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Challenges and developing solutions for increasing the benefits of IL-2 treatment in tumor therapy

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Summary

Interleukin-2 (IL-2) is a cytokine with pleiotropic effects on the immune system. Systemic IL-2 treatment has produced durable responses in melanoma and renal cancer patients, but unfortunately this is effective only in a fraction of patients. Moreover, IL-2 treatment also engenders serious side effects, which limit its clinical utility. It is now appreciated that IL-2 not only stimulates NK and effector T cells but also has a critical role in the generation and maintenance of regulatory T cells, which act to dampen immune responses. Thus, successful immunotherapy of cancers using IL-2 has to address two fundamentally important issues: (1) how to limit side effects yet be active where it is needed, and (2) how to preferentially activate effector T cells while limiting the stimulation of Tregs. Strategies are now being developed to address these critical obstacles that may lead to a renaissance of IL-2 therapy.

Keywords

IL-2; IL-2 receptor; tumor immunotherapy; tumor microenvironment1

Perspective

IL-2 is a cytokine with potent and pleiotropic effects on the immune system. Exploiting these effects has been a longstanding goal of tumor immunologists. IL-2, due to its role as a growth factor and critical signaling molecule of immune cells, has long served as a paradigm for cytokine research [1]. There are several excellent reviews on IL-2 which examine the molecular biology of its expression, its role in immune cell signaling and immune development, as well as the structural biology of cytokines and their receptors [2-4]. In the current review, we focus on the key biological features of IL-2, how these features affect its use as a therapeutic reagent, and current directions to improve its effectiveness in tumor immunotherapy.

Key features of the biology of IL-2

IL-2 was one of the first cytokines discovered and characterized and, based on its ability to stimulate the growth of T cells, was termed T cell growth factor (TCGF) [5]. Subsequently, it was shown that IL-2 can also support the growth and expansion of NK cells as well as other immune cells such as B cells at varying stages of development or activation [3].

Perhaps surprisingly, it also plays a role in activation induced cell death of effector T cells [6]. IL-2 plays a multifaceted and complex role in the generation, expansion, and maintenance of CD8 effector cells. IL-2, along with other cytokines, is required for optimal induction and differentiation of CD8 effector cells and may help determine the type of memory cells (central vs. effector) generated [2,7-11]. Interestingly, in some situations IL-2 can convert naive CD8 cells into memory-like cells with minimal TCR signaling [12-14].

Like many cytokines, IL-2 has evolved to act locally over short distances in an autocrine or paracrine fashion. Reflecting this property, IL-2 is found at very low levels in normal serum and exogenously introduced IL-2 has a very short serum half-life [15-21]. Paradoxically, given its role in T cell proliferation, IL-2 gene deletion or “knock-out” experiments in mice, resulted in progressive lymphoid hyperplasia and the development of autoimmune diseases. This phenotype is now thought to be due to impaired development of regulatory T cells, which require IL-2, and are essential for maintaining the proper homeostatic balance of the immune system [22-26].

Different cells express different combinations and amounts of IL-2 receptor chains which dictate much of how IL-2 functions *in vivo*

The expression of the IL-2 receptor on immune cells is highly regulated. The IL-2 receptor is a complex of several chains (α , β , and γ) in combinations that can vary depending upon the cell type and activation state. The $\alpha\beta\gamma$ trimeric complex forms the highest affinity receptor (Kd approximately 10pM). The α chain (CD25) is unique to IL-2 whereas the β chain (CD122) is shared with the IL-15 receptor. The γ chain (CD132) can be partnered with several other cytokine receptor chains and is critically involved in signaling [3,4]. The $\beta\gamma$ combination binds IL-2 with intermediate affinity (Kd approximately 1nM). The isolated α chain can bind IL-2 with relatively low affinity (Kd approximately 10nM). Importantly in terms of function, the composition of the receptors, the number, and likely signaling capacity can vary with the cell type and activation stage. The level of IL-2 receptors on resting naive T cells is relatively low. However, activated CD4 and CD8 T cells begin to express high levels of the α chain, which allows them to bind IL-2 efficiently. The α chain is expressed at higher amounts (8-10 fold) compared to the $\beta\gamma$ chains. It is believed that the α chain functions to bind IL-2 initially, localizing it to the cell surface, effectively increasing its concentration and also inducing a conformational change in IL-2 which then subsequently binds to the $\beta\gamma$ chains on the cell surface [3,4,27]. NK cells and memory phenotype CD8 cells express high levels of $\beta\gamma$ compared to naive cells and some NK cells can also express α chain after stimulation with IL-2. Perhaps most interestingly is that CD4 Tregs, which act to dampen the immune response, constitutively express high levels of α chain [23,25]. Tregs, which are now known to be a diverse set of immune cells, can act in multiple ways to down regulate many immune responses including anti-tumor responses [24,25,28-30]. However, one of the most relevant mechanisms in the cytokine context is that they can bind IL-2, potentially depriving activated T cells of IL-2, thus dampening their proliferation and maintenance [2,3,30-32]. Consistent with this concept, the addition of IL-2 in many, although not all, *in vitro* assays of Treg function alleviates their suppressive activity [25,30].

