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Immunotherapy of Cancer by IL-12-based Cytokine Combinations

Jonathan M. Weiss¹, Jeff J. Subleski¹, Jon M. Wigginton², and Robert H. Wilttrout¹

¹Cancer and Inflammation Program, Laboratory of Experimental Immunology, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702

²Merck Research Laboratories, North Wales, PA 19454

Abstract

Cancer is a multi-faceted disease comprising complex interactions between neoplastic and normal cells. Over the past decade, there has been considerable progress in defining the molecular, cellular and environmental contributions to the pathophysiology of tumor development. Despite these advances, the conventional treatment of patients still generally involves surgery, radiotherapy and/or chemotherapy and the clinical outcome for many of these efforts remains unsatisfactory. Recent studies have highlighted the feasibility of using immunotherapeutic approaches that seek to enhance host immune responses to developing tumors. These strategies include immunomodulatory cytokines, with tumor necrosis factor (TNF) alpha, type I or type II Interferons (IFNs), Interleukins (IL)-2, IL-12, IL-15 and IL-18 being among the most potent inducers of anti-tumor activity in a variety of pre-clinical studies. More recently, some exciting new cytokines have been characterized, such as IL-21, IL-23, IL-27, and their immunomodulatory and anti-tumor effects *in vitro* and *in vivo* suggest that they may have considerable promise for future immunotherapy protocols. The promise of cytokine therapy does indeed derive from the identification of these novel cytokines, but even more fundamentally, the field is greatly benefiting from the ever-expanding amount of pre-clinical data that convincingly demonstrate synergistic and/or novel biological effects that may be achieved through the use of several combinations of cytokines with complementary immune-stimulating capabilities. One cytokine in particular, IL-12, holds considerable promise by virtue of the fact that it plays a central role in regulating both innate and adaptive immune responses, can by itself induce potent anti-cancer effects, and synergizes with several other cytokines for increased immunoregulatory and anti-tumor activities. This review will discuss the anti-tumor activity of IL-12, with a special emphasis on its ability to synergize with other cytokines for enhancement of immune effector cell populations and regulation of host-tumor cell interactions and the overall tumor microenvironment.

Keywords

Interleukin 12; cytokine; immunoregulation; cancer

1. Introduction

Interleukin (IL)-12 has emerged as a promising molecule for anti-tumor immunotherapeutic protocols. Originally characterized in the early 1990s as a cytokine with the potent ability to activate T and natural killer (NK) cells [1], it has also been described to possess other key immunomodulatory effects [2,3]. Principally, IL-12 facilitates the presentation of tumor antigens through the upregulation of class I and II major histocompatibility (MHC) molecules, as well as the generation of T helper type I (Th1) immune responses. These effects are tightly

associated with the ability of IL-12 to induce expression of interferon gamma (IFN- γ). In this regard, IL-12 primes developing T cells to stably produce IFN- γ [4], and the endogenous production of IFN- γ is required for the anti-tumor effects of IL-12 administration in most, if not all, tumor studies. In our own studies, endogenous IFN- γ production is required for all of the IL-12 based immunotherapeutic regimens we have evaluated to date [5-7]. Furthermore, IL-12 exerts anti-angiogenic properties, albeit indirectly, through downstream mediators such as IFN- γ -inducible protein 10 (IP-10) and monokine-induced by IFN- γ (Mig) [8].

As a single agent, IL-12 exerts potent anti-tumor effects in many preclinical rodent tumor models. The administration of IL-12 into tumor-bearing mice can delay, reduce and, in some cases, completely inhibit tumor development [2]. As has been extensively discussed, IL-12 has demonstrated significant therapeutic efficacy in many solid tumors as well as hematological leukemias and lymphomas (reviewed in [9]). There is accumulating evidence that the anti-tumor effects of IL-12 are mediated, at least in part, by indirect mechanisms. The downstream induction of cytokines such as IFN- γ , in particular, results in the upregulation of class I and class II MHC molecules, adhesion molecules, such as Intercellular adhesion molecule-1 (ICAM-1), nitric oxide production by antigen presenting cells (APC), and the production of additional cytokines, such as Mig and IP-10, which in turn mediate anti-angiogenic effects.

Recently, the intra-tumoral injection of IL-12 has been shown to elicit significant anti-tumor activity in mice and humans. Using two different animal models, B16 melanoma in C57BL/6 mice and human melanoma xenografts in nude mice, Heinzerling et al showed that the treatment with DNA coding for murine IL-12 induced regression of all tumors through a mechanism that was completely dependent upon NK cells [10]. The therapeutic effect was associated with evident anti-neovascular effects. In humans, these same authors demonstrated the feasibility of administering plasmid DNA encoding IL-12 to patients with metastatic melanoma [11]. The therapy involved increasing doses of DNA and was generally well tolerated. Overall, 3 out of 9 patients demonstrated clinical responses that included disease stabilization and/or complete remission.

Additional studies have demonstrated the effectiveness of treating cancer patients with recombinant human IL-12 protein. In one study, cancer patients receiving increasing doses of IL-12 were profiled for changes in circulating cytokines after treatment [12]. Intriguingly, several potential immunosuppressive mechanisms were engaged upon repeated IL-12 administration, including a Th1 to Th2 shift as illustrated by an increase in IL-10 expression and diminishment of IFN- γ , tumor necrosis factor alpha (TNF α) and IP-10 in the sera of patients repeatedly treated with IL-12 [12,13]. As will be discussed later, this observation indicates a potential limitation for the use of IL-12 as a single agent. In contrast, the inhibition of IFN- γ production that is observed after repeated IL-12 administration can be overcome through the combined administration of other cytokines, such as IL-2.

