

Cytokines as Adjuvants for Vaccine and Cellular Therapies for Cancer

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Abstract: Problem statement: The development of a potent vaccine that can help treat tumors resistant to conventional cytotoxic therapies remains elusive. While part of the problem may be that trials have focused on patients with bulky residual disease, the desire to maximize responses to the vaccine remains. **Approach:** The gamma (γ) family of cytokines offered a unique opportunity to support the expansion and effectors potential of vaccine-responding T-cells, as well as stimulate other effectors, such as Natural Killer (NK) cells, to become activated. **Results:** Combining vaccines with cytokines seems logical but can bring unwanted toxicity, as had been observed with interleukin (IL)-2. In addition, the nonspecific activation or expansion of unwanted cell subsets, such as regulatory T-cells, can contribute to global immunosuppression and limit vaccine responses. The development of IL-7 and IL-21 for the clinic offered the promise of enhancing anti-tumor responses but with far less systemic toxicity and no expansion of regulatory T cells. Preclinical studies demonstrated that IL-15 could also improve T-cell and especially NK-cell, responses as well. **Conclusion/Recommendations:** Future study should expand the use of vaccines with IL-7, IL-21 and hopefully IL-15 in high-risk patients and consider treatment while in a state of minimal residual disease to maximize benefit. Identifying tumors that can signal through gamma(c) cytokines will also be essential so that induction of relapse will be avoided.

Key words: Interleukin 2, interleukin 7, interleukin 15, interleukin 21, tumor vaccines

INTRODUCTION

Despite the success of multimodality therapy for cancer including chemotherapeutic agents, radiation and surgery, relapse still occurs in a large percentage of patients. Immune effectors cells represent a powerful tool for eliminating residual tumor cells. The potency of Donor Lymphocyte Infusions (DLI) in treating chronic myelogenous leukemia following Hematopoietic Stem Cell Transplant (HSCT) is a striking example of the potential of cancer immunotherapy. Clinical responses to adoptive cell therapies administered in the autologous setting further illustrate the promise of cancer immunotherapy. The development of vaccines designed to elicit adaptive immune responses, mainly from T-cells, has occurred in parallel with adoptive cell therapy, but it is evident based on the clinical data generated with vaccines thus far that approaches to increase potency will be necessary. Indeed, the great appeal of this approach is the potential to generate immunotherapies that are antigen-specific, with a particular focus on tumor-associated antigens. One of the

main drawbacks to this approach, however, is that tumor antigens represent self-proteins, which can induce tolerance in the host. Thus, vaccines have been examined in combination with other immunotherapies as a means of generating a potent T-cell response against tumor antigens while overcoming the barriers of tolerance.

Vaccines are being pursued for multiple types of cancers and are being designed using multiple approaches to enhance immunogenicity. Administration of whole cancer cells, purified peptides, or DNA vaccines have been given alone, or in combination, with professional Antigen Presenting Cells (APCs) to elicit immunogenic responses. Combinations of different types of immunotherapy with traditional modalities like chemotherapy, radiation and surgery, have been tested in clinical trials with some promise. Cytokines delivered with vaccines as adjuvants aim to improve antitumor immunity by increasing the proliferation of effectors cells and also improving their cytotoxicity or cytokine production^[1]. It has been demonstrated in preclinical models that concurrent administration of cytokines with a vaccine has the potential to enhance

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immune reactivity through the recruitment of T cells and APCs to lymphoid organs, as well as by activation of T-cells and Natural Killer (NK) cells directly. In this review, we will discuss clinically relevant cytokines that have been coupled with a vaccine or cellular therapy in preclinical models and in some cases clinical trials, to generate antitumor immune responses, with a focus on the so-called gamma(c) family of cytokines. These cytokines (Interleukin 2, Interleukin 7, Interleukin 15 and Interleukin 21) all utilize the common cytokine gamma chain for signaling and have potent effects on T-cells and NK cells, the major effectors in the anti-tumor immune response.

Interleukin 2:

Background: The first cytokine administered in a vaccine trial against cancer was interleukin 2 (IL-2). IL-2 is produced mainly by T helper cells, acts on a variety of immune cells across the innate and adaptive immune system and is known to play an important role in the initiation and maintenance of antigen-specific immune responses (Fig. 1). The biology and signaling pathways of IL-2 have been reviewed extensively^[1,2]. Because of the broad effects of IL-2 on a variety of immune cells, the specific mechanisms by which IL-2 influences the immune system to induce tumor regression are not completely understood.

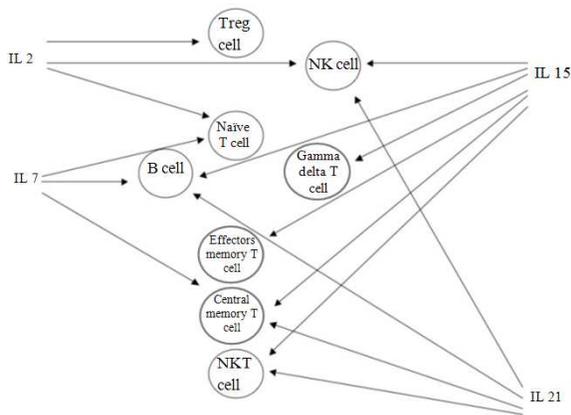


Fig. 1: Target lymphocyte populations of gamma(c) cytokines. While there is considerable overlap of cytokine activity on lymphocytes, effects can be stimulatory or inhibitory depending on the cytokine. IL-21 only increases proliferation of T cells stimulated with anti-CD3 or antigen, but can augment responses to other gamma(c) cytokines. IL-7 acts on developing B cells but not mature cells of this lineage. IL: Interleukin, NK: Natural Killer, NKT: Natural Killer-T cell, Treg: Regulatory T cell

