main source of the constant but low levels of IL-2. On immune activation, IL-2 production rapidly rises. Low IL-2 secretion by activated DCs has been suggested to provide an early IL-2 source [23], thereby supporting T cell stimulation. In parallel, activated T cells, including $CD4^+$ and $CD8^+$ T cells, start secreting large amounts of IL-2 for their own (autocrine) use and to stimulate in a paracrine fashion neighboring IL-$2R^+$ cells [6,17,18]. Interestingly, Treg cells are unable to produce IL-2, even on stimulation, unless they constitute peripherally derived ('induced') Treg cells redifferentiating to Th cells [24]. IL-2 production by activated T cells is transient and transcriptionally regulated, including silencing by Blimp1 and Aiolos (encoded by *Ikzf3*) [18]. Moreover, negative feedback appears to also exist on the cellular level involving IL-2-producing $CD4^+$ T and IL-2-consuming Treg cells [25]. Such regulation mechanisms might be key in preventing T cell overstimulation by persistent IL-2 signals in conjunction with repetitive TCR stimulation by antigen (including self- and tumor antigens), which can lead to T cell exhaustion or Fas (CD95)-mediated activation-induced cell death [26,27].

In terms of IL-2 responsiveness, *in vitro* activated human T cells have been reported to carry about 2000 high-affinity and 11 000 low-affinity IL-2Rs per cell [4,28]. Dimeric IL-2Rs are expressed at high levels on antigen-experienced (memory) $CD8^+$ T cells and NK cells, whereas low to intermediate levels of dimeric IL-2Rs are found on memory $CD4^+$ and naive T cells [6]. On TCR stimulation, T cells transiently upregulate CD25, thus expressing now trimeric IL-2Rs. In

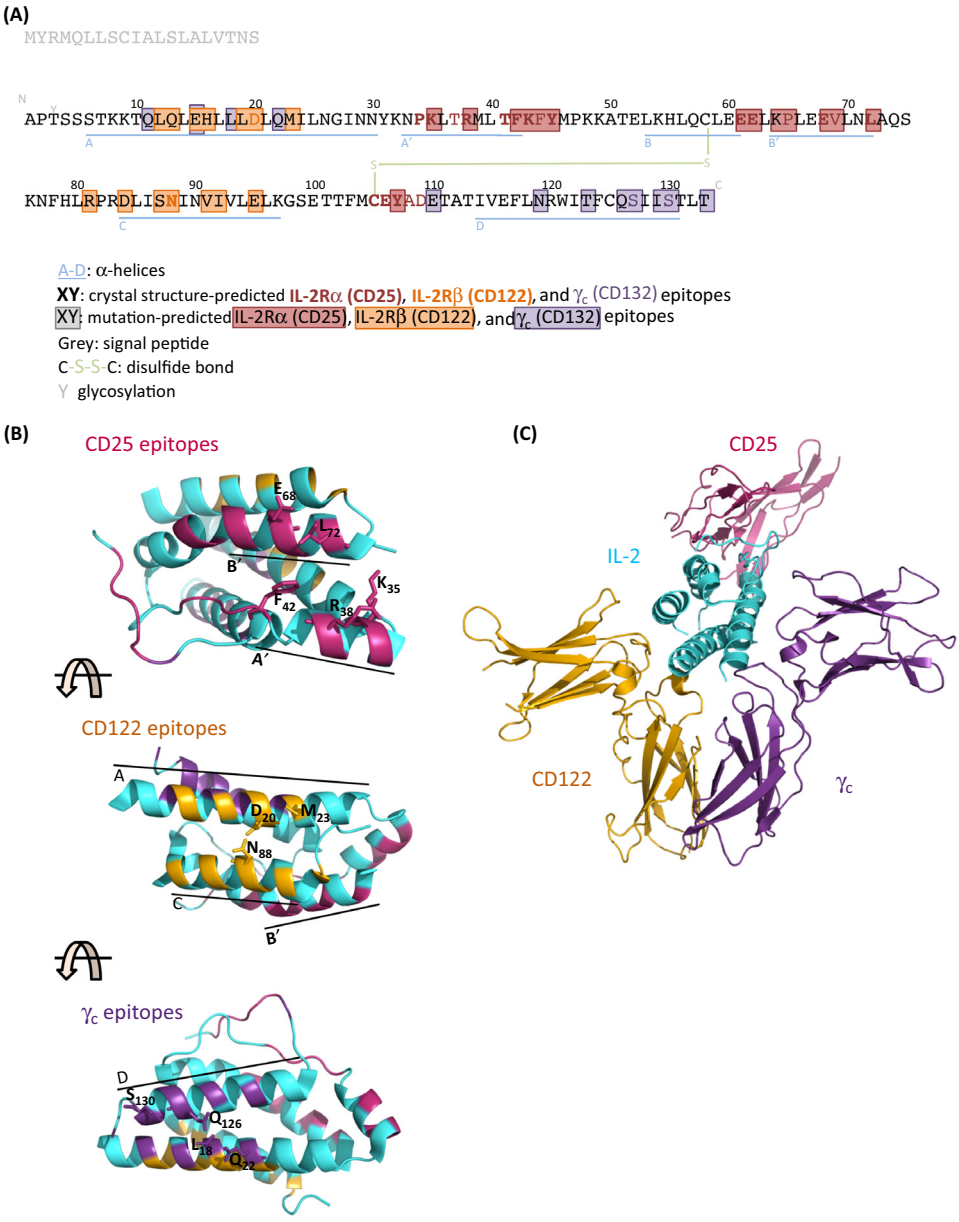

Figure 1. Sequence and Structure of IL-2 in Association with its Receptor Subunits. (A) Amino acid sequence of IL-2, indicating amino acids that interact with IL-2 receptor (IL-2R) subunits, including IL-2Rα (CD25; pink), IL-2Rβ (CD122; orange), and common γ-chain (γ_c, CD132; purple). Human IL-2 comprises 133 amino acids and weighs 15.5 kDa; mouse IL-2 measures 149 amino acids and has 16 kDa molecular mass. Human and mouse IL-2 show 57% sequence homology, yet human IL-2 can efficiently stimulate mouse IL-2Rs whereas mouse IL-2 is rather inefficient in binding to human IL-2Rs. (B) Human IL-2 (light blue) and its epitopes for CD25 (pink), CD122 (orange), and γ_c (purple) (accession code PDB: 1M47 [82]). (C) 3D structure of the quaternary IL-2–IL-2R complex, comprising IL-2 (light blue), CD25 (pink), CD122 (orange), and γ_c (purple) (accession code PDB: 2B5I [8]).