IL-12 is also distinguished by its ability to cooperate, and in some cases synergize, with the co-exposure and/or co-administration of additional immunomodulatory factors and cytokines. In one early study, the ability of IL-12 to induce IFN- γ , TNF α and GM-CSF production from T and NK cells was found to be greater when cells were co-treated with IL-12 and IL-2 [14]. In another study, IL-12 synergized with the co-administration of anti-CD28 antibodies to elicit higher levels of T cell proliferation and IFN- γ production [15]. Similarly, immunotherapies that combined IL-12 with a vaccine composed of MHC class II, CD80 transfected tumor cells could achieve the greatest degree of T and NK cell activation, IFN- γ production and overall anti-tumor activity [16]. The production of IL-12 from APC thereby forms a critical bridge between the innate and adaptive branches of immunity. In this review, we will describe recent studies *in vitro* and *in vivo* that best illustrate the promise for combination cytokine immunotherapies that involve IL-12.

2. IL-2/IL-12

IL-2 is a cytokine that exerts potent immunoregulatory effects upon effector and regulatory T cells, NK and NK-T cells (reviewed in [17]). The rationale for combining IL-2 and IL-12 therapies stems primarily from the fact that the two cytokines reciprocally upregulate each other's receptors and use separate signaling pathways to induce different but complementary biological effects. Figure 1 illustrates a schematic of the functional consequences of IL-2 and IL-12 cytokine family members interacting with their corresponding receptors. Both IL-2 and IL-12 can stimulate mitogen or CD3-activated T cells to proliferate, produce IFN- γ and kill target tumor cells. However, when applied in combination, these two cytokines synergize with each other. This synergy involves a dramatic augmentation in the biological effects of each cytokine, principally the synergistic increase in both the phosphorylation of signaling proteins and the magnitude of T cell functional responses [18].

Several pre-clinical studies have underscored the promise for IL-12/IL-2 combination therapies. Many of these studies have utilized genetic approaches, which have the particular advantage of being more cost effective and feasible when recombinant proteins are difficult to obtain in the amounts necessary for *in vivo* protocols. It is noteworthy that many of the characteristic downstream biological effects can be achieved to a similar degree using IL-12 gene approaches as are observed in response to the administration of IL-2 and IL-12 proteins. For example, the use of an IL-12/IL-2 bi-functional fusion protein that contains both IL-2 and IL-12 genes in one DNA construct resulted in similar levels of IFN- γ as compared to the administration of both cytokines as protein [19]. Functionally, the transfection of both IL-2 and IL-12 genes into the spleens of rats bearing liver tumors resulted in the significant enhancement of NK cell activity and an increase in mouse survival time with low toxicity [20]. In another study, the delivery of both IL-2 and IL-12 genes into mice bearing B16 melanoma elicited a significant reduction in tumor burden and enhancement in overall survival [21]. Combined IL-2 and IL-12 gene therapy has also been used in a murine model for head and neck squamous cell carcinoma, in which the significant anti-tumor effects in the IL-2/IL-12 treatment groups was also strongly correlated with enhanced cytotoxic T and NK cell activation [22].

Our own laboratory has shown that an IL-12/pulse IL-2 treatment regimen involving the administration of intermittent high doses of recombinant human IL-2 with repeated doses of murine IL-12 proteins leads to the complete regression of a murine spontaneous breast tumor [23] as well as transplantable mouse renal cell carcinomas [5,24]. This process is associated with the local production of IFN- γ and IP-10 that induce the recruitment and activation of CD8⁺ T cells in the tumor microenvironment. Moreover, these newly recruited T cells express Fas ligand (FasL) in an IFN- γ dependent manner, and are thereby capable of inducing the FasL-mediated apoptosis of Fas-positive tumor and endothelial cells and inhibiting tumor neo-vascularization [5]. Consistently, the IL-12/pulse IL-2 mediated tumor regression was completely lost in mice that either lacked IFN γ or expressed a disrupted Fas/FasL signaling pathway [5,23]. In this model system, we clearly demonstrate that the potent therapeutic activity of the IL-12/pulse IL-2 combination rapidly engages potent apoptotic and anti-angiogenic mechanisms that remain active during the delivery of this treatment regimen. Furthermore, we show that the immunomodulatory effects of IL-12/pulse IL-2 can be effective against established tumors as well as developing preneoplastic lesions. The IL-12/pulse IL-2 schedule has now been translated into human clinical trials at the National Institutes for Health (NIH) in patients with advanced solid tumors in clinical trials at both the National Cancer Institute (Bethesda, MD) and Beth Israel Deaconess Medical Center (Boston, MA) (reviewed in [9]). Both of these trials are ongoing, yet they have already demonstrated some suggestion of potential efficacy.

