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Functions of γ C cytokines in immune homeostasis: current and potential clinical applications

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Abstract

Cytokines that signal through receptor complexes containing the common γ (γ C) chain receptor subunit are central regulators of lymphocyte homeostasis. In this review, we discuss the four major γ C cytokines that have proven activity in or potential for immunotherapy: IL-2, IL-7, IL-15 and IL-21. Their shared and unique activities on specific lymphocyte populations suggest therapeutic applications such as enhancing lymphocyte reconstitution, expanding tumor and pathogen-specific lymphocytes, and optimizing vaccines. Because the responsiveness of individual lymphocyte subsets varies under different situations such as lymphopenia and active immune responses, understanding the dynamics of γ C-containing receptor expression is important in deciding how to achieve the most desired effect. Current understanding of γ C cytokine biology suggests several clinical applications, including their direct administration or use in generation of lymphocytes for adoptive transfer, increasing their endogenous production, and potentiating their activity by complex formation with specific antibodies or their specific receptor- α subunits. Overall, γ C cytokines have great potential, through their targeted use alone or in combination, to be an integral part of clinical interventions with enhanced efficacy and decreased toxicity.

Keywords

common gamma chain; lymphocyte homeostasis; cytokine therapy; lymphopenia-induced proliferation; adoptive cell therapy; tumor-infiltrating lymphocytes; regulatory T cells; immune reconstitution; human immunodeficiency virus

Introduction

Cytokines are endogenous proteins that mediate communication between cells, particularly cells of the immune system. The γ C family of cytokines consists of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, all of which signal through specific receptor complexes sharing the common γ receptor chain (γ C). As a result of this shared receptor usage, γ C cytokines share similar functions while also each possessing unique features. Predominant functions relate to crucial regulation of lymphocyte development, homeostasis, and function, and therefore have the

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potential to enhance lymphocyte reconstitution, expand tumor and pathogen-specific lymphocytes, and optimize vaccines. The γ C cytokine members currently of highest interest to clinical use are IL-2, IL-7, IL-15 and more recently IL-21. Clinical effects of treatment with these cytokines only partially reflect their normal endogenous function or activities in *in vitro* assays (Table 1). This review will summarize the current understanding of the normal function of these four cytokines, the cytokine receptor expression, their clinical use thus far, and the potential and limitations for future therapeutic applications.

IL-2

Normal *in vivo* functions

IL-2 is the quintessential growth factor for T cells and theoretically would be ideal for expanding T cells due to its high specificity to T cells (Table 1). *In vivo*, CD4 helper T cells, and to a lesser extent CD8 T cells and DCs, are the source for IL-2, which is used mostly in an autocrine and paracrine manner as a growth factor for nearby T cells. IL-2 mediates its effects by binding to a heterotrimeric receptor complex composed of the IL-2R α , IL-2R β , and γ C subunits. Since IL-2 has a low affinity for the IL-2R β and γ C heterodimeric complex, most *in vivo* responses require the presence of all three subunits. IL-2R α (also designated CD25) lacks a cytoplasmic domain and does not contribute to IL-2 signaling; instead, its main function is to increase the affinity of the β/γ C complex for IL-2 by approximately 100-fold [1]. Thus, the IL-2 binding specificity is dictated mostly by the expression of the IL-2R α . IL-2R α is expressed at a minimal level by naïve T cells and becomes transiently upregulated upon T cell activation, allowing a selective, IL-2 induced expansion of activated effector T cells. Since sustained signaling through the IL-2 receptor complex can result in apoptosis, the transient nature of IL-2R α expression protects the T cells from this form of over-stimulation. Besides stimulating effector T cells, IL-2 also mediates the development and homeostasis of regulatory T cells (Tregs). Tregs constitutively express elevated levels of IL-2R α compared to conventional T cells [2] (Figure 1). Accordingly, in a pool of unstimulated T cells, the Tregs are the most sensitive to IL-2 stimulation. Among IL-2R α -negative cells, IL-2 preferentially stimulates cells with the highest IL-2R β expression, *i.e.* previously activated T cells or T cells with a memory phenotype (Figure 1).

IL-2 use in clinical setting

HIV—Because of its proliferative effect on T cells, recombinant IL-2 has been tested in a number of clinical trials to increase CD4 T cell counts in HIV-induced CD4 T cell lymphopenia [3-6] (Table 2). Intermittent subcutaneous IL-2 treatment for 5 day cycles increased CD4 T cell counts in HIV patients, irrespective of concurrent highly active antiretroviral (HAART) therapy [4]. Importantly, IL-2 therapy appeared to confer clinical benefit by lowering opportunistic infections and extending patient survival[3,4]. Mechanistically, IL-2 in this setting increased both naïve and memory CD4 T cells, mostly through profound inhibition of their apoptosis [5]. In addition, IL-2 increased numbers of T cells with a classical Treg phenotype (CD4+CD45RO-CD25+foxP3+); however, these cells were not strongly suppressive in direct *ex vivo* assays, leaving their function *in vivo* an open question [6]. Currently, the clinical benefit of intermittent IL-2 for HIV patients with low CD4 T cell counts is being rigorously tested in phase III clinical trials.