How does the biology of IL-2 influence its use as a therapeutic reagent?

It has long been appreciated that the immune system has the potential to destroy neoplastic cells if harnessed correctly. Based on its ability to enhance the proliferation, activation, and differentiation of T cells and NK cells, IL-2 was one of the first cytokines tested in patients and successfully used to treat cancers. In a seminal study, high dose IL-2 was used to treat renal and melanoma cancer patients (Table 1) [33]. In this study, 7% of both melanoma and renal cancer patients achieved complete regression as defined as the disappearance of all measurable tumor and a similar fraction (10% of melanoma and 13% of renal cancer patients) had partial regressions as defined as 50% or greater decrease of all lesions lasting at least 1 month without any increase in tumor burden or generation of any new tumors (Table 1) [33]. How high dose IL-2 functions mechanistically *in vivo* is still not well understood, although it likely reflects not only the activation of immune cells including NK and T cells, but also due to effects at the tumor site [15,34]. IL-2 is now FDA approved for the treatment of renal cancer and melanoma patients. Perhaps most impressively, many of the complete remissions achieved with IL-2 seemed durable [35,36], with ongoing complete responses reported in one study from 39 to 148 months [35]. The durability of the response may reflect the generation of T cell memory and could be a general feature of cancer immunotherapy. In this light, other immunotherapies, such as antibody blocking of anti-CTLA-4 (e.g. Ipilimumab) and antibodies to PD-1/PD-L1 are showing clinically significant anti-tumor responses in recent trials [37-41]. These immunotherapy approaches may result in immunologic memory and thus also have potential for long-term durable responses. However, the durability of these responses, the overall survival benefits, as well as other important questions such as the possibility of re-treatment, needs to be addressed in larger clinical trials with extended follow-up which will no doubt be forthcoming.

Interestingly, recent studies showed that immunization with an altered peptide ligand derived from the melanoma associated gp100 protein combined with IL-2 showed better overall clinical response (16% vs. 6%), as well as longer progression-free survival than IL-2 treatment alone, consistent with the idea that IL-2 might enhance or maintain the tumor reactive T cells (Table 1). Unfortunately, this strategy again was only beneficial in a fraction of patients [42]. While these clinical studies highlight the promise of IL-2, there are also serious side effects of IL-2 treatment including vascular leak syndrome (VLS), which limits the widespread adoption and utility of IL-2 treatment. Unfortunately, as illustrated in a study of renal cancer patients, lower amounts of IL-2 used in an attempt to limit side effects were generally less effective clinically [43]. The short half-life of IL-2 in serum, reflecting tissue biodistribution, binding to receptors, and degradation in the kidney [18-20,44], requires that large amounts of IL-2 be injected to achieve therapeutic levels. The high dose of IL-2 results in a release of cytokines sometimes termed “a cytokine storm”, thought to be a result of the widespread stimulation of NK cells and other immune cells which in turn produce a variety of inflammatory cytokines [15]. These cytokines in turn have been suggested to mediate many of the unwanted side effects and were also thought to contribute to VLS. This cytokine storm is reminiscent of some features of septic shock although there are a number of key differences, and a critical one is that the deleterious effects of IL-2 appear to be reversible [34]. Interestingly, studies in mice have indicated that pulmonary endothelial cells

themselves can also express low levels of high affinity IL-2 receptors which was suggested to be a major contributor to the vascular leak syndrome observed [45].