The contrasting effects of IL-12 alone and in combination with IL-2 in the clinic are highlighted by two separate phase I trials where patients with metastatic renal cell cancer or malignant melanoma were treated with escalating doses of IL-12 [25,26]. At the maximum tolerated dose of 500 ng/kg via the intravenous route, one patient in the earlier trial exhibited a partial response and two patients had prolonged disease stabilization after 6 or 8 cycles of rhIL-12 administration, respectively [25]. The chronic administration of IL-12 was well-tolerated and was shown to efficiently induce IFN- γ production. Interestingly, there was an attenuation of IFN- γ production midway through the first cycle, and this could be partially overcome through the co-administration of IL-2 along with IL-12 [25]. Strategies involving the addition of IL-2 to IL-12 treatment regimens may thus be able to lengthen the duration of immune stimulation by IL-12 *in vivo* and perhaps reduce the effective dose of IL-12 to more easily tolerated ranges. In a follow-up Phase I trial using twice-weekly intravenous administration of human IL-12 plus low-dose IL-2 in melanoma or renal cell carcinoma patients, the IL-2/IL-12 combination therapy was again well-tolerated with rapidly reversible toxicities including fever or chills, anemia, fatigue, nausea or vomiting and orthostatic hypertension being most commonly reported [26]. In this later study, there was one partial clinical response and two pathologic responses among the melanoma patients evaluated. The inclusion of IL-2 along with IL-12 was associated with a significant expansion of natural killer (NK) cells and a significant increase in IFN- γ and IP-10 expression, both of which are key immunomodulatory cytokines (further discussed later) involved in regulating tumor vascularization as well as the recruitment, activation and priming for cell killing of effector immune cells, such as NK and cytotoxic T cells.

IL-2 and IL-12 combination therapies can also be used for the *ex vivo* enhancement of anti-tumor priming of lymphocyte cultures. In a further characterization of the effects of IL-12/IL-2 therapy upon leukocyte function, one early study treated cancer patients' lymphocyte cultures with IL-2 and IL-12 for at least 10 days and generated significant pools of lymphokine-activated killer and cytotoxic T cells that had augmented cytolytic activity against tumor targets, as compared to those cultures treated with either IL-2 or IL-12 alone [27]. Certainly, the effects of IL-2 and IL-12 combination therapy *in vitro* suggest that there may be opportunities for the treatment of cells with IL-2 and IL-12 *ex vivo* to enhance their cytolytic and anti-tumor activities.

3. IL-15/IL-12

IL-15, another member of the common γ_c family (Figure 1), shares some biological activities with IL-2 that also makes it potentially useful for therapies against cancer. These include stimulating the proliferation of NK cells and activated T cells and supporting the expansion of effector T cells (Reviewed in [17,28,29]). However, biological differences exist between IL-15 and IL-2 that may make IL-15 a better candidate for cancer immunotherapy. IL-2 potentiates activation induced cell death (AICD) of CD4⁺ T cells [30,31], while IL-15 protects cells from IL-2 induced AICD [32]. Furthermore, IL-2 was found to be important in maintaining Treg cells that suppress T cell responses [33-35], while IL-15 activates the proliferation of memory CD8⁺ T cells [36].

Because IL-2 is a potent adjuvant given with IL-12 (see IL-2/IL-12 section) and because IL-15 shares many biological properties with IL-2, yet has important beneficial differences from IL-2, it is reasonable to expect that IL-15, when used as an adjuvant with IL-12, would induce significant anti-tumor activity. Indeed, it was found that the intra-tumoral delivery of IL-12 and IL-15 induced significant tumor regression in a B16F10 experimental melanoma model [37]. The combination of IL-12 plus IL-15 induced enhanced anti-tumor activity over either cytokine alone that was correlated with the reciprocal up regulation of each cytokine's receptors through the synergistic induction of IFN- γ . Additionally, it was found that IL-12 plus IL-15

stimulates anti-tumor activity in peritoneal macrophages through the synthesis of nitric oxide [37]. A role for macrophages in the anti-tumor activity of IL-12 plus IL-15 was also found using nude mice that had been xenotransplanted with N592, a MHC class I negative small lung cancer cell line. In this model, complete rejection of N592 tumor cells engineered to secrete IL-12 and IL-15 was found to be independent of T or NK cells [38]. In addition, these authors found that the anti-tumor activity of TS/A, a murine adenocarcinoma, engineered to secrete both IL-15 and IL-12, was independent of IFN- γ [39]. Although the anti-tumor activity of IL-15 and IL-12-secreting TS/A or N592 tumor cells was found to be independent of IFN- γ and NK cells, another group using a methylcholanthrene-induced fibrosarcoma line found the anti-tumor activity from exogenous IL-15 and IL-12 was dependent on NK cells and partially dependent on IFN- γ [40]. Thus the mechanism for the anti-tumor activity of IL-15 plus IL-12 combination therapies may depend on the type of tumor, its location and/or the delivery mode of the cytokines.

Perhaps the biggest potential for IL-12 and IL-15 combination in cancer therapies will be the use of IL-12 to prime and IL-15 to boost and maintain the anti-tumor response. In this way, one takes advantage of each cytokines' biological functions, namely the ability of IL-12 to rapidly activate the innate response and that of IL-15 to potently stimulate the proliferation of T cells and maintain memory CD8⁺ T cells. One report studied this rationale using a neuroblastoma mouse model. The sequential vaccination of mice with Neuro2a cells engineered to secrete IL-12 followed by Neuro2a cells engineered to secrete IL-15 gave better protection to mice challenged with Neuro2a than did giving cells expressing either cytokine alone or both at the same time [41]. These authors went on to show that sequential delivery of IL-12 and IL-15 expressing cells also cured mice in a therapeutic setting of established tumor. Depleting CD8⁺ cells eliminated the protection of this therapy, suggesting clonal expansion of tumor CTL [41].

4. IL-7/IL-12

IL-7 is another member of the IL-2 family of cytokines (see Figure 1) that is critical for normal B and T cell lymphopoiesis and, similar to IL-15, regulates the homeostasis of survival and proliferation of naïve [42] and memory [43] CD8⁺ T cells [17]. IL-7 has been used to enhance CTL generation against murine sarcomas [44] and mediate the reduction in lung metastases in a murine RCC model [45]. Although few studies have investigated the combined use of IL-12 with IL-7 for anti-tumor therapies, there is good rationale for such investigations. IL-12 acts directly on CD8⁺ T cells to enhance their IL-7 mediated proliferation [46]. Furthermore, IL-7 could stimulate lymphocyte proliferation and was particularly effective at synergizing with IL-2 for the restoration of cytolytic activity of lymphocytes against autologous tumors [47]. Thus, the addition of IL-7 to regimens of IL-12 or IL-12 plus IL-2 may result in more optimal proliferative responses and enhanced cytolytic activity against tumors.