Melanoma and Renal cancer—Early after its discovery as a T cell growth factor, IL-2 was hypothesized to boost the immune system's natural abilities to eliminate tumors. After promising results in animal models followed by clinical trials in a variety of cancers, IL-2 was approved for the treatment of metastatic melanoma and renal cancer (Table 2). High-dose IL-2 therapy results in a modest clinical response rate of 14% [7]. IL-2 therapy is of continued interests in part because about one-third of the clinical responses are complete, durable

remissions. The reason for the selectivity of this response among patients is not known and emphasizes a need to better understand the mechanism underlying IL-2-induced tumor regression. Animal studies and some observations in patients point to a predominant role for CD8 T cells and possibly NK cells in IL-2-mediated tumor regression.

Despite its modest success, there are number of drawbacks in the use of IL-2 therapy, the most notable being its high toxicity. In particular, high dose IL-2 induces a vascular leak syndrome resulting in multiple organ edema, blood flow perturbations, and hypotension [8]. Unfortunately, less toxic lower-dose regimens resulted in lower response rates [9]. The mechanism for the vascular symptoms is not completely clear and may involve direct effects on endothelial cells [10-12]. In contrast, mouse studies have implicated IL-2-mediated effects on NK cells as a contributing factor [13]. Interestingly, while effects on T cells are believed to be important for IL-2 responses, T cells become less abundant in the peripheral circulation of patients receiving IL-2. This temporary T cell loss is thought to occur because IL-2-responsive cells leave the circulation and/or alternatively undergo activation-induced cell death [11,12, 14]. IL-2 tends to drive CD8 T cells to a terminally differentiated effector stage, in which they possess a high capacity for cytotoxicity and effector cytokine secretion and preference for peripheral, (non-lymphoid) tissues, but also a greatly reduced capacity to proliferate and a high propensity towards apoptosis [15]. This is another drawback of IL-2 treatment, as these T cell characteristics are not ideal for long-term tumor control and protection against recurrence. Lastly, as in mice, IL-2 treatment promotes the accumulation of Tregs in humans [16,17].

While IL-2 treatment enhances the anti-tumor effects of endogenous CD8 T cells and NK cells, this ability of IL-2 has also been utilized to expand tumor-specific T cells induced by vaccination (Table 2). Indeed, since cancer vaccine-induced T cell responses in humans tend to be weak, members of the γ C cytokine family have been investigated for their ability to enhance the generation of Ag-specific effector and memory cells in mouse models with some encouraging results [18]. Currently, IL-2 and IL-7 are the only γ C cytokines yet tested in humans for this purpose. The findings on IL-2 were reported a decade ago in a study demonstrating that the addition of peptide-based vaccination against the melanoma antigen, gp100, doubled the clinical response rate to IL-2 therapy [19]. This finding was recently substantiated by a large retrospective study showing an increased response rate from 13 to 22% by the addition of gp100 vaccination to high-dose IL-2 therapy while gp100 vaccination by itself was essentially ineffective [20]. Interestingly, although that IL-2 treatment specifically lowered the number of vaccine-induced CD8 T cells in the circulation, it still synergized with peptide vaccination to induce tumor regression [19,21]. Overall, the modest success of IL-2 used in combination with vaccination has led to further studies. As such, a large randomized, prospective multi-center trial is almost completed to determine the exact clinical benefit of gp100 peptide vaccination as an addition to IL-2 therapy in metastatic melanoma.

Ex vivo uses

Adoptive cell therapy (ACT)—In addition to their *in vivo* use, cytokines can be used *ex vivo* to expand or differentiate specific immune cells. *Ex vivo* expansion can circumvent complex immunoregulatory mechanisms present *in vivo* and allows more precise control over the specific functions of the cells prepared for infusion. For this procedure, tumor-infiltrating lymphocytes (TIL) are isolated from a surgically removed melanoma lesion, grown short-term in the presence of IL-2, and then subjected to a rapid expansion with anti-CD3 antibody to mimic antigenic stimulation through the TCR, PBMC as feeder cells, and IL-2. This typically results in a 1000-fold expansion of the initial culture, yielding 10-100 billion cells. Meanwhile, the patient is conditioned with a short-term, non-myeloablative fludarabine/cyclophosphamide-based regimen to temporarily deplete endogenous lymphocytes. The cultured TIL are then infused and patients receive high-dose IL-2. In a cohort of 35 patients,

this regimen resulted in a response rate of 51% [22] (Table 2). Considering that the only two currently FDA-approved treatments for metastatic melanoma, high dose IL-2 and the cytotoxic agent dacarbazine, both have a response rate of approximately 15%, the results with ACT have generated a strong interest in further improvement of this regimen.