addition to recently activated T cells, high levels of trimeric IL-2Rs (precisely, high CD25 levels plus intermediate levels of dimeric IL-2Rs) are found constitutively on thymus-derived ('natural') $CD4^+$ forkhead box p3 $(Foxp3)^+$ Treg cells [29], whereas type 2 innate lymphoid cells (ILC2) and certain nonimmune cells (such as pulmonary endothelial cells) have been reported to carry low to

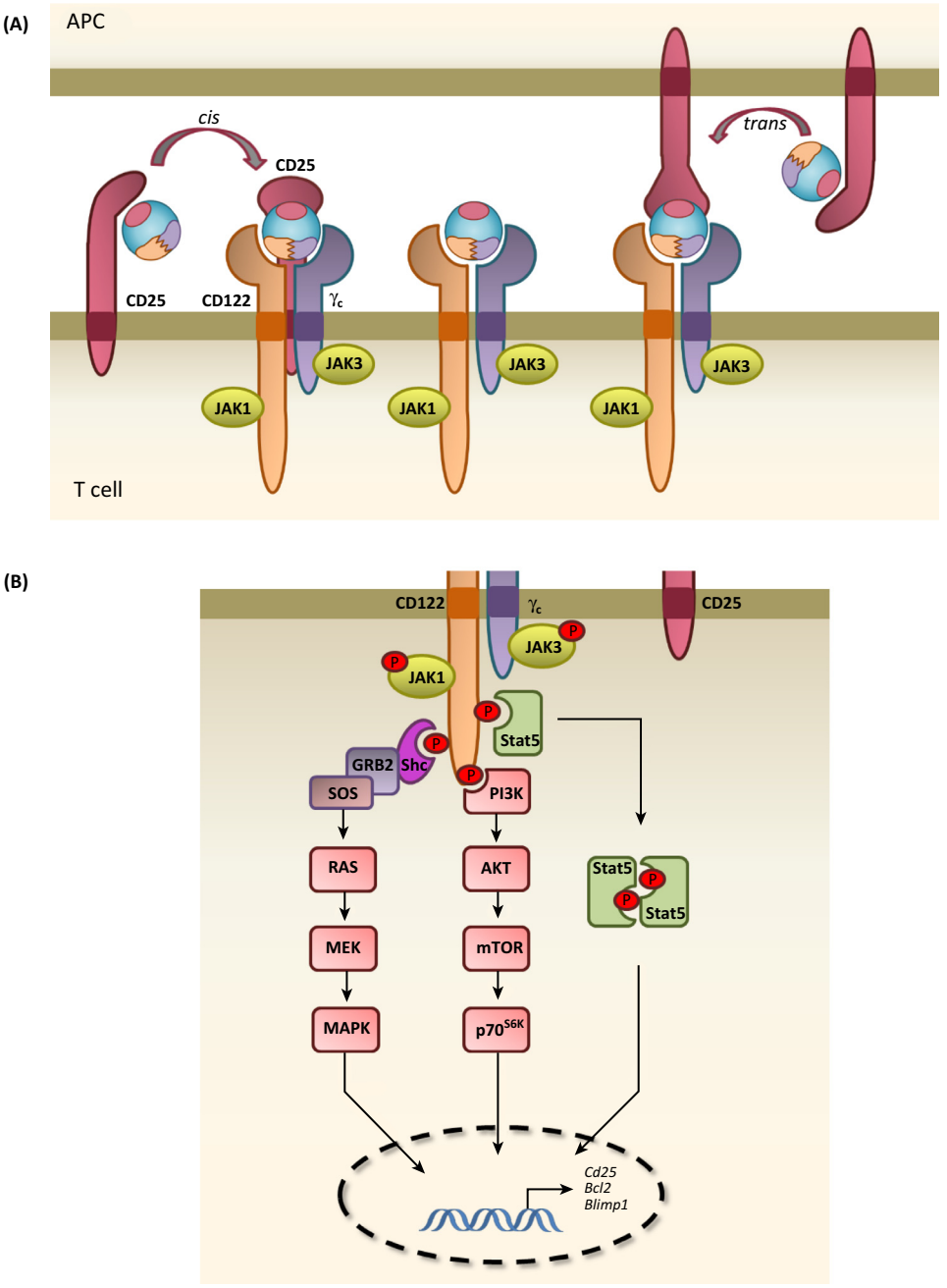

Figure 2. IL-2 Receptor (IL-2R) Binding and Signaling. Cartoon of IL-2 interacting with its receptor subunits, including IL-2Rα (CD25), IL-2Rβ (CD122), and the common γ-chain (γ_c, CD132), as well as signaling pathways following the interaction of IL-2 with various IL-2R subunits. Binding of IL-2 to CD122 and γ_c causes heterodimerization of the cytoplasmic tails of these receptor subunits and activation of Janus kinase 1 (JAK1) and JAK3 (associated with CD122 and γ_c, respectively) [18]. Activated JAK1 and JAK3 exert kinase activity on key tyrosine (Y) residues of CD122, which subsequently allows recruitment of the adaptor protein SHC and of STAT1, STAT3, and STAT5 (including STAT5A and STAT5B). Phosphorylated STAT5A and STAT5B then oligomerize forming STAT5 dimers and tetramers before undergoing nuclear translocation, where they bind to key target genes responsible for cell activation, differentiation, and proliferation. SHC in turn serves as a platform for activating the Ras–Raf–MEK–ERK mitogen-activated protein kinase (MAPK) pathway. Additionally, IL-2R triggering activates the phosphoinositide 3-kinase (PI3K)–AKT–mammalian target of rapamycin (mTOR)–p70 S6 kinase pathway.

very low levels of trimeric IL-2Rs [30,31]. Furthermore, activated mouse and human B cells express functional trimeric IL-2Rs and proliferate *in vitro* on stimulation with human IL-2 (hIL-2) [32,33]. Interestingly, DCs have been reported to express CD25 and to use CD25 to present IL-2 in *trans* to T cells expressing dimeric IL-2Rs [23,34] (Figure 2). Whether DCs carry CD122 is controversial, and stronger evidence suggests that DCs do not express CD122 under steady-state conditions [6,34].