5. IL-21/IL-12

The T cell-derived cytokine, IL-21, is an important regulator of NK and T cell function that bridges innate and adaptive immune systems. Both the IL-21 cytokine and receptor possess sequence homology to IL-2 and IL-15 [48] and the widespread lymphoid distribution of IL-21 receptor implicates this cytokine in a broad array of immune regulating activities. While some cellular responses to IL-21 are similar to those of IL-2, it is becoming increasingly clear that IL-21 exerts unique cellular effects as well. IL-21 promotes the maturation of NK cells from bone marrow progenitors and activates human peripheral NK cells [48]. It also promotes NK expansion and maturation, and enhances CD8⁺ T cell-mediated effector functions [49]. The systemic treatment with IL-21 also promotes NK cell-mediated anti-metastatic activity. Using a plasmid DNA vector encoding IL-21, Wang et al demonstrated the dose-dependent inhibition

of subcutaneously-administered B16 melanoma and MCA205 fibrosarcoma with no apparent toxicity [50]. Furthermore, the depletion of NK cells was found to completely inhibit the anti-tumor effects of IL-21.

The biological effects of IL-21 warrant investigation into its potential to synergize with IL-12. Intriguingly, the treatment of the NK-92 cell line with IL-21 resulted in a significant upregulation in the mRNA expression of IL-12 receptor beta 2, IL-18 receptor (alpha and beta), IL-2 receptor alpha, and IFN- γ genes, as well as other Th1-related immune response genes [51]. Furthermore, the pretreatment of NK cells with IL-21 strongly enhanced IL-12-induced STAT4 DNA binding. These results suggest that IL-21 can play a key role in enhancing IL-12 signal transduction and may cooperate for increased IFN- γ production. Recently, Smyth, et al treated mice sequentially with adoptively transferred alpha-galactoceramide (α GalCer)-stimulated dendritic cells (DC) followed by systemic IL-21 administration to achieve a significant reduction in MCA-induced sarcomas and metastases [52]. Although the treatment of mice with IL-12 followed by IL-21 was not as effective as treatment with α GalCer pulsed DC plus IL-21 in this model, it will be interesting to evaluate the anti-tumor efficacy and T/ NK cell responses when the two cytokines are administered concomitantly or when IL-21 is administered before IL-12, similar to the IL-12/pulse IL-2 regimen currently underway in the clinic.

6. IL-18/IL-12

Interleukin-18, first identified as IFN- γ inducing factor [53], is a member of the IL-1 superfamily of proinflammatory cytokines. Figure 2 illustrates a schematic depiction of the functional consequences of IL-18 and IL-12 cytokine family members interacting with their corresponding receptors. IL-18 induces IFN- γ production, promotes Th1 cell development and NK activation [54], making this cytokine potentially beneficial as an immunotherapeutic agent against cancer. Indeed, IL-18 has been effective in various experimental tumor models [55-57]. However, IL-18 is also implicated in tumor progression. High serum levels of IL-18 are detected in many patients with progressive cancer [58]. Additionally, blocking IL-18 in a B16 melanoma model reduced mouse tumor burden [59].

The administration of IL-18 in combination with IL-12 overcomes limitations observed with using these cytokines alone. Furthermore, IL-12 induces the upregulation of the IL-18 receptor in an IFN- γ -dependent manner [60]. While studies show IL-18 can be pro-angiogenic, (reviewed in [58]), the administration of SCK murine mammary carcinoma cells co-expressing IL-18 and IL-12 to mice reduced tumor burden and inhibited angiogenesis [61]. Th1 cytokines are usually thought to improve anti-tumor responses while Th2 cytokines are often associated with suppression of anti-tumor activity. IL-18 was found to induce Th2 cytokines when given with IL-2, but when given with IL-12, Th1 cytokines predominated [62]. Furthermore, lymph node cells from MCA tumor bearing animals treated with IL-18 were unable to produce IFN- γ , while IL-12 induced the suppressive Th2 cytokine, IL-10. However, when these cells were stimulated with IL-18 in combination with IL-12, a synergistic induction of IFN- γ was observed with an attenuation of IL-10 expression [63]. Similar findings are also reported in cancer patients. The repeated administration of IL-12 to cancer patients revealed elevated serum IL-10 with declining levels of IFN- γ [13], whereas IL-18 treated peripheral blood mononuclear cells from cancer patients were unresponsive for the production of IFN- γ [64]. Our lab has found that giving IL-18 in combination with IL-12 to tumor bearing mice synergistically induced a prolonged serum level of IFN- γ , while tumor bearing mice treated with IL-18 or IL-12 alone induced minimal serum IFN- γ that was rapidly attenuated [65]. In one recent cell-based tumor vaccine study, the intra-tumoral injection of DC engineered to secrete both IL-12 and IL-18 mediated the highest levels of IFN- γ production by CD8⁺ T cells as well as the complete rejection of murine sarcomas [66].