Dual functional properties of IL-2 is a formidable challenge to therapy

From the standpoint of using IL-2 as a therapeutic for tumor treatment, one of the most complicating and vexing issues is that IL-2 can act on both Tregs as well as effector T cells. As a result, some studies have used IL-2 to enhance anti-tumor immune responses and other studies have used IL-2 to dampen autoimmune responses. As indicated earlier, Tregs constitutively express high levels of the IL-2 receptor α chain whereas conventional naive T cells do not. Some recent studies have used low dose IL-2 either alone, or in combination with other agents in an attempt to preferentially activate Tregs with the rationale that Tregs could be suppressive directly and might also effectively compete for the low available amount of IL-2 given their high affinity IL-2 receptor expression and thus dampen autoimmune responses [23,46]. While many studies have used IL-2 in conjunction with other immunotherapies, because they used IL-2 alone, some recent studies are particularly relevant (Table 1). Two reports of small groups of patients, one dealing with steroid resistant chronic GvH [47] and the other with hepatitis C virus HCV induced vasculitis [48], revealed that low dose delivery of IL-2 improved these clinical conditions, consistent with the idea that Tregs were being activated to dampen over-exuberant immune response (Table 1). In the case of chronic GvH disease patients, IL-2 treatment restored many aspects of regulatory T cell homeostasis supporting this concept [49]. Interestingly, in a study of 8 melanoma and renal cancer patients, high dose IL-2 treatment resulted in increased levels of Tregs which may be one of the factors that limit the efficacy of this treatment [50]. Future trials with larger numbers of patients are needed to better understand the paradoxical and complex role of IL-2 *in vivo*. Importantly for tumor immunotherapy, these results taken together revealed that IL-2 can either enhance or reduce immune responses by preferentially stimulating particular cell populations depending upon the dose of IL-2 used. Unfortunately, as highlighted recently, using IL-2 to alter the balance of effector/Tregs *in vivo* is complex, tenuous, and not easily predictable particularly when translating treatments from preclinical mouse models to patient populations [23,51,52]. The varying genetic composition inherent in the patient population, differences in the underlying disease, and importantly differences in the ongoing immune response such as the ratio of T effectors to Tregs may alter the response to IL-2 treatment. As is now crucial for many cancer therapies, an important area of research is the search for biomarkers, such as genetic polymorphisms or the expression of tissue antigens or serum proteins, that might be used to determine prospectively which patients would benefit from IL-2 treatment. While exciting and provocative, these results underscore both the promise and the pressing need to effectively direct the action of IL-2 to particular immune cell compartments.

Strategies to enhance effectiveness of IL-2

Initial approaches to improve effectiveness of IL-2 sought to increase its very short half-life *in vivo*. For example, one previous study showed that the serum half-life of IL-2 introduced i.v. is a bi-phasic event with phase I (biodistribution throughout the body) resulting in $t_{1/2}$ of approximately 7 minutes and phase II (extravasation from plasma into tissue) at

approximately 60 minutes [21] and other studies reported qualitatively similar values [18,20]. One strategy to enhance this short half-life employed genetic engineering to fuse IL-2 to human albumin. This fusion protein, termed Albuleukin, when delivered i.v., increased the serum half-life of IL-2 in mice to 7.75 hours compared to 19 minutes with IL-2 and also reduced tumor growth in the mouse Renca kidney tumor model as well as a mouse hepatic melanoma model [53]. A preliminary meeting report of a clinical trial carried out with Albuleukin by Human Genome Sciences also showed a increased half-life of approximately 14 to 15 hours [54] but Albuleukin was not taken forward after the acquisition of Human Genome Sciences by GlaxoSmithKline. While one can envision many practical advantages from enhancing the half-life of IL-2, with the benefit of hindsight, it is not clear how this approach by itself would reduce side effects or preferentially stimulate particular immune cell subsets. A conceptually related strategy that has often been used to improve the half-life of biologically active molecules, is to genetically fuse the molecule of interest to the Fc region of immunoglobulin. However, it is now clear that such fusions often do not simply increase half-life. Indeed, in the case of IL-2, the unique chimeric structural properties of the IL-2/Fc fusion were subsequently exploited to down-regulate immune responses through a variety of mechanisms including targeting activated effectors which express high levels of IL-2 receptors (reviewed in [55]). Such reagents are now being used in conjunction with other strategies to prolong graft survival [55-57]. Importantly, these studies reveal the complexities that can arise from using functionally active IL-2, particularly in the context of chimeric molecules *in vivo*.