A variety of preclinical models demonstrated potential therapeutic benefit of combining IL-2 with vaccines, leading to the study of IL-2 in clinical trials. The antitumor effect of IL-2 is believed to be mediated by lymphocyte expansion and augmentation of effector cell function^[2]. However, while IL-2 enhances the activity of both NK cells and T-cells, it can also expand regulatory T-cells (Tregs), which contribute to tumor-associated immunosuppression^[3]. The Food and Drug Administration approved IL-2 as a single agent for use in patients with metastatic Renal-Cell Carcinoma (RCC) (1992) and metastatic melanoma (1998). IL-2 has also shown efficacy as an adjuvant for infectious vaccine therapy. As will be outlined below, objective tumor responses have been observed in clinical trials combining cancer vaccines with IL-2. An informative commentary on the history of IL-2 treatment for melanoma is available^[4]. This review will explore IL-2 as a prototypic cytokine that acts as an adjuvant to vaccine therapy by examining the relevant clinical trials in melanoma, where there is the most clinical experience in adults, as well as in pediatric solid tumors. A review of the experience with IL-2 and vaccines in RCC is discussed elsewhere^[5]. Given the diversity of clinical trial designs using IL-2 as an adjuvant, we will also discuss dosing, timing of administration and reported objective response rates as potential factors that may impact efficacy.

Clinical Trials with Vaccines and IL-2 in Melanoma:

When examining the literature on IL-2 given with therapeutic vaccines for melanoma, studies vary by the type of vaccine administered, IL-2 dose, timing of IL-2 administration in relation to the vaccine and length of therapy (Table 1). The earliest data for systemic IL-2 following vaccination was reported by Rosenberg *et al.*^[6] in 1998, who used high-dose IL-2 immediately following vaccination with a modified gp100 (a relevant melanoma tumor-associated antigen) peptide vaccine in 31 patients with melanoma. A 42% objective response rate was reported, while the vaccine alone had a 5% response rate^[6]. This initial observation led to the development of further trials with more advanced vaccines, but served as a “proof of principle” study that vaccines combined with a cytokine can improve objective tumor response rates over vaccines alone. Another report by this group with a different peptide vaccine and IL-2 demonstrated a 38% objective response rate, although it was not documented whether they were Partial Remissions (PRs) or Complete Remissions (CRs), nor how long responses were maintained^[7]. However, other trials in melanoma incorporating peptide vaccines with IL-2 have shown either response rates similar to that seen with IL-2 alone (about 15-25%), or no responses at all^[8,9]. One trial did not report a specific response rate^[10].

Table 1: Analysis of melanoma trials where patients received both IL-2 and a vaccine

Vaccine	IL-2 Dose/route	Timing/length of IL-2 therapy	Objective response rate	References
Peptide (Day+0)				
g209-2M	720,000 IU kg ⁻¹ IV	Start day+1 or +5 until grade 3-4 irreversible toxicity (tolerance)	42%	[6]
g209-2M	720,000 IU kg ⁻¹ IV	Same as above (6-10 doses)	38%	[7]
Tyrosinase + gp100 + tetanus helper	3,600,000 IU m ⁻² SC	Group 1: Start day+7 Group 2: Start day+28.	Not reported 0%	[10] [8]
g209-2M	5,000,000 IU m ⁻² SC	Daily for 6 weeks in both groups	16%	[9]
g209-2M	600,000 IU kg ⁻¹ IV	Start day+1 for 5 days on: repeated every 21 days Weeks 1 and 3 for trial one Weeks 7 and 9 for trial two Weeks 1, 4, 7 & 10 for trial three		
DC or PBMCs	720,000 IU kg ⁻¹ IV	Start day+0 for 3 days	0%	[12]
DC + MART-1 + g209-2M				
DC + tumor lysate	700,000 IU SC	Start day+0, 3 times/week	0%	[14]
Tumor/DC hybrid cell	3,000,000 IU SC	Start day+0 for 6 days	0%	[15]
DC + tumor lysate	2,400,000 IU m ⁻² SC	Start day+1 for 3 days	0%	[16]
Tumor/DC hybrid cell	3,000,000 IU m ⁻²	Start day+1 for 5 days	10%	[17]
PBMC + g209-2M	720,000 IU kg ⁻¹ IV	Start day+0 until tolerance (4-11 doses)	0%	[18]
DC + tumor lysate	3,000,000 IU SC or 360,000 IU kg ⁻¹ IV	Low: Start Day+0 for 4 days High: Start Day+0 for 9 doses	0%	[13]
DC + tumor lysate or DC + peptide cocktail	1,000,000 IU m ⁻² SC	Start day+1 for 5-14 days	0% (lysate) 22% (peptides)	[11]
Other				
Adenovirus + MART-1 or Adenovirus + gp100	720,000 IU kg ⁻¹ IV	Start day+1 until tolerance	16%	[20]
Fowlpox virus + g209-2M	720,000 IU kg ⁻¹ IV	Start day+0 up to 12 doses every 4 weeks	50%	[21]
SRL172 (Mycobacterium)	6,000,000 IU SC	Start day+0 for 3 days	19%	[23]
DNP + BCG + tumor	3,000,000 IU SC or 720,000 IU kg ⁻¹ IV	Low: Start day+0 for 5 days every 14-21 days High: Start day+0 for 2 weeks, 1 week rest, then repeat once	42%	[19]
Tumor plasma membrane on silica beads	1,750,000 IU m ⁻² SC	Start day+5 from vaccine for 1 week	0%	[22]