The low but constant IL-2 production during resting conditions serves primarily for the development and homeostatic survival of Treg cells, which in turn maintain peripheral immune tolerance by dampening (autoreactive) effector T cells [15,17,35]. Treg cell dependence on IL-2 signaling is maybe best illustrated in IL-2- or IL-2R subunit (CD25 or CD122)-deficient animals and patients, which all lack Foxp3$^+$ Treg cells and develop systemic autoimmunity and inflammatory bowel disease. In IL-2R-deficient mice, this pathology can be prevented by adoptive transfer of wild-type Treg cells [36,37]. Following immune stimulation, local IL-2 concentrations increase several-fold, especially in secondary lymphoid organs, causing proliferation of NK cells as well as memory and recently activated effector T cells. Timed IL-2 signals are also required for optimal primary and secondary CD8$^+$ T cell responses [6,38,39] as well as for effector-to-memory transition in CD4$^+$ T cells [40]. Furthermore, IL-2 in concert with polarizing cytokines (pcs) (in parentheses) facilitates differentiation of naive Th cells to interferon-γ-producing Th1 (pc: IL-12), IL-4-, IL-5-, IL-9-, and IL-13-secreting Th2 (pc: IL-4), and peripherally derived ('induced') Treg [pc: transforming growth factor-β (TGF-β)] cells, while conversion of Th cells to IL-17A-, IL-17F-, and IL-22-producing Th17 (pcs: IL-6 and TGF-β) and follicular Th (pcs: IL-6 and IL-21) cells is curtailed by IL-2 signaling [6,18].

According to the current view, low doses of IL-2 [i.e., 1 500 000–3 000 000 international units (IU) once daily in humans or 15 000–30 000 IU or lower once daily in mice] preferentially stimulate Treg cells, although some effector T and NK cells might become activated as well [7]. Low-dose IL-2 might thus be suitable for the treatment of autoimmune and chronic inflammatory diseases such as systemic lupus erythematosus (SLE), type 1 diabetes, and cryoglobulinemic vasculitis [11,15,16,41,42], as well as graft rejection and chronic graft-versus-host disease [12,43], as these conditions have been reported to often feature lower IL-2 signaling and a relative Treg to effector T cell deficiency [7]. In line with this, a recent study showed, in patients with SLE, a relative reduction of Treg to effector T cells, with Treg cells displaying lower CD25 levels; moreover, cultured (but unstimulated) peripheral blood mononuclear cells and CD4$^+$ T cells from SLE patients showed deficient *ex vivo* IL-2 production [16]. Administration of a 5-day course of low-dose IL-2 to five SLE patients led to preferential stimulation of Treg over effector T cells, thus restoring Treg to effector T cell balance [16], as also observed in other pathologies [7,43]. According to a recent publication on human Treg cells from healthy controls and patients with type 1 diabetes, Treg cells seem to be especially poised to sense low concentrations of IL-2, as assessed by measuring phosphorylated STAT5, in part due to their high expression of CD25 and γ_c as well as their further transcriptional upregulation of CD25 and Foxp3 rapidly on IL-2; conversely, about tenfold higher IL-2 concentrations were needed to induce STAT5 phosphorylation in other lymphocytes, with memory CD4$^+$ T cells and CD56^{high} NK cells being the next-most-sensitive subsets, followed by naive CD4$^+$ and memory CD8$^+$ T cells and naive CD8$^+$ T and CD56^{low} NK cells [15].

Conversely, high-dose IL-2 (i.e., 600 000–720 000 IU/kg body weight three times daily for up to 14 doses per cycle in humans or 100 000 IU or higher once or twice daily in mice) has been used for immunotherapy against metastatic cancer [5] as high doses of IL-2 stimulate antitumor cytotoxic lymphocytes, including effector T and NK cells, presumably, once trimeric IL-2Rs on Treg cells have been saturated with IL-2. High-dose IL-2 treatment leads to 15–19% objective clinical response, including 7–9% complete response, in patients with metastatic melanoma or

metastatic renal cell carcinoma [5]. Other diseases where IL-2 immunotherapy has been tried comprise inherited and acquired immune deficiencies, the latter including chronic HIV infection, and other viral infections [44–46]. As an alternative to low-dose versus high-dose IL-2, various selective IL-2 formulations have been generated, including IL-2/mAb complexes and mutant IL-2 molecules (IL-2 muteins), which are discussed in the next sections.

IL-2/mAb Complexes

IL-2/mAb complexes (also termed IL-2 complexes or IL-2/anti-IL-2 mAb complexes) consist of IL-2 plus a particular anti-IL-2 mAb. The anti-IL-2 mAb can bind to and potentiate endogenous or recombinant IL-2 [6,9].

Two functionally distinct IL-2/mAb complexes are distinguished [6,9,47]. Use of the anti-mouse IL-2 (mIL-2) mAb S4B6 (i.e., mAb clone S4B6-1 or other S4B6-like mAbs, including JES6-5H4 for mIL-2 and MAB602 for hIL-2) generates IL-2/mAb complexes that preferentially stimulate cells expressing high levels of dimeric IL-2Rs, most notably memory (and memory-phenotype) $CD8^+$ T cells and NK cells. Conversely, IL-2/mAb complexes comprising IL-2 plus the anti-mIL-2 mAb JES6-1 (i.e., mAb clone JES6-1A12 or anti-hIL-2 mAb 5344) selectively activate cells carrying high levels of CD25 in addition to CD122 and γ_c. For example, five to seven daily injections of IL-2/S4B6 complexes (using ~15 000 IU IL-2) expand memory $CD8^+$ T cells and NK cells by 20–40-fold, while on such treatment $CD25^+$ $Foxp3^+$ Treg cell counts increase by two- to fivefold only [9,31,47]. This selectivity correlates with CD122 expression on memory $CD8^+$ T and NK cells (both $CD122^{high}$) versus Treg cells (expressing intermediate levels of CD122). Moreover, by avoiding contact with $CD25^+$ endothelial cells and by reducing the IL-2 dose needed for clinical efficacy, IL-2/S4B6 complexes exert less endothelial cell damage and vascular leak syndrome (VLS) in the lungs and liver of treated animals [31] (see also the section on IL-2 muteins). Given these properties, IL-2/S4B6 complexes could be used therapeutically in metastatic malignancies and chronic viral infection. In support of this notion, several groups have reported favorable results using IL-2/S4B6 complexes either as a monotherapy or in combination with another agent (such as Toll-like receptor ligand, agonist anti-OX40 mAb, or peptide vaccine) in various cancer models, including B16 melanoma, Lewis lung carcinoma, MC38 colon carcinoma, MCA-205 sarcoma, and TRAMP-C1 prostate carcinoma [31,48–53]. Moreover, IL-2/S4B6 complexes have shown efficacy in models of acute and chronic infection [54,55].