Another unique mechanism associated with anti-tumor activity induced by IL-18 plus IL-12 was the divergent regulation of NK cells and potentially suppressive NK-T cells. We found that the administration of IL-18 plus IL-12 to mice bearing RCC in their liver significantly reduced the number of tumor nodules, which was associated with the diminution in the detection of liver NK-T cells and an increase in liver NK cells [65]. The depletion of NK cells abrogated the anti-tumor activity of IL-18 plus IL-12, demonstrating their effector function. Interestingly, the depletion of NK-T cells or the use of mice deficient in NK-T cells led to an increase in anti-tumor activity in the liver, elucidating a suppressive role for these cells that is removed with IL-18 plus IL-12 treatment. Immunotherapeutic approaches, such as IL-12 plus IL-18, which enhance NK cell function while concurrently eliminating or altering NK-T cells may be effective in the treatment of cancer in the liver. Although IL-18 plus IL-12 therapy shows promise, severe toxicity has been reported. It was found that giving therapeutic doses of IL-18 in combination with IL-12 resulted in systemic inflammation and mortality [57,67]. The toxicity was found to be mediated by massive levels of systemic IFN- γ and the removal of NK cells abrogated this toxicity [67]. Strategies to ameliorate the toxicity associated with this therapy will need to be developed before IL-18/IL-12 combinations will be feasible for use in the clinic.

7. Granulocyte-macrophage colony-stimulating factor (GM-CSF)/IL-12

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is another good candidate for combinatorial cytokine immunotherapy. GM-CSF regulates hematopoietic progenitor cell differentiation and proliferation, and it plays a particularly important role in the maturation of professional APC, including monocyte/macrophages and DC [68]. GM-CSF also enhances the capacity of APC to process and present antigen, which in turn leads to the activation of cytotoxic T cells, increased IFN- γ production and, ultimately, tumor regression [68].

Although GM-CSF acts through a completely different mechanism than IL-12, both cytokines are able to elicit significant anti-tumor responses in several different preclinical tumor models. Indeed, the dual targeting of Th1-mediated T cell differentiation (IL-12) along with APC activation (GM-CSF), might achieve more potent anti-tumor immunity. Two recent studies describe the synergistic suppression of liver tumor growth using GM-CSF in combination with IL-12. In one of these studies, the combined gene delivery of both cytokines reduced the growth of hepatocellular carcinoma in mice beyond the effects of either cytokine alone and only the combination led to robust cytotoxic T cell-mediated tumor cell lysis *in vitro* [69]. In a recent study, Chang et al injected adenoviruses expressing IL-12, GM-CSF or both cytokines into mice bearing orthotopic liver tumors. As single agents, IL-12, but not GM-CSF, was able to exert therapeutic anti-tumor effects. However, the co-administration of both GM-CSF and IL-12 resulted in a synergistic increase in tumor-infiltrating effector cells, IFN- γ production and reduced tumor burden [70]. The combination of GM-CSF and IL-12 has also been applied in several other rodent models, including intracranial glioma [71], transgene-induced spontaneous mammary tumors [72] and metastatic lung and colon cancers [73]. In each of these studies, tumor eradication was dependent upon both T and NK cells and correlated with the infiltration of these cell types into the tumor. The intra-tumor coinjection of IL-12 and GM-CSF may also overcome immunosuppressive and regulatory mechanisms. Using microspheres to co-deliver IL-12 and GM-CSF directly into established lung carcinomas, Kilinc, et al showed the T cell-dependent regression of established primary and metastatic lung tumors through a mechanism that included the activation of effector and memory CD8⁺ T cells accompanied by the concomitant loss of Tregs in the tumor environment [74]. Thus, GM-CSF and IL-12 combinations are capable of overcoming tumor-associated immune suppression.

GM-CSF/IL-12 has been used in multiple myeloma patients, where the combined actions of both cytokines led to a reduction in circulating tumor B cells [75-77]. Idiotype-specific immune

responses were observed in the majority (75-85%) of patients treated with both GM-CSF and IL-12, as compared to 20-30% of the patients treated with IL-12 alone, and were inversely correlated with the frequency of CD4⁺/CD25⁺ T regulatory cells. These results indicate that GM-CSF and IL-12 combination therapy holds promise for the eradication of myeloma and possibly other tumor cells as well, by expanding and activating the critical effector T, NK and antigen presenting cells while perhaps regulating the development of suppressor cells.

8. IFN alpha/IL-12

Interferon alpha (IFN- α) has been used both as an anti-viral and anti-tumor agent, due to its potent ability to suppress tumor cell growth and angiogenesis. IFN- α also induces the proliferation of Th1 T cells and maturation of APC. IFN- α and IL-12 are both macrophage-derived cytokines that enhance innate and Th1 immune responses by up-regulating the expression of interferon regulatory factor (IRF) family of transcription factors [78]. However, the cooperative nature of these two cytokines extends beyond mere similar biological effects. There is good evidence that IL-12, which is well known to induce IFN- γ production, can lead to the production of additional soluble factors that enhance IFN- α signaling. For example, the pretreatment of HT144 melanoma cells with conditioned media from IL-12 treated PBMC exhibit elevated levels of Jak-STAT signaling that was dependent upon IFN- γ and these tumor cells were more sensitive to subsequent IFN- α administration [79]. In murine tumor models, enhanced anti-tumor effects have been observed using IFN- α and IL-12 together. Using a MC38 colorectal cancer cell line that had been virally transduced to express either IFN- α or IL-12, Eguchi et al demonstrated an additive anti-tumor effect when a combination of MC38 cells expressing both IFN- α and IL-12 was used [80]. IFN- α expressing tumor cells were correlated with CD8⁺ T cells infiltrating the tumors and long-lasting tumor specific immunity whereas IL-12 expression was associated with the activation of NK cells. In another study, mice bearing renal or colon carcinomas were treated with plasmid DNAs encoding IL-12 and IFN- α [81]. Those mice treated with IL-12 alone showed a rejection rate for renal tumors of 58% and colon tumors of 17%, whereas 0-25% of both types of tumors were rejected using IFN- α alone. In marked contrast, those mice that had been treated with the combination of IFN- α plus IL-12 had 100% tumor rejection of a mouse RCC and a 50% incidence of colon tumor rejection. Those mice that had rejected tumors were protected against subsequent tumor rechallenge and the tumor infiltrating leukocytes of these mice demonstrated a more activated phenotype.