Many attempts were also made to minimize side effects of systemic IL-2 treatment by introducing IL-2 directly into the tumor microenvironment. Local delivery of cytokines is appealing compared to systemic delivery for a variety of reasons. It takes advantage of the natural biology of cytokines that have evolved to act locally in a paracrine or autocrine fashion. Local expression also dramatically minimizes many of the side effects of systemic delivery of cytokines. This has been achieved in the past in experimental settings using a variety of techniques including gene transfection, viral delivery, or the delivery of free or encapsulated cytokines [58-63]. For example, in one study, local expression of even barely detectable amounts of IL-2 could reduce tumor growth, increase CD8 and NK cells at the tumor site, and change the entire cytokine milieu [64]. Nevertheless, it is very challenging to translate this strategy of local expression of cytokines in tumors to treatments for patients. Most attempts to directly extend this approach are based on the rationale that treating one or a few accessible sites would engender a vigorous systemic immune response that would be able to eliminate even untreated tumor sites. This would be critical in the case of metastases where there are frequently numerous disseminated tumors that are often inaccessible. Unfortunately however, the general finding is that the treated tumor site is almost always more affected than the distal unmanipulated tumor sites. This indicates that it is not simply the generation of an immune response that is important, but that the alteration of the cytokine tumor microenvironment also plays an essential role in the efficacy of this approach. Indeed, there is a growing appreciation that the tumor microenvironment plays a critical role in the efficacy of anti-tumor immune responses [65].

New approaches for enhancing efficacy or reducing side effects of IL-2

There are a number of approaches that are currently being developed or are in clinical trials that attempt to either enhance the efficacy of IL-2 or reduce unwanted side effects. Given that successful cancer treatment often depends upon the treatment of metastases, systemic delivery of the cytokine has major conceptual benefits. However, at the same time, systemic delivery engenders many deleterious side effects so there is a critical need to develop a means to minimize these side effects, yet retain the ability to treat widespread metastases. For tumor immunotherapy, it will also be essential to develop strategies that can preferentially activate relevant immune subsets, such as T effectors and NK cells, while limiting the activation of regulatory T cells. There are now strategies with substantial promise that attempt to address these important hurdles to effective immunotherapy as we outline below.

Antibody-cytokine fusions

One approach aimed at increasing efficacy attempts to deliver IL-2 to tumor sites by genetically fusing cytokines to antibodies, or antibody components such as a single chain variable fragment (scFv), that bind tumor associated antigens (TAAs) (reviewed in [66-68]). In one such approach, IL-2 was fused to an antibody reactive with a TAA (ganglioside GD2, expressed by many melanomas and neuroblastomas) [69]. The IL-2 is conjugated to the antibody in such a way that it remains active, and can bind IL-2 receptors (Figure 1A). In this situation the antibody diffuses throughout the body but eventually accumulates at the tumor site due to the binding of the antibody to the GD2 antigen on the tumor. As a result, the local concentration of the cytokine is increased at the tumor site. Such treatments might also be expected to synergize with any direct therapeutic effect of the antibody mediated by the antibody itself or through interaction with innate effectors such as NK cells that express Fc receptors. Preclinical studies in mice as well as clinical trials in patients with neuroblastoma revealed that T cells and NK cells were important in the anti-tumor effects [70-73]. Interestingly, it appears that therapeutic efficacy of these complete antibody-IL-2 fusions, may not be simply due to enhanced delivery of cytokine to the tumor. Rather, the biological activity of the antibody-IL-2 fusions appears in part to be due to the increased killing of tumor cells as a result of increased conjugate formation due to binding of IL-2 receptors on immune cells [73,74]. Thus in essence, the IL-2 conjugated antibody forms a “bridge” between cytotoxic cells and the target tumor cells. Consistent with this mechanism is that direct anti-tumoral injection of the antibody is even more effective than systemic delivery [75,76]. It is important to note that there are numerous variations of the antibody conjugated cytokine approach. These variations include using antibodies targeting different TAA, varying the Fc region to improve half-life, incorporating altered forms of IL-2 or even other immunomodulatory cytokines [66,77]. However, it is noteworthy that in all studies in which IL-2 conjugated antibodies are used, the cytokine is of necessity always biologically active. This might result in unwanted systemic effects as the dose increases, could result in binding to effectors not at the tumor site potentially decreasing its effective concentration, and may also activate Tregs. The biological functions of these chimeric molecules is thus complex and it will be critical to evaluate which of the possible mechanisms of action and biological properties are most important *in vivo*. The results from well-designed clinical