For IL-2/JES6-1 complexes, a short course of three to seven daily injections of these complexes increases $CD25^+$ $Foxp3^+$ Treg cell numbers by 7–15-fold, while $CD8^+$ T and NK cells are not significantly affected by IL-2/JES6-1 complexes [9,47,56]. IL-2/JES6-1 complexes are also stimulatory for ILC2 that express trimeric IL-2Rs, which contributes to IL-5 production and eosinophilia [30,57]. IL-2/JES6-1 complexes have shown promising results in the prevention of pancreatic [56] and skin [58] allograft rejection as well as in the treatment of several autoimmune and inflammatory diseases in mice, including type 1 diabetes in nonobese diabetic mice [59], experimental autoimmune encephalomyelitis (a model of multiple sclerosis) [56], experimental myasthenia [60], collagen-induced arthritis [61], dextran sodium sulfate-induced acute colitis [13], and T cell-mediated allergic airway disease [62]. Interestingly, administration of IL-2/JES6-1 complexes also improved the pathology of some metabolic, cardiovascular, and degenerative disorders, such as murine obesity-induced inflammation and insulin resistance ('type 2 diabetes') [63], atherosclerosis in high-fat diet-fed apolipoprotein E-deficient animals [64], and the mdx mouse model of Duchenne muscular dystrophy [65], that feature inflammatory infiltrates and are thus amenable to suppression by $CD25^+$ $Foxp3^+$ Treg cells.

Mechanistically, IL-2/mAb complexes are characterized, compared with IL-2, by increased *in vivo* potency and selectivity toward $CD122^{high}$ versus $CD25^{high}$ cells. Regarding their enhanced

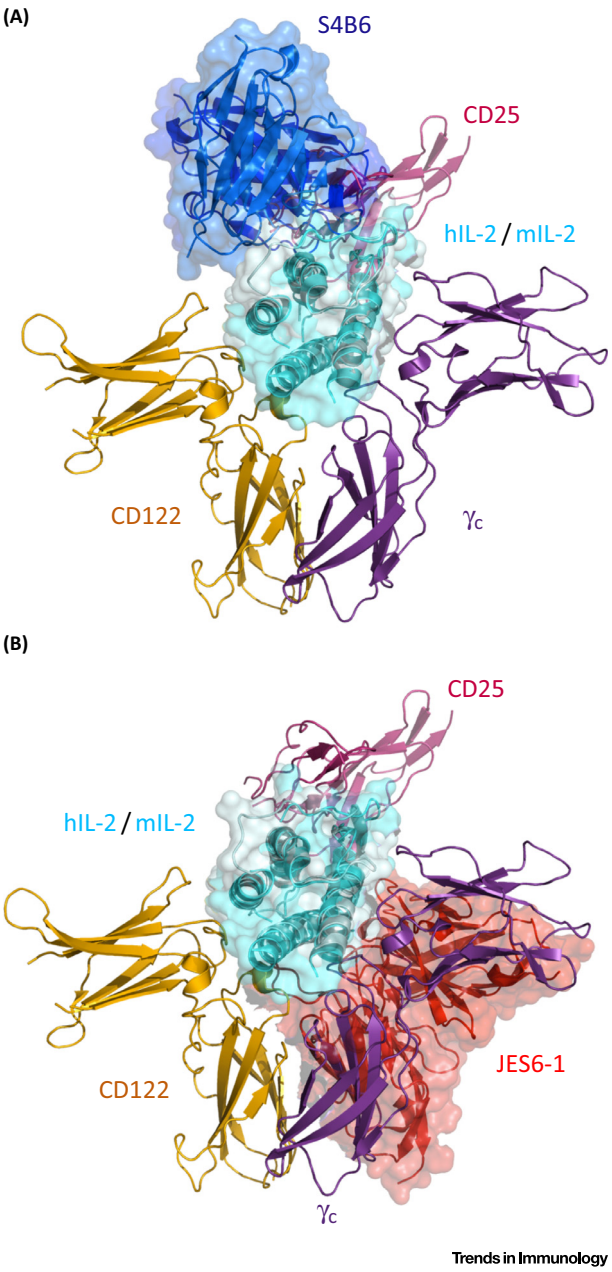

Figure 3. 3D Structure of IL-2/mAb Complexes. (A,B) The complexes formed by mouse IL-2 with Fab fragments of anti-mouse IL-2 monoclonal antibody (A) S4B6 (clone S4B6-1; accession code PDB: 4YUE [13]) or (B) JES6-1 (clone JES6-1A12; accession code PDB: 4YQX [13]) overlaying the human IL-2–IL-2R complex (accession code PDB: 2B5I [8]).

potency, IL-2/mAb complexes display an increased biological $T_{½}$ compared with IL-2 [47]. Unmodified IL-2 is rather short lived on intravenous injection ($T_{½} \sim$ 3–5 min in mice and 7–10 min in humans) due to its rapid clearance in the kidneys, probably by degradation, followed by delayed clearance [66–68]. The association of IL-2 with larger molecules, including serum albumin, liposomes, and irrelevant mAbs or mAb fragments (i.e., unrelated mAbs specific for antigens other than IL-2), increases the lifespan of IL-2 [69]. Similarly, association of IL-2 with S4B6 or JES6-1 increases IL-2 $T_{½}$ by about 10–20-fold [47,70]. This $T_{½}$ increase depends on neonatal Fc receptors and is crucial for the activity of IL-2/JES6-1 complexes and, to a lesser extent, IL-2/S4B6 complexes; contrarily, Fc receptors for IgG (FcRγ) do not appear to play a significant role in the bioactivity of IL-2/mAb complexes [47,70]. Nevertheless, similar to

CD25-mediated *trans*-presentation of IL-2 by DCs to T cells [34], IL-2/mAb complexes might bind to FcRγs on DCs and become presented in *trans* to T cells, although this has not been formally proven. Of note, the anti-IL-2 mAbs belong to various IgG subclasses – with S4B6 being rat IgG2a, JES6-5H4 rat IgG2b, JES6-1 rat IgG1, MAB602 mouse IgG2a, and 5344 mouse IgG1 [9,47] – suggesting that, for these mAbs, subclass plays a subordinate role.