IV: Intravenous; SC: Subcutaneous

While it could be argued that peptides are inefficient as vaccines since they need to be expressed by an APC and presented with costimulatory signals to get a proper effector response, studies have also examined IL-2 given with a dendritic cell (DC) vaccine. In these studies, the vaccine is potentially capable of presenting a melanoma antigen directly and IL-2 could facilitate expansion of any vaccine-responding cells. Unfortunately the response rates in these trials were quite poor^[11-17]. One group even adopted a similar approach using Peripheral Blood Mononuclear Cells (PBMC), instead of DCs, to patients pretreated with other vaccines and saw no objective clinical responses^[18].

In contrast to using APCs, one report using a dinitrophenyl-modified autologous melanoma cell vaccine as either a primary treatment or as an adjuvant showed 42% objective tumor regression (2CR, 8PR) following combination with IL-2, lasting for a median

duration of 6 months (range 3-50 months)^[19]. Viruses have also been explored as a means of presenting over expressed melanoma antigens and whereas adenovirus vaccines yielded no responses^[20], another report using a recombinant fowlpox virus encoding a minigene construct encoding a single, modified melanoma epitope yielded a 50% response rate (3CR, 3PR) when given with IL-2^[21]. Regarding nonviral approaches, using melanoma plasma membranes on silica beads showed no responses^[22] and giving only heat-killed Mycobacterium without any tumor antigens was no better than giving IL-2 alone^[23]. Thus, no controlled randomized vaccine trial ± IL-2 have reported that IL-2 improves responses to a tumor vaccine and in a multitude of non-controlled trials response rates to tumor vaccines remain low, whether or not IL-2 is co-administered. Moreover, a meta-analysis of the vaccine trials at the National Cancer Institute (NCI) demonstrated that melanoma vaccines in general,

when given with IL-2, do no better than giving IL-2 alone^[24].

Clinical trials with pediatric cancers: The clinical experience in pediatrics with IL-2 and vaccines has been more limited, with neuroblastoma the most extensively studied. Investigators have used IL-2-secreting autologous neuroblastoma cell lines as vaccines and a transgenic chemokine-cytokine (lymphotactin-IL-2) vaccine generated in autologous and allogeneic neuroblastoma cell lines^[25-27]. Except for a few patients with PRs or CRs, all of the response rates were disappointing. Importantly, very recent research has demonstrated that IL-2 combined with GM-CSF and a monoclonal antibody against GD2-expressing neuroblastomas can lead to enhanced event-free and overall survival in a phase III trial^[28]. This data demonstrates the efficacy of IL-2 as an adjuvant to monoclonal antibody therapy rather than as an adjuvant for T-cell active vaccines and illustrates the potential effectiveness of cytokines in cancer immunotherapy in the context of a large, well-designed clinical trial conducted at multiple centers through a cooperative oncology group.

In addition to neuroblastoma, Dagher *et al.*^[29] reported in 2002 no clinical benefit of a PBMC vaccine pulsed with tumor peptides given with IL-2 in children with recurrent Ewing sarcoma and alveolar rhabdomyosarcoma. A follow up study by Mackall *et al.*^[30] in the same patient populations gave autologous T-cells and DCs pulsed with peptides derived from tumor-specific translocation breakpoints and was reported in 2008 using different doses of IL-2 administered to three cohorts. Immune responses to the translocation breakpoint peptides occurred in only 39% of patients. There was a 43% 5 year overall survival for patients initiating immunotherapy, which is higher than would be predicted for patients with this level of high-risk disease, although no differences were seen in cohorts that received or did not receive IL-2. Additional data generated from this trial definitively demonstrated that *in vivo* administration of IL-2 results in expansion of Tregs. Zhang *et al.*^[31] showed that CD4⁺ CD25⁺ Tregs underwent homeostatic peripheral expansion during immune reconstitution, that IL-2 therapy expanded this subset and that this expansion was further augmented by lymphopenia.

In a leukemia trial that included 7 children with high-risk acute myeloid leukemia or Acute Lymphoblastic Leukemia (ALL) in cytologic remission, patients received up to 6 subcutaneous injections of a tumor vaccine consisting of leukemic blasts admixed with skin fibroblasts transduced with adenoviral vectors

encoding IL-2 and CD40 ligand. Eight patients remained disease-free for a range of 27-62 months after treatment, with a 5 year overall survival of 90%^[31]. Thus, IL-2 therapy is well tolerated in pediatric patients despite being heavily pretreated for their primary disease and may enhance the cytotoxicity of both T-cell and non-T cell subsets, but Treg expansion remains an issue.