studies that examine immunologic and genetic parameters have been, and will continue to be, critical in understanding the mechanisms of action and thereby improving the effectiveness of IL-2 antibody fusions [66,70,71,78,79].

Strategies to direct the immune response mediated by IL-2

An intriguing set of papers showed that anti-IL-2/IL-2 complexes had interesting and rather unexpected effects. Much earlier studies had indicated that complexes of antibodies and cytokines might extend their half-life and biological function of the cytokine [80,81]. However, it later became evident that not all anti-IL-2/IL-2 complexes behaved the same and thus it was not solely a function of half-life. For example, injection of certain monoclonal anti-IL-2/IL-2 complexes into mice (e.g. IL-2 and S4B6 antibody) resulted in the proliferation of CD8 memory phenotype cells whereas others (e.g. IL-2 and JES6-1 antibody) appeared to preferentially activate CD25⁺ Tregs compared to conventional T cells and NK cells [82,83]. This has been interpreted to mean that some monoclonal antibodies such as S4B6 bind to certain epitopes on IL-2 that block the interaction with particular components of the IL-2 receptor (for example the α chain) and therefore preferentially stimulate cells which express high levels of the $\beta\gamma$ receptor (memory phenotype CD8 and NK cells) (Figure 1B). Recent fine mapping mutational analysis of monoclonal antibody binding sites on IL-2 are consistent with this concept [84]. Further, studies indicate that these anti-IL-2/IL-2 complexes also had lower levels of side effects such as vascular leak syndrome [45]. Perhaps most significantly, studies in experimental mouse models of cancer revealed that the anti-IL-2/IL-2 strategy using the S4B6 anti-IL-2 antibody, either by itself or as part of a more complex treatment scheme, had beneficial anti-tumor effects [45,85-88]. While there are a several potential issues in translating this strategy directly to patients, particularly with respect to consistent formulation and stability of these antibody/IL-2 complexes, their development is a very important milestone. Collectively these studies are very significant since they strongly indicate that if one could preferentially stimulate particular immune cell subsets with IL-2, it would increase its anti-tumor effects.

Mutational strategies to enhance IL-2 efficacy

A conceptually related strategy employed a very different approach to stimulate particular immune cell subsets. A molecular biology approach was used to create IL-2 mutants, which had different binding properties for the IL-2 receptor components (Table 2). Initial mutational approaches to improve IL-2 efficacy for effector T cells focused on enhancing binding to the α chain of the IL-2 receptor [89-91]. This was based in part on the rationale that effector T cells express high levels of the α chain and thus would be preferentially stimulated. A related complementary strategy, created mutants that had impaired binding to the β chain, in an attempt to avoid stimulating $\beta\gamma$ -expressing cells such as NK cells but that would retain the ability to activate effector T cells, which express high levels of the α chain [92]. This was anticipated to be beneficial since NK cells were thought to mediate many of the negative side effects of systemic IL-2 treatment. Intriguingly, this was not as successful as envisioned [93]. Although not evident at the time, it is now appreciated that some of these IL-2 mutants initially designed to enhance effector T cells might actually down-regulate immune responses *in vivo* when delivered systemically due to the global stimulation of

Tregs that express the α chain component of the high affinity IL-2 receptor. Such a strategy might be employed to down-regulate T cells in autoimmune diseases such as Type I diabetes by preferentially activating Tregs [23,52,94].