A more recent study showed that the addition of whole-body irradiation to further reduce endogenous lymphocytes further increased response rates to 72% [23]. Lymphodepletion presumably enhances the response by removing cell that compete for γ C cytokines that mediate homeostasis. The resultant increased level of γ C cytokines actually induces a form of T cell proliferation called lymphopenia-induced proliferation (LIP), which is discussed in more detail below. Lymphodepletion, besides removing competition for cytokines, possibly functions by depleting Tregs that hinder the anti-tumor function of the transferred TIL, especially in a setting of concurrent IL-2 administration which promotes Treg proliferation. Due to this strong capacity to preferentially expand Tregs, IL-2 may not be the most favorable γ C cytokine for the support of transferred T cells.

As mentioned above, another potential downside of IL-2 is its tendency to favor the generation of T cells with an effector memory phenotype. Although effector memory T cells have strong cytolytic effector function and tend to preferentially locate to non-lymphoid tissues including tumors, their more limited capacity for long-term persistence may prevent them from completely eliminating the tumor and providing long term immune surveillance against recurrence. This notion is supported by the observed correlation between persistence of transferred T cells and favorable clinical outcome [24]. Therefore, therapy generating a mixture of effectors with strong immediate anti-tumor activity along with more long-lived memory cells for continued tumor surveillance may be ideal, but it is currently unclear how best to generate such a mixture. It is clear, however, that various γ C cytokines differentially induce and expand these different T cell subsets, offering an avenue for their specific therapeutic use in the generation of tailored T cell populations for adoptive transfer (Figure 1).

The *ex vivo* expansion of T cells for adoptive therapy is not limited to the use of tumor-specific effector T cells. Adoptive immunotherapy is also being employed to control infections, restore immunity, and treat autoimmunity. *Ex vivo* expanded, donor-derived CMV and EBV-specific CD8 T cells have successfully been used to help control established infections in patients receiving stem cell transplants during a time when patients are most susceptible to infections [25,26]; this strategy is also being attempted for HIV [27]. In attempts to restore CD4 T cell numbers in HIV, one study expanded patients' isolated CD4 T cells with IL-2 in the presence of antiviral agents followed by retroviral transduction with a gene that confers HIV resistance giving the CD4 T cells a selective growth advantage[28]. A year after adoptive transfer of these modified cells, CD4 T cell numbers were increased, correlating with the presence of gene-modified T cells. In many types of autoimmune conditions, the presence of functional Tregs inversely correlates with severity of disease; hence there is a great interest in increasing Treg numbers *in vivo* [29,30]. As with tumor-specific T cells, culture conditions for the optimal *ex vivo* expansion of functional, antigen-specific Tregs are incompletely defined. Currently, the attempts to expand human Tregs *ex vivo* have utilized IL-2 [31]. Determining whether these *ex vivo* generated Tregs have clinical efficacy in inhibiting autoimmunity and GVHD is ongoing.

IL-7

Normal functions *in vivo*

Under normal circumstances, IL-7 is primarily produced by stromal cells of the thymus and BM and mediates the development and homeostasis of naïve and memory T cells. As IL-7 is involved in the survival of very early thymocytes, its absence results in a profound T cell

deficiency [32,33]. In the periphery, IL-7 promotes the survival of both naïve and memory T cells [34,35]. Therefore, IL-7 may be utilized to boost *de novo* T cell production during T cell deficiency caused by immune system disorders, diseases such as HIV infection, or clinical interventions such as chemotherapy (Table 1).

Under baseline conditions, the level of constitutively produced IL-7 is appropriate to maintain a normal pool of T cells; however, when T cell numbers decrease because of disease or treatment (i.e. infection with HIV and some other viruses, irradiation or chemotherapy) the amount of IL-7 available per T cell increases. This results in an enhanced survival signal through the IL-7R complex and, if sufficiently strong, also a proliferation signal. Therefore, T cells transferred into a lymphopenic environment undergo a unique type of proliferation called lymphopenia-induced proliferation (LIP) [36]. This is one of the few situations where naïve T cells undergo proliferation in the absence of overt antigen stimulation. Actually, IL-7 likely lowers the threshold of T cell activation allowing their stimulation by self-antigen/MHC[37]. Along with this proliferation, the T cells acquire some phenotypical characteristics of activation and/or memory and thus are more responsive to antigens than naïve T cells. Thus, in addition to enhancing thymic T cell output, IL-7 can increase T cell numbers in a thymus-independent manner.

IL-7 is an attractive cytokine for immunotherapy as it acts on a broad array of T cells. Expression of IL-7R α is similar among CD4 and CD8 T cells and among naïve and memory T cells, with some reports showing slightly higher levels on memory T cells than on naïve T cells [34,35,38] (Figure 1). Upon T cell activation, IL-7R α is downregulated and then regained during the differentiation into memory T cells [34] (Figure 1). Since T cell death is a major event concurrent with the generation of memory T cells and IL-7 can enhance T cell survival, IL-7 treatment during this phase has been tested for its ability to over-ride T cell death and increase the generation of memory T cells. Whereas exogenous IL-7 can boost the generation of memory T cells, these effects are often short-lived, apparently due to an inability of IL-7 to alter the programming of events set in motion earlier in the immune response [39]. Similar to activated T cells, Tregs constitutively have decreased IL-7R α and as such undergo minimal expansion in lymphopenic conditions or after IL-7 administration [40]. This may contribute to the increased incidence of autoimmune phenomena after disease or treatment-induced lymphopenia.