Limitations of IL-2: There remains considerable debate about the dosing, schedule and timing of IL-2 administration in relation to vaccination. In general, high dose IL-2 appears to be associated with better clinical responses in melanoma (Table 1), although there is minimal data directly comparing IL-2 dosing. What is well documented is that higher doses of IL-2 cause appreciable toxicity, namely capillary leak^[2]. In addition, elevation of IL-6 has been associated with IL-2-induced mental depression^[32] and IL-2 therapy can result in autoimmune toxicities, including vitiligo, Type I diabetes or autoimmune thyroiditis^[33], perhaps indicative of the potential for these cytokines to induce tumor responses against self-antigens. IL-10 production may be a direct result of Treg stimulation and may hamper antitumor effects. In addition, IL-2 mediates activation-induced cell death, which could also hamper T-cell responses to a vaccine^[2]. Lastly, there is some concern that IL-2 may signal tumors themselves^[34-42], particularly lymphoid-derived cancers and some adult carcinomas (Table 2).

In summary, most vaccines tested with IL-2 do not do better than IL-2 alone, although there are many factors that can affect outcome, including the type of vaccine, dose and schedule of IL-2 and antigen targeted. Importantly, the relative expansion of Tregs may also be hampering responses. Future research will need to address limiting expansion of this subset. In addition, almost all of the patients treated in these trials had measurable and often bulky, tumors at the time of enrollment. It is possible that vaccines and IL-2 may work better in a Minimal Residual Disease (MRD) setting and future work should focus on using these two modalities as a means to minimize relapse. With regards to scheduling and timing of IL-2, in most clinical trials, IL-2 is usually given at the same time or following vaccination (Table 1). IL-2 might be more effective if given before vaccine administration, so the milieu will promote proinflammatory immune responses^[10], or if IL-2 is delayed until after the T-cell contraction phase^[43]. Overall, the relatively low rate of clinical responses to vaccines with IL-2, regardless of dosing or schedule, indicates that IL-2 may not be optimal as an adjuvant.

Table 2: Malignancies implicated to utilize gamma(c) cytokines

Cytokine	Malignancy	Evidence	References	
IL-2	Hodgkin disease	IL-2R ⁺ or CD25 ⁺ by IHC	[34,35]	
	B- and T-cell lymphomas	IL-2 ⁺ or IL-2R ⁺ or CD25 ⁺ by IHC	[34-37,42]	
		IL-2mRNA in CD25 ⁺ cells		
		T-cell leukemias	IL-2 can signal and promotes proliferation	[38]
		B-cell CLL	Aberrant expression CD25mRNA	[39]
		Head and neck carcinoma	CD25 ⁺ and CD122 ⁺ by antibody and mRNA analysis	
			IL-2 induces proliferation of CD25 ⁺ cells	[40,41]
	Gastric carcinoma	Surface IL-2 ⁺ and CD122 ⁺ and intracellular IL-2 ⁺ by flow cytometry	[40]	
IL-7	Squamous cell lung carcinoma	Anti-CD25 treatment induces G1 arrest and induces apoptosis <i>in vitro</i>	[37]	
	Acute B-cell leukemia	Surface IL-2 ⁺ and CD122 ⁺ and intracellular IL-2 ⁺ by flow cytometry	[58,60,61,64]	
		IL-2 ⁺ and CD25 ⁺ by IHC		
		IL-7R mRNA ⁺ , IL-7R protein ⁺ and shows <i>in vitro</i> kinase activity		
		Growth inhibition by rapamycin reversed by IL-7		
		CD127 is alternatively spliced		
		Acute T-cell leukemia	Notch1 binds to IL-7R promoter, regulates IL-7R transcription and CD127 expression	
		Hodgkin disease	IL-7R ⁺ by flow cytometry and IHC	
			IL-7 stimulates growth in colony assays	[62]
		Lung carcinoma	IL-7 prevents apoptosis in serum-free assays	
IL-15		IL-7R mRNA ⁺ , IL-7R protein ⁺ and <i>in vitro</i> kinase activity	[58,59]	
		IL-7 induces VEGF-D and promotes lymphangiogenesis	[64]	
		IL-7R is alternatively spliced	[105,106]	
		Brain tumors	IL-15 stimulates proliferation	[107]
		Large granular leukemia	IL-15 induces all known signaling deregulations	
		CLL	IL-15 causes receptor signaling, proliferation and prevents apoptosis	
			Cells stimulated with CD40 exhibit increased expression of IL-15R	
		Pediatric ALL	High IL-15 expression correlates with CNS involvement	[111]
		Cutaneous T cell lymphoma	IL-15 can signal and promote proliferation	[42]
		Renal cell carcinoma	IL-15R ⁺ by flow cytometry and RT-PCR	[109]
IL-21		IL-15 can signal IL-15R	[108]	
		Head and neck carcinoma	IL-15Ra ⁺ by RIA	
		T cell leukemia	IL-21R ⁺ by flow cytometry and RT-PCR	[140]
		Hodgkin disease	IL-21 induces signaling and proliferation	
			IL-21 ⁺ by flow cytometry, RT-PCR and IHC	[156,157]
			IL-21R ⁺ by flow cytometry	
		B- and T-cell lymphomas	IL-21 signals STAT5 to cause proliferation	[140]
		IL-21 protects cells from apoptosis		
	Multiple myeloma	IL-21R ⁺ by flow cytometry	[158]	
		IL-21 causes proliferation		
		IL-21 signals and is a growth factor via an IGF-1 autocrine loop		

IHC: Immunohistochemistry, CNS: Central Nervous System, RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction, RIA: Radioimmunoassay

Interleukin-7:

Background: IL-7 is produced by a variety of cell types and tissues, but not by lymphocytes themselves and serum IL-7 levels are inversely correlated with lymphocyte counts. IL-7 is involved in the maintenance and survival of alpha-beta T cells, the development of B cells and gamma-delta T cells and may play a role in the biology of DCs and monocytes^[44,45] (Fig. 1). IL-7 does not appear to support NK cells. Thus, IL-7 plays a critical role in lymphocyte homeostasis as indicated by markedly diminished lymphocyte counts in IL-7 and IL-7 receptor gene deleted mice and the severe combined immunodeficiency associated with IL-7 receptor mutations in humans. An extensive review of

IL-7 biology and signaling can be found elsewhere^[44,46,47], but this review will focus on potential clinical utility of recombinant human (rh) IL-7 as an agent for immunorestitution or as adjuvant therapy for vaccines or adoptively transferred T cells. Preliminary data thus far demonstrates that IL-7 therapy enhances immune reconstitution, but without stimulating Treg expansion or inducing capillary leak, as occurs with IL-2.

Clinical trials with IL-7: The first rhIL-7 phase I trial reported 12 patients with metastatic cancers. The four tested doses were 3, 10, 30 and 60 µg kg⁻¹ given subcutaneously every 3 days for a total of eight doses.

Patients also received the melanoma antigen peptide vaccines gp100 and MART-1 in incomplete Freund's adjuvant subcutaneously. The therapy was well tolerated and no MTD was reached. While no anti-tumor effects were observed, rhIL-7 was given for limited time combined with a limited number of vaccines, therefore limiting the conclusions that could be drawn regarding its capacity to enhance vaccine responses. Of note, CD4⁺ and CD8⁺ T-cell subsets increased in this trial in a dose-dependent manner. However, there was a relative decrease in regulatory T cells, making this cytokine distinct from IL-2. There was also an increase in B cell precursors in the bone marrow of some patients, but no changes in B cell numbers were noted peripherally^[48].

The second rhIL-7 phase I trial reported 16 patients with refractory malignancies using the same doses as the first trial, but IL-7 was given every other day for 14 days and no vaccines were administered. The therapy was well tolerated and no MTD was reached. No anti-tumor effects were observed, however rhIL-7 increased both CD4⁺ and CD8⁺ T cells, including central memory subsets, in a dose-dependent fashion and these increases lasted for weeks after discontinuation of the cytokine. The mechanism of expansion appeared to be augmentation of peripheral cycling with a propensity for cycling of naïve populations. While enhanced thymic output could not be definitively discerned, rhIL7 induced increased T cell repertoire diversity as measured by spectratyping, presumably due to enhanced cycling of recent thymic emigrants. Notably, Tregs were not increased, making this clearly cytokine distinct from IL-2^[49].

Lastly, a phase I trial using melanoma cells engineered to express IL-7 lead to an increase in melanoma-reactive T cells in three out of six patients. Minor antitumor responses were observed in two patients^[50]. While this trial is not the equivalent of giving IL-7 directly, it further demonstrated that IL-7 is well tolerated in vivo and is effective in mediating effector T cell expansion without associated Tregs.

Potential antitumor applications of IL-7: While IL-7 alone does not seem to eliminate tumors directly, rationale combination with other immunotherapies may be beneficial. In preclinical models, IL-7 therapy potently enhances vaccine-mediated immunity^[51,52]. Combining intralesional IL-7 with other therapies, such as Radiofrequency Ablation (RFA), induces immune responses to breast tumors, inhibits tumor development and lung metastasis and reduces myeloid-derived suppressor cells^[53]. Combining IL-7 with local hyperthermia also enhances anti-tumor activity in mice

with melanoma^[54] and combining IL-7 and lymphocytes results in prolonged survival from colon cancer^[55]. In a preclinical neuroblastoma xenograft model, combining IL-7 and gamma-delta T cells with an anti-GD2 antibody significantly improved survival^[56]. Thus in both adult and pediatric solid tumor models, IL-7 has the capability to be an effective adjuvant. Recently, adjuvant IL-7 was shown to improve vaccine mediated survival in a spontaneously occurring murine tumor model via enhanced Th17 differentiation and reduced T cell-intrinsic inhibitory networks^[51].

Finally, IL-7 may have utility after allogeneic HSCT, where it may enhance Graft-Versus-Leukemia (GVL) effects by potentiating alloreactive T cells^[57]. Thus, the available preclinical data and limited data from clinical trials would indicate that via multiple mechanisms, IL-7 is a very promising agent to enhance overall immune competence and, potentially, tumor specific immune responses. The absence of Treg expansion and the lack of toxicity observed in this clinic would suggest that IL-7 offers definite advantages over IL-2 as an adjuvant.

Potential limitations of IL-7 therapy: A potential concern regarding IL-7 therapy is that it may signal tumors directly^[58-62], promoting growth/survival (Table 2). CD127 expression has been reported on some adult solid tumors^[58], but not on pediatric solid tumors, but it is not clear if these tumors have the capacity to signal through IL-7. IL-7 does play a role in either the initiation or maintenance of some leukemias and lymphomas and therefore will need to be used with extreme caution in immunotherapy regimens involving lymphoid malignancies. It could be that malignant tissues alternatively splice IL-7, as shown in neuronal tumors and pediatric ALL^[63,64], which suggests that some tumors could generate their own supply of IL-7 for survival or possibly use an isoform as a means of local IL-7 receptor blockade on effector cells. Some neuronal tumors also alternatively splice the IL-7 receptor, suggesting the tumor could modulate their ability to respond to exogenous IL-7^[64].