A new and essentially opposite approach was to identify mutants that exhibit enhanced binding to the β chain or that show reduced binding to the α chain of the IL-2 receptor. A recent paper [27] identified IL-2 mutants using yeast display that exhibited enhanced binding to the β chain (Table 2 and Figure 1C). Interestingly these residues, while near the region of IL-2 involved in binding to the β chain, did not appear to be directly involved in contact with the β chain but rather influenced the overall conformation of IL-2 to improve binding to the β chain. Importantly, one of these mutants, which has been termed a “superkine” or “super-2”, reflecting its enhanced agonist properties, also showed improved anti-tumor activity and exhibited less VLS compared to wild-type IL-2, an important limiting side effect of systemic IL-2 treatment [27]. The higher affinity of the mutated IL-2 for the β chain of the IL-2 receptor may be particularly important in considering how the modified IL-2 might function *in vivo*. Elegant structural and signaling studies from this same group showed that IL-2 and IL-15 signaled very similarly through their shared $\beta\gamma$ chains and that many of the signaling differences previously observed might largely be explained by differences in the affinity of the cytokine for their respective receptors due to their unique α chains [95]. For example, at the optimal concentration of each of the cytokines, RNA seq analyses of splenic CD8⁺ T cells stimulated *in vitro* revealed that IL-2 and IL-15 induced a strikingly similar pattern of gene expression [95]. It is interesting to speculate that these findings may contribute to why high dose IL-2 is more effective *in vivo* than lower doses, since it might engender a qualitatively different biological response as the dose increases, one that is similar but perhaps not identical, to that obtained with IL-15. IL-15 is currently in clinical trials but super-2 has not yet been tested in patients. It will be important and informative to determine the similarities, as well as potential differences in the anti-tumor immune response *in vivo* resulting from treatment with varying doses of super-2 or IL-15. Others have pursued an alternative strategy in which they introduced 4 mutations into human IL-2 that limit interaction with the α chain of the IL-2 receptor with the rationale that this would limit the expansion of Tregs (Table 2 and Figure 1C). Intriguingly, *in vivo* studies with this mutated IL-2 resulted in reduced toxicity and it also was more effective than wild type IL-2 in two metastatic mouse models, the 3LL-D122 model and the B16 melanoma model [96]. Taken together, these mutational studies clearly show that it is possible to structurally alter IL-2 to accentuate or reduce particular biologic properties based on the binding propensities of different cell types due to differences of the IL-2 receptor expression. This represents a very significant breakthrough in the use of IL-2 for cancer therapy.

Protease activated cytokines

A new strategy being developed attempts to reduce the side effects of IL-2 associated with systemic delivery, yet retain the ability to treat tumors that have spread. This strategy employs a fusion protein (FP) in which IL-2 (or any cytokine) is joined covalently to a specific inhibitory binding component separated by a protease cleavage site (Figure 1D). Thus the local concentration of the specific binding inhibitor (in this case the α chain of the

IL-2 receptor or an scFv that binds IL-2), with respect to IL-2 is extraordinarily high since they are physically connected on the same molecule. This dramatically favors the inhibitor binding to IL-2. However, after cleavage by proteases over-expressed locally at the tumor site (such as Matrix metalloproteases (MMPs) or Prostate Specific Antigen (PSA) a protease expressed by Prostate Cancer cells), the cytokine can become available to interact with high affinity receptors on immune cells. In the case of IL-2, these receptors are 10 to 1000 fold more avid than the specific binding of the typical scFv or the isolated α chain. Further, once stimulated, immune cells can themselves produce additional cytokines, thereby changing the cytokine milieu within the tumor microenvironment. This change in the cytokine environment may in turn help enhance an anti-tumor immune response, similar to what has been observed in experimental animal studies with cytokine gene transfected tumor cell lines. This approach has been successfully tested *in vitro* using IL-2 with either the scFv or the α chain as the inhibitory component. In addition, initial experiments with this overall strategy showed that the FP could reduce tumor growth in a mouse colon cancer model *in vivo* [97]. Interestingly, there are at least two non-mutually exclusive ways the FP might function mechanistically. In the first model, the complex of inhibitory component and IL-2 might dissociate after cleavage of the protease site, and once dissociated, the IL-2 would then become available for interaction with cellular receptors. This would have the effect of delivering active IL-2 preferentially at or near the tumor microenvironment and thus minimize side effects. However, the studies with antibodies discussed above [83,98], suggest that the IL-2/inhibitor complex after cleavage, but before dissociation, would be functional. In this second model, analogous to the antibody/IL-2 complexes described above, this non-covalently associated complex might be able to bind preferentially to cells that express particular combinations of receptors since IL-2 binding is no longer sterically hindered by the connecting linker containing the cleavage sequence. This is significant since depending upon the inhibitory component employed, it then might preferentially stimulate effector cells such as NK cells and CD8 cells, or alternatively Tregs, thus achieving an additional level of specificity, which might increase its efficacy. Thus, this protease activated cytokine strategy has the potential to reach all tumor sites when delivered systemically while reducing toxicity, and may even increase the efficacy of immunotherapy by preferentially altering the tumor microenvironment and enhancing particular subsets of immune cells.