The combined use of IFN- α and IL-12 has been applied to patients with advanced or metastatic cancers. Two separate Phase I studies have reported on the co-administration of IFN- α and IL-12 in patients with metastatic renal cell carcinoma or malignant melanoma. In one study, patients received via the intravenous route 100-500 ng/kg IL-12 on day 1 followed by 1-10 million units of IFN- α (subcutaneous route) on days 2-6 [82]. This combination of IL-12 and IFN- α was administered sequentially with minimal toxicity and there was a marked increase in IFN- γ production and IFN- α -induced Jak-STAT signal transduction in patient PBMC. In another study, patients received escalating doses (100-500 ng/kg) of IL-12 via the subcutaneous route twice weekly and IFN- α (1-3 million units) via the subcutaneous route three times a week in 4 week cycles [83]. In this case, dose-limiting toxicity was observed and generally included grades 3 and 4 hepatotoxicity and neutropenia/leucopenia. At the recommended doses of 500 ng/kg IL-12 and 1 million units IFN- α , an increase in IFN- γ , IP-10, Mig, and IL-5 was detected in patients' PBMC and 3 patients (17%) had partial anti-tumor responses.

9. Chemokines and anti-angiogenic cytokines plus IL-12

Although the anti-tumor effects of IL-12 are broadly attributed to its effects upon innate and adaptive immune cells, IL-12 expression also indirectly leads to the inhibition of angiogenesis.

IL-12 induces the production of IFN- γ , which in turn stimulates the production of IFN- γ inducible protein-10 (IP-10) as well as Mig. It has been known for quite some time that these two chemokines are key elements of the anti-angiogenic effects of IL-12 [84-87]. Although IP-10 and Mig are chemoattractants for T lymphocytes and NK cells, their ability to inhibit angiogenesis is dependent upon NK cells, independent of T cell recruitment, and even persists in nude mice that lack T cells [88]. The newly recruited NK cells appear to play a pivotal role in the anti-angiogenic effects of IL-12 plus IP-10 since the antibody-mediated neutralization of these cells reversed the anti-angiogenic effect of IL-12 [89]. Furthermore, NK cells contribute to the secretion of additional IFN- γ that amplifies local IP-10 expression and further activates them to kill neighboring endothelial cells and impair neovascularization.

Given the strong anti-tumor effects of anti-angiogenic chemokines such as IP-10 and Mig, considerable efforts have been undertaken to boost their anti-tumor effects by using them in combination with IL-12. In one recent study, Keyser et al administered plasmid DNA encoding IP-10 and/or IL-12 into mice bearing malignant melanoma (B16F10) and Lewis lung carcinoma [88]. As expected, IL-12 expression led to a significant level of tumor regression even at low doses. As compared to IL-12, IP-10 by itself achieved a much less robust anti-tumor response, even at considerably higher doses. In contrast, the combination of both IL-12 and IP-10 achieved the most significant anti-tumor effects that were particularly associated with significant T cell infiltration into the tumors along with a proposed increase in NK cell activation. Similar results were achieved through the intra-tumor administration of adenoviral vectors encoding IP-10 and IL-12 [90,91]. In these studies, the co-localization of adenoviruses encoding IL-12 and IP-10 synergized for powerful anti-tumor responses, generation of tumor-specific cytotoxic T cell responses and T cell infiltration into the tumors. These responses were predominantly lost when CD4⁺ and CD8⁺ T cells were simultaneously depleted, or when NK cells were depleted from mice. From a clinical perspective, it is particularly noteworthy that the inclusion of IP-10 could reduce the amount of IL-12 needed to achieve therapeutic responses, which would be anticipated to reduce IL-12 mediated toxicity [90].

IL-12 has been combined with other anti-angiogenic factors, such as Angiostatin [92], vasostatin [93], and soluble receptors for vascular endothelial growth factor (VEGF) [94]. Similar to the studies involving IP-10+IL-12, the combination of IL-12 with each of these factors was also able to achieve increased tumor regression that was generally associated with reduced microvessel density in tumor sections as well as T cell mediated protection against subsequent tumor rechallenge. In these settings, the anti-angiogenic factors may limit the tumor size by attacking its vasculature, while IL-12 can then elicit specific T cell responses against tumor antigens. These dual-pronged approaches may hold considerable promise for the treatment of many solid tumors.

10. Conclusion and Expert Opinion

Single cytokine agents that usually target defined compartments of the immune system often fail to exert potent and durable immunomodulatory effects. In this regard, although IL-12 by itself exhibits efficacy in some murine pre-clinical models [2] and in the clinic (reviewed in [9]), there is increasing evidence for counter-regulatory (i.e. immunosuppressive) measures that may limit its overall usefulness as a cancer therapeutic. In this regard, the attenuation of IFN- γ , TNF α and IP-10 expression after repeated IL-12 administration is particularly instructive. These cytokines, particularly IFN- γ , are critical for the effective activation and engagement of cellular components of the innate and adaptive immune system, including NK cells, cytotoxic T cells and APC. Indeed, we have found that the endogenous production of IFN- γ is required for the anti-tumor effects of all immunotherapeutic regimens we have evaluated to date [5-7].