While IL-7 has been shown to enhance GVL responses after allogeneic HSCT, it may also exacerbate Graft-Versus-Host-Disease (GVHD)^[65,66]. Thus, the use of IL-7 in the allogeneic setting may be most effective in the setting of T cell-depleted grafts^[67]. IL-7 over expression has been described to create an osteoclastogenic microenvironment within the bone marrow, which promotes the commitment of precursors towards the osteoclast lineage, leading to bone loss^[68]. Lastly, additional pre-clinical work has shown that IL-7 therapy may generate a suppressive DC that does not

present antigen effectively^[45]. Careful selection of tumors along with close monitoring of bone density and the development of autoimmunity may be necessary in future trials. However, the overall experience with IL-7 thus far would indicate that clinical trials with this cytokine in multiple settings, including as a vaccine adjuvant, are warranted.

Interleukin-15:

Background: IL-15 is constitutively expressed by a variety of cell types and tissues, but in contrast to IL-2, is mainly membrane bound. A thorough review of IL-15 biology and receptor physiology has been described elsewhere^[1,2,69]. IL-15 and IL-2 exhibit similar immune effects and share the IL-2 receptor subunits IL-2Rbeta and IL-2Rgamma(c), but each cytokine has a separate alpha receptor (Ra). One unique feature of IL-15 is the requirement for cross presentation by IL-15Ra in order to induce optimal biologic activity. Unlike most cytokines that function as soluble mediators, IL-15 appears to function primarily as a “cell associated” molecule and therefore is highly dependent upon an available reservoir of IL15Ra⁺ cells for optimal biologic activity. It is possible that this feature of IL-15 biology will have important implications for how best to utilize this cytokine as a therapeutic agent^[70].

IL-15 is required for the differentiation of NK cells^[71] and plays a role in maintaining and expanding CD8⁺ T cells (particularly memory subsets)^[72-74], NK cells^[75], NKT cells^[76,77], interferon-killer DCs^[78,79] and gamma-delta T cells^[80,81]. In addition to effects on T cells and NK cells, IL-15 may also sustain B cells^[82] and convert polymorphonuclear cells into APCs^[83] (Fig. 1). Finally, there has been a number of reports exploring NK cell-DC cross talk and IL-15 is presented by DCs to NK cells to enhance survival^[84,85]. Based upon these properties, investigators have incorporated IL-15 with or onto artificial APCs to expand NK cells^[86-88]. IL-15 has not been tested in clinical trials yet, but is being developed by the NCI for clinical use^[2]. Thus, exploration of preclinical data is warranted to gain a full understanding of the potential impact in the clinic.

IL-15 and preclinical tumor models: While IL-15 may have direct anti-tumor effects, most studies demonstrate that it acts as an adjuvant to enhance anti-tumor immunity, as was described with IL-2 and IL-7. IL-15 is superior to IL-2 in lung adenocarcinoma models^[89] and can improve immunity against colon cancer^[90,91]. Moreover, IL-15 treatment increased amphoterin (HMGB1) secretion by colon cancer cells, which was associated with a depletion of tumor-associated macrophages^[92]. In melanoma, IL-15 can

mediate regression of established tumor^[93] and may also enhance tumor-resident CD8⁺ T cells rather than attract newly infiltrated T cells^[94]. When combined with IL-7 or IL-12, it may even be better than the “gold standard” of IL-2 to enhance T cell-mediated killing of melanoma^[95,96]. NK cells show enhanced killing of Ewing sarcoma cells after IL-15 administration^[97]. RFA of breast tumors combined with intralesional IL-15 (and IL-7) inhibits tumor development and metastasis^[53]. IL-15 enhances NK cell cytotoxicity of human glioblastoma cells, which are resistant to freshly isolated NK cells^[98]. IL-15 also can reverse the unresponsiveness to the antigen WT-1 in prostate cancer lines, leading to restored expansion and gamma interferon production of WT1-specific T cells^[99]. Thus IL-15 may enhance anti-tumor immune responses to a wide variety of pediatric and adult malignancies.

There have been numerous preclinical studies exploring IL-15 as a vaccine adjuvant. Although the majority has been in infection models, a number of reports of the adjuvant effect of IL-15 have been published. One important observation is that IL-15 can revert tolerant T cells to become effectors^[100]. Adjuvant use of IL-15 can enhance vaccine responses to both dominant and subdominant, tumor antigens^[101]. Recently, IL-15 administered after a gene-modified vaccine resulted in enhanced anti-tumor activity in a murine melanoma model^[96]. After allogeneic HSCT, IL-15 seems to upregulate NK cell activating receptors^[102] and administration of IL-15 with IL-2 enhanced NK-DLI-mediated GVL^[103]. Importantly, although IL-15 is thought to act primarily on mature T cells, it may prove to be beneficial after T cell-depleted HSCT^[104]. Based on the available pre-clinical data, IL-15 would appear to be well suited as an adjuvant to cancer vaccines.

Potential limitations of IL-15 therapy: The main limitation is that IL-15 is not yet readily available. Despite the fact IL-15 was cloned in 1994, rhIL-15 remains under development by the NCI^[2]. Some caution should be expressed with IL-15 administration in certain tumor types^[42,105-108], since there is both evidence of IL-15R expression and involvement in tumoral progression (Table 2). Normal kidney expresses a functional IL-15 receptor^[109] and human RCC expresses an IL-15R that seems to be directly involved in renal tumoral progression^[110]. In pediatric ALL, high IL-15 expression correlates with CNS involvement^[111], but those children with high IL-15Ra expression have a significantly better probability of survival at 5 years^[112]. Mice that have transgenic overexpression of IL-15 also develop a fatal large granular leukemia^[113].