Expert commentary and 5-year perspectives

IL-2 is one of the first clinically significant forms of tumor immunotherapy. However, the contrasting effects of IL-2 on the immune system have confounded the application of IL-2 for tumor immunotherapy. Moreover, the negative side effects associated with systemic delivery of IL-2 as well as other cytokines, which have generally evolved to act locally, have severely limited the efficacy and utility of cytokines in a clinical setting. However, a greater appreciation and understanding of the dual and sometimes antithetical nature of IL-2 on immune responses, has revealed exciting new avenues for improved treatment of cancer. As described in this review, there are a number of new strategies being developed and many of these have the potential to be combined in creative ways to improve efficacy and also reduce side effects of IL-2 therapy. It is anticipated that in the next 5 years, strategies that limit

these problematic side effects and target different immune cell populations will be developed and tested not only in experimental models but also in initial clinical trials. Results from these trials will be important in guiding the development of subsequent modifications of IL-2 mediated strategies. These new approaches have the promise to be clinically significant even when used as single agents. Importantly, we predict that new strategies will be also be integrated with targeted molecular therapies to achieve synergistic and durable effects since these strategies target different vulnerabilities of the tumor. This may be particularly important in the context of the targeted therapies in which the responses can be dramatic but where resistance can eventually develop (e.g. inhibitors that target mutated BRAF).

A number of important issues translating advances of IL-2 therapy to patient care still remain, including determining the dose and timing of these new reagents, the immunogenicity of the novel molecules, and their effective combination not only with other treatments and also with each other. We believe it is very likely that effective immunotherapy will include not only one of the novel forms of IL-2 treatment as outlined in the review, but will be combined with other immunologic approaches, such as active vaccination, antibody treatments to block inhibitory pathways, or adoptive cell transfer of *ex vivo* expanded TAA reactive T cells or genetically modified T cells (such as ones modified to express chimeric antigen receptors). Given the plethora of exciting new approaches being developed, determining which immunologic combinations will be most effective, although challenging, should yield great clinical benefit. In this regard, there is a pressing need to develop new approaches to assess the activity of combined treatments in patients, since traditional means of assessing combinatorial strategies greatly expand the size of clinical trials. Nevertheless, the recent advances in understanding and manipulating IL-2 raise the very real possibility of a renaissance in the use of IL-2 for treatment of cancer. Indeed it may be possible to teach an “old” cytokine new tricks.

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Key Issues

IL-2 has pleiotropic effects on the immune system

IL-2, like many cytokines, has evolved to act locally in a paracrine or autocrine fashion

High dose systemic IL-2 can be extremely effective in a fraction of cancer patients

Different immune cell subsets express different combinations and amounts of the IL-2 receptors

Activated effector T cells and regulatory T cells (Tregs) both express the α chain component of the high affinity IL-2 receptor

IL-2 has dual opposing effects on immune responses by promoting NK cells and T effector cells but also enhancing Tregs

Establishing new strategies that preferentially target specific immune cell subsets is critical

Finding ways to deliver IL-2 systemically while avoiding unwanted side effects will be essential in developing effective tumor therapy

Figure 1. Strategies for enhancing the effectiveness or reducing the toxicity of IL-2

A. A schematic of an immunocytokine, which targets a tumor expressing a tumor associated antigen (TAA). In this illustration, IL-2 is genetically fused via a gly-ser linker to an anti-GD2 monoclonal antibody, which recognizes the GD2 ganglioside (black triangles) that is expressed by many melanomas and neuroblastomas. In this context the IL-2 is constitutively biologically active.