IL-7 use in clinical setting

Due to its function during thymic development and peripheral T cell homeostasis, IL-7 has potential to enhance T cell reconstitution, ACT, and vaccination efficacy. To date, only the effects of IL-7 in altering T cell populations in humans with complete immune systems have been studied; these results have been documented in two recent reports [41,42] (Table 2). In the first study, four cohorts of patients (3 each) were each given different doses of IL-7 every 3 days for 21 days [41]. Absolute numbers of CD4 and CD8 T cells increased in a dose-dependent manner over the treatment period, without significant changes in the numbers of B cells, NK cells, CD4+Foxp3+ Tregs, neutrophils, eosinophils, and basophils. At the highest dose, the expression of the IL-7R α chain (CD127) on T cells tended to be decreased, likely because of previously described feedback downregulation [43]. B cell progenitor levels in bone marrow were increased but did not translate in increased peripheral B cell levels. Toxicity of IL-7 was mild, including transient fevers and mild erythema at the injection site.

The second study used a similar protocol and more extensively examined the effects of IL-7 on T cell subsets and TCR repertoire [42]. As T cell expansion during LIP favors T cells with an increased affinity to self-peptide [37], it was important to determine whether administration of IL-7 to patients skew the T cell repertoire towards self-reactivity. In addition, it was speculated that IL-7 might have a limited ability to boost T cell production from the thymus

in an elderly population due to thymus atrophy. Interestingly, IL-7 treatment in humans induced a preferential expansion of naïve T cells resulting in a broader T cell repertoire than before treatment, and this effect was independent of age [42]. IL-7 administration increased both CD4⁺ and CD8 T cell counts at a similar rate, maintaining the CD4/CD8 T cell ratio. This increase correlated with an increase in T cell proliferation as well as increased levels of the anti-apoptotic bcl-2 protein [42]. There was a trend toward preferential increase in naïve (CD45RO⁺) T cells over memory (CD45RO⁺) T cells. These T cells were functional; in fact IL-7 treatment increased their capacity to proliferate upon *in vitro* stimulation by up to 10-fold. In this same study, a comparison was also done with IL-2 treatment. While IL-7 selectively promoted accumulation of CD8 T cells with a more naïve phenotype, IL-2 markedly boosted the absolute and relative number of Tregs and CD8 T cells with effector functions.

As a part of this study, the melanoma patients received a gp100 and MART-1 peptide vaccine along with the IL-7 treatment [41]. This showed no evidence of enhanced gp100 or MART-1-specific immunity, possibly because unlike IL-2, IL-7 favors the expansion of naïve CD8 T cells over effector CD8 T cells which downregulate IL-7R α expression after activation by antigen [43].

While IL-7 had mild side effects, five out of 12 patients showed low-level induction of IL-7 specific antibodies [41], possibly due to the lack of glycosylation of this bacterially produced form of the IL-7 protein. The antibodies had low neutralizing activity *in vitro* and did not appear to inhibit endogenous IL-7 levels in the patients, as none of them experienced a post-treatment decrease in lymphocyte counts as might be expected if endogenous IL-7 were neutralized [41]. Nevertheless, a fully glycosylated IL-7 protein is currently being manufactured for use in future studies.

The ability of IL-7 to induce proliferation in normally non-proliferating T cells may also be of use in the treatment of HIV patients. One problem with HIV is its ability to remain sequestered inside T cells, which are normally not proliferating. Because it induces T cell proliferation, IL-7 also upregulates latent HIV replication [44]. Therefore, IL-7 induced virus purging by concurrent retroviral treatment may help reduce viral burden in these patients.

IL-7 may also be of use in the setting of ACT in cancer or viral disease. Here, the ability of IL-7 to maintain the survival of antigen-experienced, memory-type T cells may be of use in the later phase of ACT. Since re-expression of IL-7R α does not occur until after cessation of TCR stimulation, allowing *ex vivo* expanded T cells to rest without TCR stimulation before their adoptive transfer may increase their responsiveness to endogenous or exogenous IL-7. The limited expansion of Tregs by IL-7 are a further argument for inducing lymphopenia prior to infusion of tumor-specific T cells and/or administration of IL-7 to support infused cells after ACT. Indeed, IL-7R α expression on adoptively transferred, melanoma-specific CD8 T cells directly correlated with their ability to proliferate and persist *in vivo* in an animal model of ACT (Acquisition of full effector function *in vitro* paradoxically impairs the *in vivo* antitumor efficacy of adoptively transferred CD8 T cells [45]).