Another mechanism for the superagonistic *in vivo* activity of IL-2/mAb complexes could be enhanced IL-2R signaling due to crosslinking of two dimeric IL-2Rs by an IL-2 complex, structural changes in IL-2 on mAb binding, or prolonged interaction of IL-2 with its receptor. IL-2R crosslinking has so far not been formally disproved. Yet, compared with IL-2, testing of IL-2/S4B6 complexes *in vitro* on $CD25^{-}$ YT-1 human NK cells induced decreased STAT5 phosphorylation [13] and lower proliferation of wild-type and $CD25^{-/-}$ memory-phenotype mouse $CD8^{+}$ T cells [9]. These data would rather argue against IL-2R crosslinking by IL-2/S4B6 complexes. For IL-2/JES6-1 complexes, STAT5 phosphorylation was reduced in $CD25^{+}$ YT-1 human NK cells compared with IL-2 *in vitro* [13], although another report using *in vitro* stimulation of total mouse splenocytes suggested that, compared with IL-2, IL-2/JES6-1 complexes led to increased STAT5 phosphorylation in $CD4^{+}$ T cells [71]. In line with the former finding, activation of $CD25^{+}$ murine $CD8^{+}$ T cells *in vitro* led to about tenfold lower proliferation with IL-2/JES6-1 complexes compared with IL-2 [9]. Conversely, *in vivo* IL-2/JES6-1 complexes led to prolonged and increased phosphorylation of STAT5 in Treg cells compared with IL-2 [61].

In its simplest embodiment, a mechanistic model for the selectivity of different IL-2/mAb complexes should explain the following observations [6,9]: (i) IL-2/mAb complexes are formed using *in vitro* 'neutralizing' anti-IL-2 mAbs; (ii) S4B6 and JES6-1 bind to different IL-2 sites; (iii) IL-2/S4B6 complexes mediate CD122-dependent but CD25-independent responsiveness of cells; and (iv) stimulation of cells by IL-2/JES6-1 complexes is strictly CD25 dependent. Based on functional *in vivo* and *in vitro* data, it has been proposed that the different functional properties of IL-2/S4B6 and IL-2/JES6-1 complexes are a result of the mAbs binding to different IL-2R-binding sites of IL-2 [6,9]: association of IL-2 with S4B6 sterically impedes binding to CD25 by covering IL-2's CD25 epitope, thus biasing IL-2/S4B6 complexes to preferentially stimulate $CD122^{high}$ cells, including memory $CD8^{+}$ T and NK cells; conversely, complexing IL-2 with JES6-1 occludes IL-2's CD122 and/or γ_c epitope, thus rendering IL-2 dependent on contact with CD25 and directing IL-2/JES6-1 complexes to selectively stimulate $CD25^{high}$ cells, such as Treg cells.

Recent structural data not only provide evidence for the steric aspects of the abovementioned model but also suggest mild antagonistic and allosteric forces at work (Figure 3). Thus, the data show that S4B6 covers CD25-interacting epitopes on IL-2, particularly those that lie on helices B and C of IL-2, which harbor some of the residues important for CD25 binding (Figure 1). However, S4B6 does not appear to contact the mouse counterparts of hIL-2 residues K35 and R38 (cf. mIL-2/S4B6 complex structure accessed at accession code PDB: 4YUE [13]). Both K35 and R38 have previously been shown to be intimately involved in the association of hIL-2 with human CD25 (Figure 1), with R38 representing the residue with the largest estimated buried surface with CD25 and most numerous van der Waals and hydrogen-bond contacts with CD25 [72]. Hence, these data suggest that S4B6's footprint on IL-2 does not fully overlap with that of CD25.

Moreover, S4B6 affected the binding of IL-2 to its receptor in two additional ways. Firstly, based on structural comparison of the mIL-2/S4B6 complex with unbound hIL-2 and hIL-2 in complex with human CD25, S4B6 binding caused mIL-2 to undergo a slight conformational change in its helix C leading to increased affinity to CD122 [13], similar to what has been reported for human CD25-bound IL-2 [8]. Evidence for the relevance of this conformational change comes from the

study of H9 and D10 hIL-2 muteins (also termed IL-2 'superkines'; see next section), which structurally replicated the said change in helix C and showed 200-fold increased affinities to CD122, led to more STAT5 phosphorylation in $CD25^-$ YT-1 human NK cells, and enhanced proliferation of human naive $CD4^+$ T cells *in vitro* [51]. In line with this hypothesis, immobilized monovalent mIL-2/S4B6 complex showed six-times-higher affinity to monomeric mouse CD122 compared with immobilized mIL-2 [13], which, however, is considerably lower than the binding affinity to CD122 of the IL-2 superkines [51]. Furthermore, the authors reported a small steric clash between S4B6 and CD122, presumably antagonizing, at least in part, the affinity-enhancing effects described above [13]. Nevertheless, the net result of these somewhat opposing effects was increased binding of IL-2/S4B6 to CD122; however, surprisingly, this did not translate into enhanced STAT5 phosphorylation [13] or T cell proliferation *in vitro* [9], which is unlike the case of IL-2 superkines.

Based on the recent crystal structure of the IL-2/JES6-1 complex, steric effects at the interfaces of IL-2 with CD122/γ_c and with CD25, as well as allosteric effects affecting IL-2's CD25-binding site, have been put forward [13]. The data demonstrated that hydrogen bond contacts existed between mIL-2 residues Q36 and E37 (corresponding to hIL-2 residues Q22 and M23; Figure 1) and JES6-1 [13], thereby causing steric competition between JES6-1 and CD122 and γ_c, respectively. While these aspects of the structure confirm the previously postulated model, the proposed steric competition between JES6-1 and CD25 and allosteric changes in IL-2's CD25 epitope are both unexpected and somewhat counterintuitive, considering that IL-2/JES6-1 complexes preferentially stimulate $CD25^+$ cells. Thus, the authors reported that JES6-1 distorted the entire CD25-binding AB loop of mIL-2 by contacting mIL-2 residues K49 and R52, which in hIL-2 correspond to residues K35 and R38, which are central to CD25 interaction. This latter finding makes it difficult to understand how IL-2/JES6-1 complexes are still able to bind to and stimulate $CD25^+$ cells, especially considering the low-affinity interaction of IL-2 with CD25 compared with the estimated 10–100-fold higher affinity of JES6-1 to IL-2. However, the authors offered a model whereby, in cells with very high CD25 expression levels, CD25 displaces JES6-1 from IL-2 by mass action, thus liberating IL-2 to engage CD122/γ_c. This effect is further enhanced by a positive feedback loop involving IL-2 signaling-mediated transcriptional upregulation of CD25 on responding cells [6,13,73] (cf. also Figure 2).

