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Interleukin 12: Stumbling Blocks and Stepping Stones to Effective Anti-Tumor Therapy

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1. Introduction

Interleukin-12 (IL-12) is a heterodimeric pro-inflammatory cytokine long recognized to have properties capable of mediating immune effector functions in a manner compatible to enhancing endogenous anti-tumor immune responses. For this reason the cytokine has garnered significant interest from investigators in the field of immune mediated anti-cancer therapies. While the exact mechanisms whereby IL-12 mediates pro-inflammatory endogenous anti-tumor responses remains to be fully elucidated, pre-clinical murine tumor models demonstrate unequivocal anti-tumor benefit mediated by IL-12 (1-5). Preclinical studies demonstrate that the mechanisms of IL-12 mediated anti-tumor endogenous immune responses seen in these models are likely to be complex and multifactorial. Beyond the ability of IL-12 to induce an inflammatory Th1 CD4+ T cell response, studies have demonstrated the ability of IL-12 to enhance CD8+ T cell cytotoxicity. Additionally, preclinical studies have shown IL-12 to recruit and activate innate cytotoxic NK cells and modulate a pro-inflammatory macrophage phenotype. Further, studies have shown that T cell secretion of IFNγ mediated by IL-12 may reverse T cell anergy and confer effector T cell resistance to immune suppressive Tregs. The ability of IL-12 to activate the adaptive as well as the innate immune systems, but also further modulate the otherwise immune-hostile tumor microenvironment, suggests that the cytokine may serve as a potent immunotherapeutic agent. Significantly, pre-clinical murine tumor models have largely validated these predictions. These promising pre-clinical studies consequently spurred on a series of clinical trials treating patients with a variety of tumors with intravenous infusions of recombinant IL-12. Unfortunately, these studies have yielded only modest tumor responses in the context of associated severe and unforeseen toxicities. The IL-12 related toxicities seen in these early clinical trials served to markedly dampen enthusiasm for this cytokine as a potential anti-tumor therapeutic reagent in the clinical setting. However, subsequent clinical trials conducted utilizing direct infusion of IL-12 into accessible tumor sites has resulted in

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promising anti-tumor responses in the absence of toxicities induced by systemic infusion seen in earlier studies. Thus, based on these clinical trials, the potent anti-tumor effects of IL-12 can best be harnessed by restricting its administration directly into the tumor microenvironment. Therefore, optimal utilization of the anti-tumor efficacy of IL-12 may be realized utilizing novel approaches whereby the cytokine is delivered directly to the site of the tumor with limited systemic distribution to avoid previously observed toxicities. In this chapter, we review the biology of IL-12 and the predicted mechanisms whereby this cytokine may mediate anti-tumor endogenous immune responses. We further discuss preclinical studies to support the utilization of IL-12 in cancer therapy as well as clinical trial data, which, in part, have tempered enthusiasm for IL-12 as an effective anti-tumor reagent in the clinical setting due to associated toxicities. Finally, we present and discuss previously published approaches to overcome systemic toxicity through targeted delivery of IL-12 directly into the tumor microenvironment.

2. Basic biology and immune stimulatory effects of IL-12

IL-12 is biologically functional as a heterodimeric molecule consisting of an α and β chain, where covalently-linked p35 and p40 subunits together form the active molecule, IL-12p70 (6-8). The p35 subunit is expressed ubiquitously but only phagocytic cells produce the p40 subunit, therefore functional IL-12p70 is only produced by activated antigen-presenting cells (APCs), neutrophils and macrophages (9). The IL-12 receptor (IL-12R) is composed of two subunits, β1 and β2, and is expressed predominantly on dendritic cells (DCs), T cells and natural killer (NK) cells. The IL-12R mediates signal transduction through the Janus kinases (JAKs) but these pathways will not be discussed here (10).