IL-15

Normal *in vivo* functions

IL-15 shares many functions with IL-2, largely because both cytokines signal through two of the same receptor subunits, i.e. IL-2/15R β , and γ C. Despite the sharing of two receptor subunits, IL-15 has distinct functions *in vivo*. In mouse studies, IL-15 has been shown to be important for the development and homeostasis of memory CD8 T cells, NK cells, NK T cells, and certain subsets of intestinal CD8 T cells [46,47]. Cell proliferation and survival induced by IL-15 are major components by which IL-15 mediates its developmental and homeostatic effects. The

activities of IL-2 and IL-15 appear more similar *in vitro* than they are *in vivo* due to the nature of the culture conditions used, including cytokine concentrations, use of homogenous cell populations, and overriding mechanisms that confer cytokine specificity *in vivo*. Similar to IL-2R α , the IL-15R α contributes if any signals associated with IL-15-mediated functions and instead mainly functions to confer specificity [48]. Unlike IL-2R α , the IL-15R α has a very high affinity for IL-15 that is not further increased in the presence of the IL-2/15R β / γ C complex. The affinity of IL-15 for the IL-2/15R β / γ C complex is moderate and allows IL-15 to signal through this heterodimeric complex in the absence of IL-15R α . Whereas this can occur *in vitro* by stimulating with soluble IL-15, it likely does not normally occur *in vivo* as soluble IL-15 is rarely found in the circulation or tissue fluids.

Whether IL-15 utilizes the three receptor subunits as a heterotrimeric complex is questionable. It now appears that IL-15R α binds and then presents or delivers IL-15 to target cells, a mechanism called trans-presentation [49]. A trans-presenting cell expresses both IL-15 and IL-15R α , which associates intracellularly and are shuttled to the cell surface where the complex stimulates neighboring cells through their IL-2/15R β / γ C receptor complex. Therefore, due to the high affinity of IL-15R α for IL-15, soluble IL-15 given *in vivo* would likely be bound up by empty IL-15R α that could then be delivered via trans-presentation. Because of this unique mechanism utilized by IL-15, the expression of IL-15R α does not correlate to cell responsiveness and rather is an indicator of a cell capable of presenting IL-15 to neighboring cells [50-52]. As IL-15 signals are mediated through the IL-2/15R β / γ C complex, the expression level of IL-2/15R β is a good indicator of responsiveness to IL-15 [53] (Figure 1). All lymphocytes express both the IL-2/15R β and γ C, but CD8 T cells, particularly memory CD8 T cells, NKT cells, and NK cells express the highest levels of IL-2/15R β chain making these cells particularly sensitive to IL-15 [53] (Figure 1).

As the model of trans-presentation would imply, coordinate expression of both IL-15 and IL-15R α is required for a cell to trans-present IL-15 and this appears to be true based on functional studies using mouse models [54,55]. Since both the amount of IL-15 produced by cells and the reagents available for its detection are limited, the precise identity of the cells providing IL-15 *in vivo* remains under investigation. Many cell types express IL-15 transcripts, but detection of protein has been difficult. This difficulty relates to both the tight regulation of IL-15 protein production along with the fact that the predominant form of IL-15 is bound to cell surface IL-15R α [49,56]. As IL-15 impacts lymphocyte development and homeostasis during the steady state, IL-15 protein is probably constitutively produced at low levels, similar to IL-7. Upregulation of IL-15 mRNA and protein is induced by many pathogens via TLR stimulation and type I IFNs in DCs, monocytes, and macrophages [53,57,58]. As a consequence, these are also the cell types thought to be the main producers of IL-15.

***In vivo* treatment**

To date, *in vivo* studies of IL-15 have been limited to animals (Table 1). This may seem surprising considering human IL-15 was cloned in 1994 [59] and has shown strong therapeutic activity in multiple settings in animal models of human disease including cancer [60]. The lack of human trials is partially due to difficulties in producing sufficient quantities of IL-15 protein and to restricted commercial licensing [61]. In mice, *in vivo* treatment with rIL-15 has many of the same effects as rIL-2 in that it induces the proliferation of T cells, with a preference towards memory CD8 T cells and NK cells (Table 1). Although similar to IL-2 in stimulating CD8 T cells, IL-15 could have advantages as it is predicted not to boost Treg levels as observed for IL-2 (Table 1). In addition, although the half-life of IL-15 in the circulation is shorter than that of IL-2, the functional activities of IL-15 *in vivo* may be more sustained because it is stabilized upon binding to IL-15R α and thus maintained on the cell surface. Once production and licensing hurdles are overcome, treatment with IL-15 may be used similar to IL-2 with

possibly increased efficacy and reduced side effects. Indeed, rIL-15 is currently being produced at the NCI for the purpose of human clinical trials [62].

IL-21

IL-21 is another immunomodulator in the γ C family primarily produced by CD4 T cells, particularly TH17 CD4 T cells and NKT cells. IL-21 signals through a heterodimeric complex that is composed of the IL-21R and the γ C[63]. IL-21R is present on many cell types such as CD4 T cells, CD8 T cells, NK cells, B cells, DC, macrophages and epithelial cells [63,64] and its expression tends to be upregulated upon activation (Figure 1). It does not appear to play a major role in the development of immune cells as IL-21R deficient mice do not display major immune cell deficiencies. They do, however, have reduced levels of IgG1 and IgE [65], highlighting its ability to modulate B cell functions. In general, IL-21 appears to primarily modulate the function of mature lymphocytes. For CD8 T cells, IL-21 has little effect on *in vitro* T cell proliferation, but can synergize with IL-15 or IL-7 [61,66]. The synergistic effect of IL-21 with other γ C cytokines has also been observed with other cell types and highlights the limited understanding of the underlying mechanism. IL-21 is unusual in that it has been reported to have biphasic effects. For example, at low doses it promotes, but at high doses inhibits NK cell proliferation [67].