Collectively, the recent structural data confirm the steric aspects of the proposed model and further suggest allosteric effects that, especially in IL-2/S4B6 complexes, appear to fine-tune the mechanistic steps of action of IL-2/mAb complexes, which allows the formulation of a framework for achieving selective IL-2 immunotherapy (discussed in more detail in the Concluding Remarks). In addition to IL-2/mAb complexes, studies on selective IL-2 muteins lend support for such a model, as outlined below.

IL-2 Muteins

An alternative approach to selective IL-2-mediated stimulation is the generation of IL-2 muteins. Initially, research in this area was driven by the search for IL-2 muteins with reduced toxic adverse effects. At that time, IL-2-induced toxic effects were thought to rely primarily on activated NK cells secreting proinflammatory cytokines, such as tumor necrosis factor-α, and vasoactive mediators, which in turn led to endothelial damage and VLS [69]. To this end, IL-2 muteins were generated with decreased binding affinity to CD122, such as BAY 50-4798 (containing an N88R mutation of IL-2; see also Figure 1) and Selectikine (harboring a D20T mutation of IL-2) [74,75]. However, when tested in clinical trials such IL-2 muteins did not show decreased toxicity in patients [10,76]. More recent data showed that NK cells are implicated in VLS; however, the more proximal events of endothelial cell damage and VLS include the direct binding of IL-2 to $CD25^+$ pulmonary endothelial cells [31]. Thus, use of IL-2/S4B6 complexes or of the IL-2 muteins R38E, R38G, R38W, and F42A (all predicted or shown

to impair binding to CD25) disfavors contact with endothelial cells and significantly reduces VLS [10,31,77].

Capitalizing on these findings, more recent efforts aimed at generating IL-2 muteins with decreased affinity to CD25 (such as 'no-∝ mutein' [78], GA501, and GA504 [79]). An alternative approach was taken based on the structural data of hIL-2 bound to CD25 [72] and the human quaternary IL-2–IL-2R complex [8], which suggested that binding to CD25 led to the