Initially, IL-12 was described as “Natural killer-stimulating factor” and “cytotoxic lymphocyte maturation factor” and has since been reported to have important effects on the generation of an adaptive immune response (6, 11). It is a potent activator of NK cells, with IL-12 stimulation resulting in enhanced NK cell mediated cytotoxicity (9). The effects of IL-12 on T cells include enhanced cytotoxicity and CD4+ T cell differentiation into type-1 helper T cells (Th1) (12-14). It has also been demonstrated that IL-12 could provide “signal 3” for T cell activation, where signal 2 provides co-stimulation and signal 3 upregulates the expression of the lytic protein Granzyme B, leading to increased cytotoxic effector function and overcoming tolerance (15, 16). IL-12 also mediates significant effects on T cell proliferation. In a murine model of adoptive transfer, OT-1 T cells were inoculated into an irradiated syngeneic mouse and exhibited increased homeostatic proliferation when supported with injection of IL-12 (17). Further studies have demonstrated that T cell expansion was augmented with IL-12 as a result of decreased apoptosis. This was found to be due to decreased Fas expression, increased expression of anti-apoptotic FLIP proteins and inhibition of caspase activation (18). Additionally, it has been reported that IL-12 is important in the fate of CD8+ T cells where IL-12 promotes differentiation into functional effector cells and inhibits memory T cell formation (19).

An additional, but no less important outcome following IL-12 production is the induction of Interferon (IFN)-γ from B, T and NK cells (20-22). IL-12 has positive feedback loops whereby IL-12 stimulates DCs to produce more IL-12, thereby stimulating IFNγ production resulting in additional IL-12 produced by monocytes (23, 24). This IFNγ is able to further activate
Innate and adaptive immune systems as well as influencing the tumor microenvironment, as discussed below. Many of these IL-12 mediated anti-tumor effects are abrogated upon neutralization of IFN-γ thereby demonstrating the importance of this pro-inflammatory cytokine in IL-12 mediated immune stimulation (3, 4, 25).

3. IL-12 in the tumor microenvironment

The wide-ranging effects of IL-12 have profound impacts upon the tumor microenvironment; acting directly on tumor cells, influencing the surrounding tumor stroma/structure, and modulating infiltrating immune cells. These effects, detailed below, mediate the recruitment of lymphocytes, activation of tumor infiltrating lymphocytes, as well as direct effects on tumor cells to decrease angiogenesis which combine to result in tumor eradication or inhibition.

Direct effects of IL-12 on tumor cells may include the ability of IL-12 to up-regulate expression of molecules that induce immune recognition and death of tumor cells. It has been documented that adenoviral mediated expression of IL-12 in human osteosarcoma cells or chemo-resistant breast cancer cells increased expression of Fas, and subsequent apoptosis of tumor cells (26). This was postulated to be a function of the IL-12R β1 chain activating NF-κB, a signaling effect that is thought to be absent in lymphocytes ensuring that IL-12 stimulation of T and NK cells does not result in Fas up-regulation or apoptosis. In a mouse model of mammary adenocarcinoma, it was demonstrated that IL-12 induced IFN-γ led to increased surface MHC expression on tumor cells (2), increasing the presentation of tumor-associated antigens to the immune system and resulting in increased endogenous anti-tumor immune responses.

IL-12 is widely reported to increase IFN-γ expression, which is responsible for mediating effects directly on the tumor cells, including upregulation of inducible nitric oxide synthase (iNOS) and indolamine 2,3-dioxygenase (IDO) genes, as well as increased MHC expression. Utilizing a murine model of fibrosarcoma, systemic injection of IL-12 was found to induce IFN-γ, which in turn mediated increased expression of IDO and iNOS mRNA by tumor cells (3). The products of these genes may slow the growth of tumor cells. IDO is thought to influence tumor growth through the deprivation of tryptophan (27). This was also demonstrated in a study employing a murine model of spontaneous breast cancer treated with systemic IL-12 injection (1).