Besides directly stimulating tumor growth, IL-15 may also enhance endogenous immunosuppressive pathways. Umbilical cord blood-derived Tregs stimulated with IL-2 and IL-15 express higher levels of Cytotoxic T Lymphocyte Antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor superfamily member number 18 (GITR), membrane bound Transforming Growth Factor (TGF)-beta and FOXP3, leading to higher production of IL-10 and TGF-beta^[114]. Even Tregs from peripheral blood can be generated and sustained partially with IL-15 in the absence of IL-2^[115,116]. After allogeneic HSCT, trans-presentation of donor-derived IL-15 is needed for acute GVHD but not for GVL effects^[117]. Given the described effects of IL-15 in sustaining memory T cells, there is concern that IL-15 could potentiate GVHD by supporting alloreactive memory T cells^[118] and IL-15 has been shown to exacerbate xenogeneic GVHD^[119]. Lastly there is an extensive literature on the effects of IL-15 on total body fat mass as well as promoting autoimmunity^[69]. As is the case with IL-7, there is great promise for enhancing anti-tumor immunity with IL-15, but the potential to signal tumors and possibly Tregs, as well as to the potential for induction of autoimmunity, remain valid concerns.

Interleukin-21:

Background: IL-21 is homologous to IL-15, but the receptor for IL-21 is comprised of a unique subunit designated IL-21Ra and the IL-2Rgamma(c)^[120,121] and there is no evidence that IL-21 requires trans-presentation for biologic activity. IL-21Ra is expressed on most mature lymphocyte populations (Fig. 1). Production of IL-21 is restricted to activated CD4⁺ T helper cells^[122,123]. IL-21 appears to play important roles in modulating responses of lymphocytes to other cytokines. While IL-21 alone does not affect receptor expression, IL-21 can synergize with IL-2 to up-regulate several surface receptors, including NKG2A, CD25, CD86 and CD69^[124]. In certain tumor models IL-21-enhanced tumor rejection is NKG2D dependent^[125], however IL-21 does not support NK cells and in fact, has been shown to limit NK cell expansion and induce apoptosis^[126,127]. IL-21 alone does not induce T cell proliferation, however IL-21 can enhance the effects of other stimuli of proliferation, such as other gamma(c) cytokines^[128-130]. IL-21 also has a role in B cell proliferation^[128] but may uniquely also induce B cell apoptosis^[131]. IL-21 has also been shown to induce IL-10 production in models of lupus, suggesting that like IL-2, it can also contribute to immunosuppressive activity^[132]. Thus, the available pre-clinical data would suggest that IL-21 may work

best in combination with other gamma(c) cytokines in the adjuvant setting.

Clinical trials with IL-21: There have been 3 clinical trials with IL-21. In a phase I trial of 43 patients with metastatic melanoma and RCC, IL-21 was administered in two 5-day cycles on days 1 through 5 and 15-19, of a treatment course. Doses ranged from 3-100 ug kg⁻¹ dose⁻¹ and an expanded cohort was treated at the MTD, estimated to be 30 ug kg⁻¹. Twenty-eight patients were treated in the expanded cohort. Twelve patients received up to five additional two-cycle courses of treatment without cumulative toxicity, except for one patient with reversible grade IV hepatotoxicity. Antitumor activity was observed in both melanoma (1CR, 4%) and RCC (4PR, 21%)^[133].

In another open-label, two-arm, dose escalation phase I trial of IL-21 involving 29 patients with metastatic melanoma, dose levels from 1-100 ug kg⁻¹ were utilized in two parallel treatment regimens: Thrice weekly for 6 weeks (3/week) or three cycles of daily dosing for 5 days followed by 9 days of rest (5+9). The MTD was also 30 ug kg⁻¹ for both regimens. One PR was observed after the 3/week regimen and became a CR 3 months later^[134].

In a phase II, open-label, single-arm, two-stage trial study of IL-21 (30 ug kg⁻¹ dose⁻¹) was administered in 8 week cycles (5+9) in patients with metastatic melanoma. No toxicity was observed and the best tumor response included 1 CR and 1 PR, both with lung metastases^[135]. Pharmacodynamic studies show that IL-21 affects the serum levels of several cytokines, chemokines, acute-phase proteins and cell adhesion proteins in a dose-dependent fashion^[136]. In the (5+9) regimen, IL-21 induced a dose-dependent decrease in circulating NK cells and T cells, followed by a return to baseline in resting periods. In both CD8⁺ T cells and NK cells, up-regulation of perforin and granzyme B mRNA was observed. Finally, cytotoxicity assays showed that IL-21 enhanced the ability of NK cells to kill sensitive targets *ex vivo*^[137].