Although IL-21 can act on many immune cells, the predominant effect of IL-21 administration is a potent anti-tumor effect mediated by NK cells and CD8 T cells (Table 1). Interestingly, in one study, the anti-tumor effects of IL-21 were predominately mediated through NK cells while in other studies, the effect was mediated by CD8 T cells [39,68-71]. These discrepancies could be due to differences in cytokine delivery and/or particular tumor type and/or its localization. In studies comparing the anti-tumor effects of IL-2, IL-15, and IL-21, IL-21 had the most dramatic effect in enhancing animal survival. Although IL-21 treatment predominately affects CD8 T cells and NK cells, the recently recognized ability of IL-21 to skew T helper responses toward inflammatory TH17 will surely be an important consideration for future clinical trials. In multiple studies, the differentiation of TH17 cells over Tregs promoted by IL-21 has been implicated in inflammatory diseases such as Systemic Lupus Erythematosus, Multiple Sclerosis, Celiac disease, Crohn's disease, and type I Diabetes [72,73].

There are currently 2 published reports on the clinical use of rIL-21: a phase I study in melanoma patients and one in metastatic melanoma and renal cell carcinoma patients [74,75]. Both reports show some efficacy in both melanoma (1/24 complete response and 1/29 partial response) and renal cell carcinoma (4/19 partial response). The most prominent toxicities were flu-like symptoms and rash similar to those observed in patients treated with other cytokines such as rIL-2, rIL-12 and rIL-18 [7,76-78]. A transient lymphopenia was also common similar to what has been observed in human studies using IL-2 and mouse studies using IL-15. Altogether, these results suggest that the highest dose of rIL-21 (30 μ g/kg) is well-tolerated and has some anti-tumor activity in melanoma and renal cell carcinoma. The overall response rate in this population was slightly lower than the ~15% response rate routinely observed with high-dose rIL-2[7,9]. Considering these were phase I dose-escalation trials and most patients received a suboptimal dose of rIL-21, the response rate may increase as more patients are treated with higher doses.

Complexed cytokines and cytokine agonists

During investigations dissecting the mechanism of IL-15 trans-presentation, it was discovered that the complex of IL-15 bound to the IL-15R α induced a robust proliferation in CD8 T cells, NK cells, and NKT cells. In addition, it induced naïve CD8 T cell activation, effector differentiation, and memory development [79,80]. In addition to inducing a surge in CD8 T cells and NK cells, IL-15/IL-15R α complexes promoted tumor destruction by boosting the

proliferation of tumor-resident CD8 T cells [80]. Increasing doses of free IL-15 could only induce a fraction of the response that the complexed IL-15 induced, likely due to increased stability of the complexed cytokine. In addition, compared to free IL-15, the IL-15/IL-15R α complex has a 20-fold higher affinity for the IL-2/15R β / γ C complex [81]. While the effect was most dramatic for IL-15, enhanced *in vivo* activity of IL-2 and IL-7 on T cell proliferation was also observed when these cytokines were allowed to form complexes to their respective R α subunits (K.S. Schluns, T. Stoklasek, and L. Lefrancois, unpublished observations). These findings open up a new avenue of exploration that may yield improved cytokine reagents that may achieve an enhanced response with a reduced amount of cytokine.

Similarly, antibodies that form complexes with cytokines have been shown to enhance cytokine activity. Treatment with anti-IL-2 antibodies *in vivo* was found to increase proliferation of memory-phenotype CD8 T cells. While originally it was believed that this paradoxical effect was due to IL-2 deprivation of Tregs, it was more recently found that the antibody binds endogenous IL-2, forming a cytokine/antibody complex that directly stimulates CD8 T cells and NK cells. Specifically, a 100-fold increase in CD8 T cells and a 20-fold increase in NK cells was observed [82]. While the complex preferentially stimulated T cells with a memory-phenotype, naïve CD8 T cells also responded by becoming activated, obtaining effector functions, and differentiating into memory T cells. As this effect on CD8 T cells was similar to that observed with IL-15/IL-15R α complex [80,83], it suggests a comparable mechanism of stimulation. The increase in effector function was relevant as the CD8 T cells provided protection against *Listeria* infection [83] and enhanced the numbers of vaccine-induced CD8 T cells [84]. Interestingly, not all IL-2 antibodies had this stimulatory effect on CD8 T cells and NK cells: one specific anti-IL-2 antibody instead stimulated Tregs. This particular antibody is thought form a complex that preferentially promotes binding of IL-2 to the IL-2R α / β / γ C complex, favoring CD25^{hi} Tregs, while the other IL-2 antibodies promote binding to the IL-2R β / γ C complex independent of the IL-2R α , favoring non-Tregs. As such, depending on the binding specificity, IL-2 antibodies appear to utilize endogenous IL-2 to form a complex that can behave as either a more potent version of IL-2 or as IL-15.