B. Illustration of non-covalent antibody complexes used to direct the immune response mediated by IL-2 to particular immune cell subsets. By using an antibody that binds to an epitope on IL-2 (indicated by the green portion of IL-2) in such a way that it blocks binding of IL-2 to the α chain, it is proposed that $\beta\gamma$ expressing cells such as memory phenotype CD8 and NK cells are preferentially activated.

C. Illustration of the location of mutations in wild-type IL-2, which result in either enhanced binding to IL-2R β chain (blue spheres) or reduced binding to the IL-2R α chain (green spheres) which result in preferential activation of anti-tumor effectors.

D. Schematic of protease activated IL-2 fusion protein in which IL-2 is genetically fused to a specific inhibitory binding component (either α chain of IL-2 receptor or an scFv) via a gly-ser linker and a protease cleavage site. In this configuration the cytokine is inactive but after cleavage by proteases over-expressed at the tumor site, IL-2 can become biologically available either by dissociation from its inhibitor or in the context of the cleaved complex as described in the text.

Table 1

Selected summary of clinical trials illustrating the contrasting roles of systemic IL-2 in enhancing or dampening immune responses

Trial	Results	References
High dose treatment with IL-2 for melanoma or renal cancer	<p>Treatment of 283 melanoma or renal patients with high dose bolus IL-2</p> <p>Melanoma-complete response in 7% of metastatic melanoma patients, 10% had partial regression</p> <p>Real cell cancer-complete regression in 10 patients (7%) with metastatic renal cell cancer, 20 patients (13%) had partial regression.</p> <p>Significant side effects in some patients due to Vascular Leak Syndrome (VLS)</p> <p>Some complete responses were durable</p>	<p>Rosenberg et al. [33]</p> <p>Rosenberg et al. [35]</p> <p>Atkins et al [36]</p>
Analysis of Tregs in cancer patients before and after high dose treatment with IL-2	High dose IL-2 treatment resulted in 6-fold increase in the frequency of CD4+CD25hi T cells in the peripheral blood in 8 patients with either melanoma or renal cancer compared with the pretreatment levels.	Ahmadzadeh and Rosenberg [50]
Effect of high dose IL-2 treatment plus or minus vaccination with the melanoma antigen gp100	<p>A multi-center phase 3 trial involving 185 patients with advanced melanoma</p> <p>The response rate was higher and progression free survival longer with gp100 vaccine and IL-2 than with IL-2 alone</p> <p>Toxic effects were consistent with those expected with interleukin-2 therapy</p>	Schwartzentruber et al. [42]
Low dose IL-2 treatment in patients with active chronic GVHD that was refractory to glucocorticoid therapy	<p>The numbers of CD4+ Treg cells were preferentially increased in all patients</p> <p>The Treg:Tconventional ratio increased</p> <p>Of the 23 patients, 12 had major responses involving multiple sites</p>	<p>Koreth et al. [47]</p> <p>Matsuoka et al. [49]</p>
Low dose IL-2 treatment of HCV-induced vasculitis	<p>In a small study of 10 patients IL-2 treatment lead to Treg recovery and concomitant clinical improvement in patients with HCV-induced vasculitis</p> <p>The treatment did not induce effector T-cell activation, vasculitis flare, or increased HCV viremia.</p>	Saadoun et al. [48]

Table 2

Selected human IL-2 mutants with altered binding to IL-2R subunits

Result of mutation	Method to isolate	Mutations made	References
Enhanced binding to α chain	Yeast display and <i>in vitro</i> evolution	Series of mutants, e.g. M6 (V69A, Q74P, I128T) M1 (V69A, Q74P) C1 (I128T)	Rao et al. [89]
Decreased binding to β chain	Site directed mutants	Series of mutants-best characterized is N88R Also called BAY 50-4798	Shanafelt et al. [92]
Enhanced binding to β chain	Yeast display and <i>in vitro</i> evolution	Series of mutants-consensus called superkine or super-2 L80F,R81D,L85V,I86V,I92F	Levin et al. [27]
Decreased binding to α chain	Site directed mutants	Site directed alanine substitutions R38A, F42A, Y45A, E62A	Carmenate et al. [96]