Potential antitumor applications of IL-21: IL-21 may have direct anti-tumor effects. For example, the majority of Chronic Lymphocytic Leukemia (CLL) patients have surface IL-21Ra, its expression correlates with apoptosis^[107] and IL-21 counteracts the proliferative and antiapoptotic signals delivered by IL-15 to CLL B cells^[138]. In addition to its pro-apoptotic effect, IL-21 promotes NK cell-mediated antibody-dependent cellular cytotoxicity against rituximab-coated CLL cells *in vitro*^[139]. While follicular lymphoma cells show high levels of IL-21R, addition of

the cytokine inhibits proliferation and induced apoptosis^[140]. Gene-modified melanoma cells that express IL-21 grow slower than nonmodified cells *in vitro* and *in vivo*^[141]. IL-21 has also been shown to exert activities on vascular Endothelial Cells (ECs), leading to decreased angiogenesis related gene expression^[142], decreased proliferation and sprouting of activated ECs after IL-21 treatment, disturbing vessel architecture and negatively affecting vessel outgrowth. A murine myeloma cell vaccine containing IL-21 plasmid DNA induced significant tumor regression and prolonged survival^[143]. IL-21-secreting RENCA cells were efficiently rejected following subcutaneous injection into syngeneic mice^[144]. Similar results were seen in a mouse bladder carcinoma genetically modified to express IL-21^[145]. Finally, using a glioblastoma transduced to express IL-21, 100% of the animals rejected the tumor and 76% of these animals survived a subsequent tumor re-challenge, while other transduced cytokine genes were not as effective^[146].

IL-21 may also improve the potency of effector cells and other gamma(c) cytokines. For instance, administering IL-21 locally to melanoma tumors enhanced the therapeutic effects of adoptively transferred gp100-specific T cells and was synergistic with IL-2, leading to an increased proliferation of local CD8⁺ T cells and decreased accumulation of Tregs within the tumor microenvironment^[147]. IL-21 also improves expansion and effector function of gamma-delta T cells and reverses expression of inhibitory receptors. IL-21 can be combined with IL-2 to enhance gamma-delta T cell-mediated antitumor responses^[148]. Use of IL-21 and IL-2 in culture up-regulate cytokine production of activated tumor-draining lymph node cells and enhances their therapeutic efficacy against established pulmonary metastatic fibrosarcomas. Animals treated with combined IL-21 and IL-2 showed protective immunity against tumor rechallenge, with expansion of memory T cells, antibody production and significantly elevated serum levels of IFN-gamma and IL-10^[149].

Besides enhancing IL-2 therapy, IL-21 may also improve the effectiveness of other cytokines and immunotherapies. Combining alpha interferon and IL-21 increases NK cell and CD8⁺ T-cell-mediated cytotoxicity in an experimental model of RCC, leading to inhibition of tumor growth and an increased survival^[150]. IL-21 can also significantly augment IL-7-induced expansion of cytotoxic T cells, possibly by preventing the cytokine-induced down-regulation of CD127 on antigen-stimulated T cells, results which suggest that IL-21 may also play a cooperative role with IL-7 in modulating primary CD8⁺ T-cell

responses^[151]. Several monoclonal antibodies targeting TAAs also have improved antitumor activities in mice when used in combination with IL-21^[152] and human NK cells cultured with IL-21 and human breast cancer cells coated with trastuzumab showed enhanced lytic activity^[153]. Lastly, in regards to a pediatric tumor, vaccinating with IL-21-gene-modified cells in a syngeneic metastatic neuroblastoma model demonstrated a reduction of microvessels in late metastases from therapeutically vaccinated mice. A role of survivin as a tumor antigen was suggested since a specific T cell response against this antigen was induced^[154].

Interestingly, the route of IL-21 administration may be critical. Whereas both Subcutaneous (SC) and Intraperitoneal (IP) routes of IL-21 administration significantly inhibit growth of small, established RCC and melanoma tumors, only SC therapy significantly inhibited the growth of large, established tumors. Greater bioavailability and significant drainage of IL-21 to regional lymph nodes was observed following SC administration, which could account for the apparent increase in anti-tumor activity. In the RCC model, SC administration of IL-21 led to a significantly higher density of tumor infiltrating CD8⁺ T cells compared to IP^[155].

Limitations to IL-21 therapy: As with the other gamma(c) cytokines, IL-21 receptor has been observed on multiple tumor types^[156-158] and IL-21 has contributed to tumorigenesis (Table 2). IL-21 shows divergent effects depending on the cell origin: growth stimulation in B cell lymphoma cell lines and adult T cell leukemia/lymphoma cell lines but induction of apoptosis in follicular lymphoma^[140]. IL-21 has also been implicated in the pathogenesis of autoimmunity in a number of models^[159]. As with the other gamma(c) cytokines, care in selecting the relevant tumor types as well as care in not to enhance Treg activity or cause autoimmunity is warranted.

CONCLUSION

Although initial clinical trials using IL-2 as a vaccine adjuvant demonstrated only modest effects in the clinic, combination immunotherapies using newer gamma(c) cytokines to promote NK cell and T-cell expansion and effector function are promising strategies to enhance immunotherapy of tumors. A number of different vaccine strategies in both preclinical and clinical studies have shown potentiation with concomitant IL-7, IL-15 and IL-21. These results have involved various regimens of adjuvant cytokine

therapy, with differences in dosing, time of administration and schedule leading to different outcomes. Further research is needed to determine the most potent vehicles of vaccination as well as effective doses and schedules for cytokine delivery. Combinations of cytokines may be warranted. Future T cell-based immunotherapies will likely combine regimens that optimize vaccination and/or adoptive cell therapy with growth-promoting cells that can augment anti-tumor immunity while limiting autoimmunity responses. Caution needs to be exercised that tumors themselves are not signaling by these cytokines so that relapse is not promoted and patients should be monitored for autoimmunity where possible.

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