An IL-7 antibody that forms complexes with IL-7 has also been identified; these complexes were shown to have a 100-fold increased activity compared to free IL-7 [85]. *In vivo*, this complex increased thymopoiesis homeostatic proliferation of both naïve and memory CD4 and CD8 T cells [85]. The observation that three of the γ C cytokines have increased activity upon complexing implies that this phenomenon may also occur upon complexing other γ C cytokines or even other non- γ C cytokines. The finding that γ C cytokines complexed to receptor subunits or specific antibodies enhances the activity of these proteins can surely benefit immunotherapy by reducing cytokine production burden and/or enhancing endogenous cytokine responses.

Optimizing the clinical use of specific cytokines

Understanding the normal functions of the γ C cytokine members and the relative responsiveness to them of specific cell types (Figure 1, Table 1), allows the rational design of better cytokine-based immunotherapies. While there is some overlap in functions among the γ C cytokines, therapeutic fine-tuning may be achieved by choosing the appropriate cytokines based on knowledge of cytokine receptor expression, the timing of cytokine delivery, and the use of cytokine combinations. For instance, if the goal is to generate anti-tumor responses, stimulation of CD8 T cells and NK cells with IL-15 or IL-15/IL-15R α complexes may be the best choice. To reconstitute lymphopenic patients after chemotherapy or HIV infection, IL-7 therapy would seem ideal as it preferentially promotes and supports urgently needed naïve CD4 and CD8 T cells. Furthermore, the addition of IL-2 to that regiment could also restore Tregs, thus reducing the chance of developing autoimmunity-like conditions although possibly also interfering with immune-mediated viral control. As IL-21 tends to have synergistic effects

with most other γ C cytokines, its addition could potentiate a γ C cytokine treatment. These are examples of how combinations of cytokines may provide additional benefits over monotherapy. Optimal timing of cytokine administration is particularly relevant when the activation of T cells is involved as expression of individual cytokine receptors changes over the course of T cell differentiation. While IL-2 clearly enhances the expansion of T cells, continued stimulation with IL-2 is counter-productive as it can induce activation-induced cell death [86]. While Ag and IL-2 are used to expand Ag-specific T cells, it is becoming increasingly clear that a rest from Ag stimulation is important to prevent exhaustion and allow differentiation into memory-like T cells that have greatly enhanced capacity to proliferate and survive. Thus, after expansion of the desired population of T cells, they could be maintained by a secondary *in vitro* stimulation with IL-7 and/or IL-15 that would help promote the survival of the T cell clones by promoting their differentiation into long-lived memory cells.

In considering cytokine therapies, it is also important to take into consideration how cytokine responses differ in specific environments. Interestingly, IL-7 administration is most likely to work in an IL-7 deficient environment. Studies have shown that administration of supraphysiological levels of IL-7 in steady state conditions has little impact on thymopoiesis or enhancing Ag-specific memory T cells despite the expression of IL-7R α by T cells; this indicates that other homeostatic mechanisms must be in place. The advantages of IL-15 and IL-2 lie in their ability to preferentially enhance previously-activated CD8 T cells and NK cell numbers that can provide effective anti-tumor responses. However, one should keep in mind that since IL-2/15R β is expressed at similar levels on activated and memory CD8 T cells, T cells targeted in vaccine regimens or stimulated for ACT will have no competitive advantages over endogenous memory-phenotype CD8 T cells and NK cells. Other manipulations may be needed to enhance their competition.

The last few years have seen a great increase in our understanding of the specific properties of the members of the γ C cytokine family, including their the expression patterns of their specific receptors and their differential *in vivo* activities on specific immune cell subsets. Altogether, these insights make clear that the γ C cytokines should not be considered as largely redundant factors, but as individual potential therapeutics with specific clinical applications. Now that IL-2, IL-7, and IL-21 have been proven clinically safe and biologically-active, there is great opportunity, through their targeted use alone or in combination, to implement immunotherapies with enhanced efficacy and decreased toxicity.

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Figure 1. Relationship between cytokine receptor expression and cytokine responsiveness among lymphocyte subsets

Schematic depiction of relative expression of γ C cytokine receptor subunits with bound cytokine on various lymphocyte populations. Black subunits represents the γ C, blue=IL-2R α , green=IL-7R α , orange=IL-2/15R β . Thickness of the colored bars indicates the cells' relative cytokine responsiveness. Size of the receptor complex indicates relative expression level. Hatched bar indicates that some subsets are cytokine responsive.