The coordinate enhancement of multiple immunological components by combination cytokine therapies may achieve the most durable and, ultimately, successful eradication of malignant neoplasms. The rationale for IL-12 plus GM-CSF therapies, for example, is that such approaches induce both innate and adaptive anti-tumor immune responses. Indeed, several such studies have demonstrated that IL-12 plus GM-CSF combination approaches can achieve the complete eradication of disseminated cancers in a majority of treated animals [73]. In our own studies, we have co-administered agonistic antibodies to CD40 along with IL-2 and demonstrated synergistic anti-tumor responses in mice bearing RCC that required IL-12 p40 for maximal effect [6]. Here, too, the coordinated targeting of APC/DC (α CD40) and CD8⁺ T cell (IL-2) responses induced the complete regression of metastatic RCC tumors in an IFN- γ -dependent manner and through the potentiation of cellular immune responses.

As highlighted throughout this review, IL-12 is also distinguished by its ability to cooperate, and in some cases synergize, with the co-administration of additional immunomodulatory factors and cytokines. This is due, in part, to reciprocal regulation of the expression of each cytokine's receptor. In many cases, the result is a synergistic increase in the production of downstream cytokines, such as IFN- γ , whose own expression can be made to be more protracted. In this regard, it is particularly noteworthy that the inclusion of additional cytokines, such as IL-2, to IL-12 cytokine therapies is able to overcome the inhibition of IFN- γ production that is often observed after repeated IL-12 administration as a single agent [25]. Similarly, we found that the co-treatment of mice with IL-12 and IL-18 also prolonged IFN- γ expression, as compared to treatment with IL-12 alone [65]. Since transient expression of IFN- γ has correlated with poor prognosis in the clinic [25], those combinatorial strategies that correlate with protracted IFN- γ expression are likely to be most successful. Furthermore, the inflammatory cascade involves the continuous recruitment and activation of newly-recruited leukocytes and the sustained expression of IFN- γ and IFN- γ dependent chemokines (e.g. Mig and IP-10) would thereby be expected to amplify this ongoing process. Consistently, therapies that combine IP-10 with IL-12 have also achieved synergistic increases in tumor regression, mediated by the enhanced infiltration of activated CTLs into tumors accompanied by an inhibition of angiogenesis [88,90,91].

Unfortunately, many of the combination therapies that include IL-12 are associated with a considerable degree of toxicity. This involves a high level of systemic IFN- γ production [57, 67], which potently primes NK cells for enhanced cytotoxic and cell mediated-immune responses. Although IFN- γ is capable of activating many different types of leukocytes, the removal of NK cells was shown to ameliorate these toxic side effects. The local administration of cytokines, rather than systemically, is one way that this problem may be overcome. Toward this goal, studies that utilize replication-deficient viral vectors to deliver cytokine genes directly into the tumor hold considerable promise. Additionally, the potential for IL-12 to synergize with other cytokines often results in a lower effective dose for the cytokines, thereby reducing any potential toxicity. In this regard, regimens of IL-12 combined with low-dose IL-2 have been successfully tested in murine pre-clinical models with spontaneous mammary carcinoma and RCC [23,24], and in patients with metastatic melanoma and RCC [25,26]. The results from these studies have demonstrated that IL-12 plus concurrent low-dose IL-2 is well tolerated with minimal and readily reversible side effects. The significant improvement in tumor regression mediated by this therapeutic regimen has resulted in the further investigation of IL-12/pulse IL-2 therapy in additional patients with melanoma, RCC and other IL-12 responsive malignancies.

The members of the γ_c family of cytokines share some biological activities with IL-2 that also make them potentially useful for therapies against cancer [17]. IL-15, in particular, has potent effects upon the proliferation of NK memory CD8⁺ T cells [36]. However, it is the biological differences between IL-15 and IL-2 that have further highlighted the potential for IL-15 as a

better candidate for cancer immunotherapy. IL-2 potentiates activation induced cell death (AICD) of CD4⁺ T cells [30,31], while IL-15 protects cells from IL-2 induced AICD [32]. Furthermore, IL-2 is important in maintaining Treg cells that suppress T cell responses [33-35], while IL-15 does not have this effect. IL-7 is another γ_c cytokine that is particularly critical for T cell differentiation and can be used to enhance CTL generation against murine sarcomas [44]. Moreover, when IL-7 was used in combination with IL-12, there was a significant increase in CTL activity against autologous tumor cells [47]. In this combination, IL-7 exerted a major effect upon the stimulation of lymphocyte proliferation whereas IL-12 could drive lymphocytes to the Th1 pathway with increased IFN- γ production. It has therefore been proposed that IL-7 could possibly be included in IL-12/pulse IL-2 therapeutic regimens for even more optimal proliferative responses and cytolytic activities against autologous tumors [47]. Finally, IL-21, the most recently identified member of the γ_c cytokine family, also plays an important role in regulating T cell responses. IL-21 augments the IL-15 and TCR-induced proliferation of CD8⁺ T cells, and it also increases the ability of these cells to respond to co-stimulatory ligands by sustaining CD28 expression [17]. Thus, IL-21 plays an important role in fine-tuning T cell responses by working in connection with other proinflammatory cytokines. In summary, although IL-2 was initially considered to be the best candidate in the development of methodologies to boost T cell responses against cancer, it may be more appropriate to use IL-2 during the initial phases of CD8⁺ T cell priming. New reports suggest that IL-7, IL-15 and/or IL-21 may promote a more durable T cell response during the maintenance of such immunological responses.