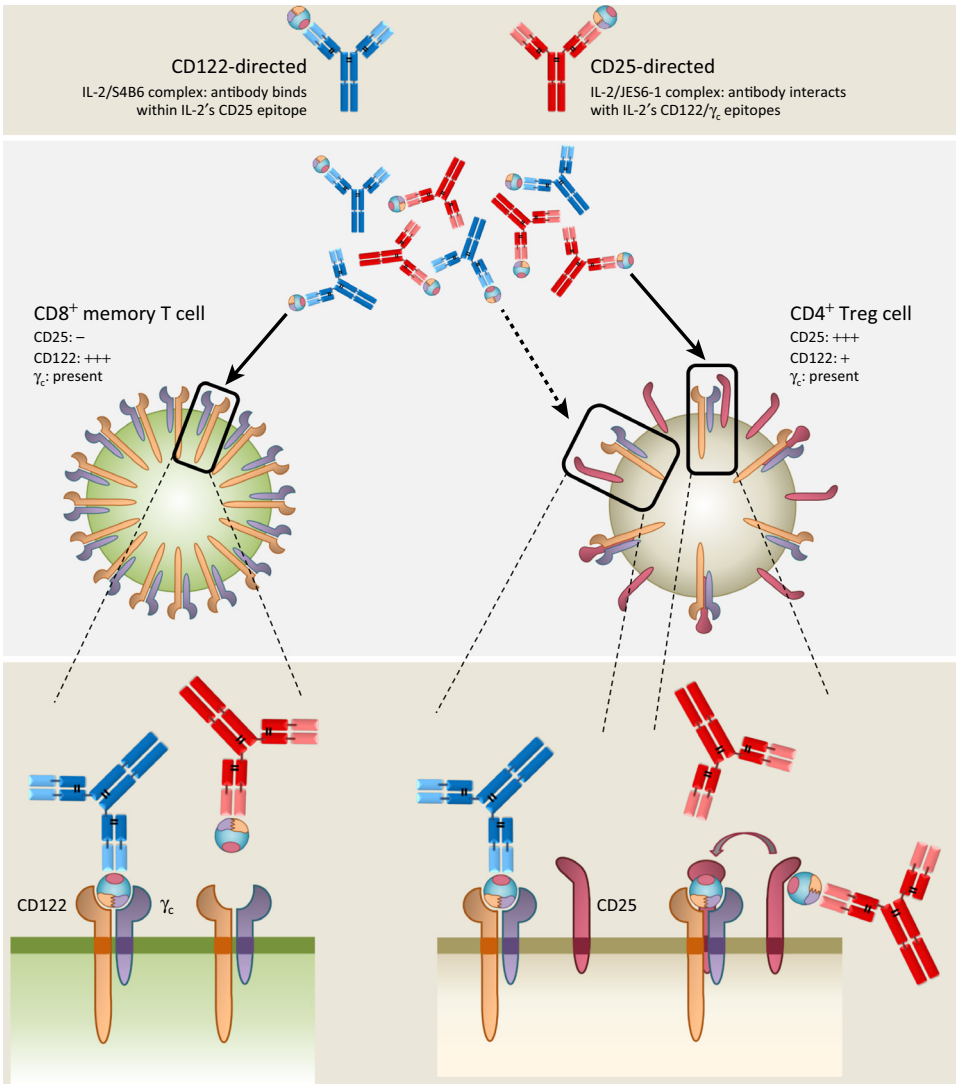

Figure 4. Framework for IL-2-Based Selective Immune Modulation, as Exemplified by IL-2/mAb Complexes. Selective immune modulation – that is, immune stimulation versus immune suppression – can be achieved using IL-2 that has been modified to (temporarily) impede IL-2's binding to certain IL-2R subunits. For example, in IL-2/S4B6 complexes (blue) the anti-IL-2 antibody binds to IL-2's CD25-binding epitope, thereby rendering IL-2 'blind' to CD25. For this and other reasons (see text), IL-2/S4B6 complexes preferentially stimulate cells expressing high levels of CD122 (in addition to γ_c, which in this model is assumed to be not limiting), including memory $CD8^+$ T cells (green) and natural killer cells (not shown). $CD4^+$ regulatory T (Treg) cells (brown) express only intermediate levels of CD122, which is one of the reasons (see text) why IL-2/S4B6 complexes are less efficient in activating Treg cells. Conversely, in IL-2/JES6-1 complexes (red), the anti-IL-2 antibody interacts with IL-2's CD122 and γ_c epitopes. As a consequence IL-2/JES6-1 complexes crucially depend on the presence of high levels of CD25, such as in $CD25^+$ Treg cells.

aforementioned structural change of helix C of IL-2 that improved its binding to CD122. Based on this hypothesis, the agonistic IL-2 muteins H9 and D10 (also known as IL-2 superkines) were generated [51]. These IL-2 muteins associated with CD122 with 200-fold increased affinity and efficiently bound dimeric IL-2Rs without the need for CD25; such binding resulted in increased STAT5 phosphorylation and cell proliferation *in vitro* and *in vivo* [51]. Use of the H9 IL-2 mutein showed increased $CD8^+$ T cell-mediated antitumor activity against solid murine tumors [51] and enhanced NK cell-mediated cytotoxicity against MHC class I-deficient RMA-S tumor cells *in vivo* [80].

Leveraging on the properties of H9, an H9 mutant that retained CD122 binding but demonstrated significantly decreased affinity to γ_c was engineered and characterized recently [14]. This was achieved by introducing four mutations into H9 (L18R, Q22E, Q126T, and S130R) and the resultant mutein was called H9-RETR. These newly introduced mutations of H9-RETR affect IL-2 residues involved in binding to γ_c (Figure 1). Interestingly, L18 and Q22 are interspaced by two residues, L19 and D20, that participate in CD122 binding, as shown by structural data [8] or the D20T IL-2 mutein Selectikine displaying reduced CD122 binding [75]; however, an H9-RE mutein containing only L18R and Q22E mutations did not show decreased signaling activity compared with H9 [14]. When used *in vivo*, an injection of H9-RETR–Fc4 (i.e., H9-RETR fused to human IgG4 Fc domain) was able to block IL-2- or IL-15-induced STAT5 phosphorylation of murine $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells. Moreover, a 10-day course of H9-RETR–Fc4 prevented acute graft-versus-host disease in a full MHC-mismatch C57BL/6 to BALB/c situation, presumably by blocking the IL-2R-mediated stimulation of activated effector T cells. *In vitro*, H9-RETR was of comparable efficacy to daclizumab, an antihuman CD25 mAb, in inhibiting the proliferation of adult T cell leukemia cells.

Hence, engineered IL-2 muteins provide interesting tools in being able to either enhance or dampen IL-2-mediated responses and might be useful for IL-2-directed immune modulation.

Concluding Remarks

Taken together, the findings on IL-2/mAb complexes and IL-2 muteins suggest a mechanistic framework for IL-2-based selective immunotherapy, as exemplified by the two functionally different IL-2/mAb complexes (Figure 4). This concept hinges on the use of qualitatively different IL-2 variants, namely CD122-biased versus CD25-directed IL-2 formulations, rather than different IL-2 doses, such as high- versus low-dose IL-2 immunotherapy. IL-2/mAb complexes have served as a paradigm for IL-2-based selective immune modulation. But how accurate is our mechanistic understanding of IL-2/mAb complexes?

A mechanistic model of IL-2/mAb complexes, including the new elements arising from the recent biophysical and structural data, needs to consider the following aspects [6,9,13]: (i) IL-2/mAb complexes comprise *in vitro* 'neutralizing' anti-IL-2 mAbs; (ii) S4B6 and JES6-1 occupy different IL-2 sites; (iii) IL-2/S4B6 complexes are CD122 dependent; (iv) IL-2/JES6-1 complexes strictly depend on CD25; (v) S4B6 partly covers the CD25 epitope; (vi) JES6-1 occupies CD122/γ_c-binding sites; (vii) S4B6 shows a mild clash with CD122; (viii) JES6-1 occupies CD122/γ_c-binding sites; (ix) S4B6 induces allosteric changes in the CD122 epitope; and (x) JES6-1 allosterically changes the CD25 epitope of IL-2. In integrating these aspects (Figure 4), it can be proposed that association of IL-2 with S4B6 (or S4B6-like mAbs) sterically hinders the binding of CD25 to IL-2 by covering (part of) the CD25-binding site of IL-2 [thus explaining abovementioned points (i) and (v)], which renders IL-2 'blind' to CD25 but dependent on CD122 [point (iii)]. Of note, maybe partial – rather than full – obstruction of the CD25 epitope by S4B6-like mAbs is important for their mechanism of action? Conversely, binding of IL-2 to JES6-1 (or JES6-1-like mAbs) occludes IL-2 residues interacting with CD122 and/or γ_c [explaining points (ii) and (vi)]; this renders IL-2 dependent on CD25 [point (iv)] and inaccessible to CD122 and/or γ_c until the IL-2/

JES6-1 complex binds to CD25 thus releasing IL-2 from JES6-1 and making it now amenable to CD122 and γ_c.