Table 1
Specific *in vitro* and *in vivo* activities of γ C cytokines in natural and therapeutic settings

	IL-2	IL-7	IL-15	IL-21
In vitro activities - low doses	-Mediates survival of T regs and NK cells	Maintains CD4 and CD8 T cell survival	-Mediates survival of CD8 T cells and NK cells	-Costimulates T cell proliferation -Expands hematopoietic progenitors -Regulates Thelper differentiation
In vitro activities -high doses	-Costimulates activation of naïve CD8 T cells - Enhances proliferation of memory CD8 T cells - Induces proliferation and effector functions of NK cells	Induces CD4 and CD8 T cell proliferation	-Induces activation of naïve CD8 T cells -Enhances proliferation of memory CD8T cells - Enhances proliferation and induction of effector functions of NK cells	-Inhibits differentiation of Tregs -Regulates CD8 T cell and NK cell proliferation and effector functions -Regulates B cell proliferation, immunoglobulin production, and plasmas cell differentiation -Inhibits DC maturation and function
In vivo activities	-Expands memory phenotype CD8 T cells, NK cells, and Tregs	-Expands naïve and memory T cells in lymphopenic environment	-Expands memory phenotype CD8 T cells and NK cells	-Expands hematopoietic progenitors - Promotes TH-17 differentiation
Therapeutic uses	-Increases CD4+ T cells In AIDS -Enhances anti-tumor responses -Expanding T cells for ACT -Suppresses autoimmune responses	-Reconstituting T cell in lymphodepleted -Maintenance of T cells in late stages of ACT	-Enhancing anti-tumor responses -Expanding CD8T cells for ACT	-Enhancing anti-tumor responses

Table 2

Selected human clinical trials with γ C cytokines

Patient population	# of patients	cytokine	dose	concurrent treatments	immunological effect of cytokine	clinical outcome	dose-limiting cytokine toxicity	remarks	Ref.
HIV infected	35	IL-2	variable: 1-18 mIU daily for 5 days for up to 28 cycles over 6-7 years	none	↑ CD4 T naïve/ Tcm/“Treg,”	not reported	not reported	↑ “Treg,” are foxP3 ^{int} ; IL-2-induced Teff?	[5,6]
HIV infected	204	IL-2	9 mIU (i.v.) daily or 7.5 mIU (s.c) twice daily for 5 days every 8 weeks up to 9 weeks	Highly Active Antiretroviral Therapy	↑ CD4 T naïve and Teff/ Tmem, ↔ CD8T	↓ new AIDS-defining event in IL-2 treatment arms	nausea, rash, fatigue, fever		[87]
melanoma	182	IL-2	2 cycles of 720,000 IU/kg (i.v.) every 8 hours up to 5 days	none	lymphocytosis	6.6% CR, 8.2% PR	hypotension, mental status changes, renal impairment, oliguria, respiratory events	toxicities rapidly reversed	[88]
renal cell carcinoma	227	IL-2	2 cycles of 720,000 IU/kg (i.v.) every 8 hours up to 5 days	none	lymphocytosis	9.3% CR, 9.7% PR	hypotension, mental status changes, renal impairment, oliguria, respiratory events	toxicities rapidly reversed	[7,88,89]
melanoma	31	IL-2	2 cycles of 720,000 IU/kg (i.v.) every 8 hours up to 5 days	gp100.209_2M peptide/IFA vaccination	↓ vaccine-induced CD8 T	3.2% CR, 39% PR	typical IL-2 toxicity; not specified	updated retrospective response rate for vaccine + IL-2 at 22% vs 13% for IL-2 alone	[7,19-21,88,89]
melanoma	43	IL-2	2 cycles of 720,000 IU/kg (i.v.) every 8 hours up to 5 days	lymphodepletio n regimen 1, adoptive T cell transfer	unclear; presumably proliferation/maintenance of transferred T cells	9.3% CR, 40% PR	typical IL-2 toxicity; not specified		[90,91]
melanoma	25	IL-2	2 cycles of 720,000 IU/kg (i.v.) every 8 hours up to 5 days	lymphodepletio n regimen 2, adoptive T cell transfer	unclear; presumably proliferation/maintenance of transferred T cells	16% CR, 56% PR	typical IL-2 toxicity; not specified		[23]
melanoma	11	IL-7	3-60 µg/kg (s.c.) every 3 days for 8 doses	gp100.209_2M and MART-1.26_2L peptide/IFA vaccination	↑ CD4 and CD8T, ↓ Treg	no objective responses	none	no evidence of vaccine enhancement by IL-7	[41]
non-hematologic, non-lymphoid cancer	16	IL-7	3-60 µg/kg (s.c.) every 2 days for 8 doses	none	↑ naïve and Tcm but not Teff CD4 and CD8 T, ↓ Treg, broadened TCR repertoire	not reported: future publication	1 hypertension, 1 ↑ aminotransferase		[42]
melanoma	29	IL-21	1-100 µg/kg thrice weekly for 6 weeks or 3 cycles of daily for 5 days, 9 days rest	none	transient lymphopenia; ↑ soluble CD25	3.4% PR	↑ aminotransferase, neutropenia, thrombocytopenia, lightheadedness, fatigue		[92,93]
melanoma, renal cell carcinoma	24	IL-21	3-100 µg/kg days 1-5 and 15-19 (i.v); additional cycles optional	none	transient lymphopenia; ↑ soluble CD25, ↑ perforin-1 and granzyme B mRNA in CD8 T	4.2% CR (melanoma); 21% PR (renal cell carcinoma)	“grade 3 laboratory abnormalities,”		[94]