IL-23 and IL-27 are structurally related to IL-12, yet they mediate functionally distinct immunologic phenotypes (Figures 1 and 2). Similar to IL-12, both IL-23 and IL-27 are capable of exerting anti-tumor effects, albeit through differing mechanisms [95, 96]. There is an increasing amount of data that suggests that these related cytokines hold considerable promise for combination cytokine therapies as well. IL-23 shares the p40 subunit with IL-12 and promotes the proliferation and IFN- γ production by memory CD4⁺ T cells [97]. IL-23 can also act directly on APC to enhance their production of TNF α and nitric oxide. Several recent reports have indicated that IL-23 can mediate potent anti-tumor and anti-metastatic activity in murine models of cancer, in some cases equally as well as IL-12 [98]. Furthermore, when administered in combination with a gp100 peptide vaccine, IL-23 specifically increased the number of vaccine-induced CD8⁺ T cells and their production of IFN- γ [99]. Similarly, IL-27 potently enhances various aspects of T and/or NK cell function *in vitro* including enhancement of proliferative responses and the production of IFN- γ [100]. IL-27 has been recently evaluated in various preclinical tumors, including murine models of colon carcinoma and neuroblastoma [101-103]. Combining either IL-23 or IL-27 with other cytokines, such as IL-2 or IL-15, may potentiate the immunoregulatory activity and anti-tumor efficacy of these cytokines for the improved treatment of cancer.

Finally, IL-12 has been included as an adjuvant for cancer vaccines, with the goal of boosting tumor-specific immune responses. The efficacy of these cancer vaccine approaches is dependent upon overcoming the poor antigenicity of many tumors by making them more recognizable by cells of the immune system. In mice, the intra-tumoral injection of IL-12-encoding DNA in combination with gp100-encoding DNA into mice bearing B16 melanoma resulted in a strong anti-tumor effect that was associated with an increase in the activation of and infiltration into tumors by DC and CD8⁺ T cells [104,105]. The adjuvant administration of IL-12 after immunization with a vaccinia virus expressing the co-stimulatory molecule B7-1 to tumor-bearing mice similarly enhanced animal survival [106]. Other recent approaches include the co-administration of IL-12 along with tumor antigen-pulsed DC [107] and the engineering of tumor cells or antigen-pulsed DC to express IL-12 alone [108,109] or in combination with IL-18 [66] for enhanced anti-tumor immunity. In humans, many studies have combined IL-12 with peptide vaccines for the improved treatment of cancers. In one recent

study, the treatment of melanoma patients with IL-12 plus a multi-peptide vaccine consisting of gp100, MART1 and tyrosinase, resulted in increased immune responses and time to relapse in the majority of patients [110]. Other studies have similarly combined IL-12 with melanoma antigens and demonstrated considerable success at boosting the frequency of circulating tumor-reactive T cells although the effects upon tumor regression was relatively more mixed [111]. Specifically, although these peptide-based vaccines can effectively generate a quantifiable T cell-specific immune response in the PBMC of cancer patients, such a response does not always correlate with a clinically evident regression of metastatic melanoma. Current immunization trials using peptides or recombinant viruses containing genes encoding the melanosomal antigens MART-1 or gp100, along with a more optimized co-administration of IL-12, are currently being further evaluated at the National Cancer Institute (Bethesda, MD) and elsewhere, that will hopefully demonstrate the feasibility of safely using IL-12 as a vaccine adjuvant for the therapy of melanoma and other solid tumors.

Collectively, studies performed to date have demonstrated that IL-12 exerts powerful anti-tumor effects by itself and that it possesses a marked ability to synergize and cooperate with many additional cytokines. Ongoing studies *in vitro* and *in vivo* are further improving our understanding of the cellular and molecular mechanisms underlying the immunoregulatory and anti-tumor consequences of these combination approaches. The results of these studies will provide a foundation for the ongoing translation of IL-12 based cytokine combination therapies to the clinic for the improved treatment of different types of cancers in humans.

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Abbreviations used in this paper

DC	dendritic cells
APC	antigen-presenting cell
IFN	Interferon
IL	Interleukin
NK	natural killer
GM-CSF	granulocyte macrophage-colony stimulating factor
Mig	monokine induced by gamma-interferon
IP-10	IFN-gamma-induced protein 10

Heterogeneity of IL-2 and IL-12 cytokine families: Receptor structure, signaling and biological effects

Figure 1.

Schematic depiction of the IL-2 and IL-12 cytokine/receptor families and their functions. IL-2, IL-7 and IL-15 are cytokines that interact with receptor complexes that contain a common gamma (γ_c) chain. Binding of the appropriate cytokines to the γ_c containing receptor primes T and NK cells for their activation and proliferation (reviewed in [17]). IL-12, IL-23 and IL-27 are heterodimeric cytokines that bind receptors with similar structure. The IL-12 family of cytokines activates T and NK cells for enhanced effector functions.

Heterogeneity of IL-18 and IL-12 cytokine families: Receptor structure, signaling and biological effects

Figure 2. Schematic depiction of the IL-18 and IL-12 cytokine/receptor families and their functions. IL-18 and IL-1 interact with receptor complexes comprising homologous alpha and beta subunits and signal through the MyD88 and IRAK adapter molecules. IL-1 and IL-18 are potent activators of NF κ B signaling which results in leukocyte activation, IFN- γ production and inflammation. IL-12, IL-23 and IL-27 are heterodimeric cytokines that bind receptors with similar structure. The IL-12 family of cytokines activates T and NK cells for enhanced effector functions.