The proposed allosteric effects in both complexes and the suggested clash between S4B6 and CD122 [13], however, suggest antagonistic forces at work. Moreover, the apparently discordant effects seen in IL-2/S4B6 complexes with increased CD122-binding affinity but decreased STAT5 phosphorylation and reduced T cell proliferation *in vitro* would suggest further complexity of the system. These aspects require future studies of IL-2 versus IL-2/mAb complexes focusing on the binding kinetics of IL-2 to its receptor subunits, internalization and degradation versus recycling of the IL-2–IL-2R complex, and qualitative and quantitative (i.e., signal duration) assessment of signaling events. Physiologically, binding of IL-2 to the IL-2R and internalization of this quaternary complex leads to the degradation of IL-2, CD122, and γ_c, while CD25 is recycled [21]. The question arises of whether, in IL-2/mAb complexes, the mAb detaches from IL-2, whether the mAb is internalized or not, and, if the mAb is internalized, whether it is recycled to the cell surface or degraded intracellularly. The former point on dissociation and internalization of the mAb has also practical consequences on whether IL-2/mAb complexes can be devised as single-molecule fusion proteins. Notably, for clinical application, there is some interest in generating a fusion protein of IL-2 complex by using a flexible linker between IL-2 and the mAb, although others do not see a problem with using IL-2/mAb complexes that can dissociate *in vivo* (Box 1) [10]. Currently, there is only one report describing a fusion protein of the IL-2/S4B6 complex; however, it remains to be tested to what extent this fusion protein still allows association of mIL-2 with S4B6's antigen-binding groove [81]. Notably, it is worth considering the use of S4B6-like mAbs to modulate endogenous IL-2 for selective immune stimulation, as shown in mice [9]; whether similar effects can be achieved with JES6-1-like mAbs remains to be explored.

Similar questions apply to CD25- versus CD122-biased IL-2 muteins, which do or do not require contact with CD25 or CD122/γ_c. Significantly, a particular issue relevant to IL-2 muteins might be immunogenicity and the generation of antidrug antibodies (see Box 1 for a detailed discussion).

Box 1. Limitations and Immunogenicity of IL-2 Formulations

Immunotherapy using IL-2 suffers from certain shortcomings [6,69]. First, IL-2 exerts both immunosuppressive and immunostimulatory actions by activating Treg cells and cytotoxic effector lymphocytes, including $CD8^+$ T cells and NK cells. Both IL-2/mAb complexes and IL-2 muteins overcome this issue by interfering with interaction of IL-2R subunits with their respective IL-2 epitopes. Second, high doses of IL-2 can lead to VLS via direct binding of IL-2 to and damage of $CD25^+$ endothelial cells and indirect damage of endothelial cells by NK cell-derived proinflammatory cytokines and vasoactive mediators. This shortcoming is addressed by the lower doses needed of IL-2/mAb complexes and some IL-2 muteins to achieve the same effects as 'wild-type' IL-2 and, in CD122-biased formulations, by avoiding contact with $CD25^+$ pulmonary endothelial cells. Third, IL-2 is short lived *in vivo* due to its rapid renal clearance, thus necessitating frequent administration. Hence, formulations with longer $T_{½}$ should be generated by coupling, or complexing, IL-2 to a mAb (or mAb fragment) specific for IL-2 or a molecule (such as a tumor antigen) enriched in the target tissue [83].

There also exist limitations that are specific to IL-2/mAb complexes or IL-2 muteins. In IL-2/mAb complexes, the administration of IL-2 plus an anti-IL-2 mAb could be seen as potentially problematic for therapeutic application as the two components, although complexed before injection, might dissociate in the patient. This should consider the following. Even if all IL-2/mAb complexes were to dissociate following injection, the amount of IL-2 would reach only 2–5% of the IL-2 dose usually administered to patients; moreover, the anti-IL-2 mAb could then bind to endogenous IL-2 and direct it to the targeted immune cells. Potentially, IL-2/mAb complexes could be linked by a flexible linker [10,81]. With regard to IL-2 muteins, immunogenicity should be considered. Drug immunogenicity is a well-known problem of biological agents [84], including IL-2. Both subcutaneous and intravenous administration of recombinant hIL-2 has been reported to cause anti-IL-2 antibodies in about 50% of patients; however, neutralizing anti-IL-2 antibodies were seen typically only in 5–10% of subjects treated [85–87]. However, the N88R IL-2 mutein BAY 50-4798 had already caused antidrug antibodies in 27% of treated patients after two cycles of intravenous infusion [76]. Hence, it is worth considering strategies to reduce the immunogenicity of IL-2 muteins; for example, by avoiding the formation of aggregates due to hydrophobicity and lack of glycosylation [85,87].

When aiming for pure CD25- versus CD122-directed IL-2 formulations, one should consider possible antagonistic interactions such as those suggested by the structural data on IL-2/mAb complexes. Notably, the structural data suggest that S4B6 is probably a suboptimal template for an ideal agonistic IL-2/mAb complex, suggesting that S4B6 partly overlaps with CD25's footprint on IL-2 and interferes mildly with CD122 binding. These features would both be unwanted when considering the design of a CD122-directing, high-affinity mimic of CD25 aimed at exerting maximal stimulation of $CD8^+$ T cells while simultaneously blocking IL-2 interaction with $CD25^+$ cells. There, one of the most important challenges will be the setup of a screening system that allows the selection of appropriate anti-IL-2 mAbs (see Outstanding Questions). Furthermore, future structural studies on IL-2/mAb complexes should ideally use S4B6-like hIL-2/mAb complexes tested on human cells and compared with the quaternary IL-2–IL-2R complex of the same species.

In conclusion, the study of selective IL-2 formulations has been instrumental in understanding some of the biology of IL-2 and has allowed us dissect the two apparently antagonistic effects of IL-2. This latter finding is perhaps the most important consequence of this research and has led to a conceptual framework for IL-2-based selective immune modulation.

Outstanding Questions

The conceptual framework of IL-2-based selective immune modulation relies on biased IL-2 formulations. Do similar concepts exist for other cytokines, particularly γ_c cytokines?

Can the necessity of *in vivo* testing for appropriate IL-2 complexes (and IL-2 muteins) be circumvented by devising a rational setup of a screening system that allows the selection of appropriate anti-IL-2 mAbs (and muteins)?

In immunotherapy, do we need to target IL-2 to a certain site and, if so, is it the secondary lymphoid organs, the target tissue, or both?

Is binding of IL-2 to its receptor subunits different in IL-2, IL-2 complexes, and IL-2 muteins?

What are the effects on internalization and degradation versus recycling of the IL-2–IL-2R complex on binding of IL-2, IL-2 complexes, or IL-2 muteins?

Do IL-2, IL-2 complexes, and IL-2 muteins lead to different qualitative and quantitative signaling events?

Can an IL-2 complex still exert its selective effects when it is covalently coupled to a mAb specific for IL-2 or another antigen? Can such a molecule still efficiently accumulate, for example at the tumor site, despite its larger size compared with IL-2?

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