An important tumor microenvironment modulatory effect by IL-12 is the inhibition of angiogenesis. Folkman and others have elegantly demonstrated the anti-angiogenic role of IL-12 against fibroblast growth factor-induced corneal neo-vascularization. This effect was abrogated upon neutralization of IFN-γ and a downstream effector, IP-10, implicating the latter as the mediator of this effect (28-31). IL-12 has further been shown to mediate down-regulation of pro-angiogenic gene vascular endothelial growth factor (VEGF)-C, as well as the pro-angiogenic proteins, VEGF and basic fibroblast growth factor (BFGF) on tumor cells and supporting fibroblast cells (32, 33). There is also evidence to suggest that NK cells and CD8+ T cells may contribute to the IL-12 mediated anti-angiogenic effect in some models (34, 35). Cytotoxic T and NK cells were shown to directly lyse epithelial cells, therefore contributing to inhibition of neo-vascularization. It is likely that both secreted factors, such as IP-10 from fibroblasts, as well as direct effects mediated by T and NK cells contribute to the overall anti-angiogenic effects of IL-12.
IL-12 may directly increase the expression of lymphocyte adhesion molecules within tumors, thereby increasing the infiltration of immune effectors into the tumor. For example, treatment of a poorly immunogeneic mammary adenocarcinoma with IL-12 resulted in increased vascular cell adhesion molecule (VCAM)-1 expression within tumors (2). This result was also reported in a murine model of breast cancer (1). In addition, a recent report documented IL-12 mediated activation of lymphoid-tissue inducer (LTI) cells, which led to the up-regulation of adhesion molecules and increased leukocyte infiltration (36). Utilizing a murine model of melanoma, it was found that LTI cells expressing the NK cell receptor NKP46, were responsible for up-regulating both VCAM-1 and inter-cellular adhesion molecule (ICAM)-1 within the tumor microenvironment. Up-regulated expression of VCAM-1 has been demonstrated to increase the migration of lymphocytes, therefore allowing increased lymphocytic infiltration into IL-12 treated tumors (37).

A consequence of increased expression of adhesion molecules is the increased infiltration of tumors with immune effector cells, as reported in several studies wherein solid tumors were treated with IL-12. In murine models of mammary adenocarcinoma, lung alveolar carcinoma, fibrosarcoma and spontaneous breast cancer, tumor masses were infiltrated with lymphocytes following systemic IL-12 treatment (1-4, 25, 38). Infiltrating cells were NK cells, CD8+ T cells, CD4+ T cells, and macrophages, depending on the model utilized. Infiltration of lymphocytes is highly important for IL-12 mediated tumor regression, as depletion of CD4 or CD8 T cells prior to treatment abrogated the anti-tumor response in several murine models (2, 4, 5).

IL-12 has further been shown to mediate activation of tumor infiltrating lymphocytes (TILs). In the context of minimal residual disease following transplant in a murine model of lymphoma, IL-12 treatment was shown to activate splenocytes, as noted by up-regulation of CD25 (39). Subsequent studies have demonstrated that IL-12 therapy mediates substantial increase in Granzyme B expression, increased proportion of IFNγ secreting CD8+ T cells, and greater levels of IFNγ secretion in previously quiescent tumor infiltrating CD8+ T cells (38). A recent report has identified the specificity of endogenous T cells activated by IL-12 in a murine model of melanoma (40). This study demonstrated that IL-12 therapy stimulated a protective CD8+ T cell response, where T cells were specific for multiple tumor associated stromal antigens. Other cells activated in response to IL-12 include B cells, as demonstrated by an increase in tumor-reactive antibodies following IL-12 treatment (2). However, the clinical relevance of these tumor reactive antibodies currently remain unclear.

IL-12 may reverse the anergic state of T cells present within the tumor microenvironment. In elegant studies, primary human lung biopsy samples were transplanted into SCID mice and treated with intra-tumoral injection of IL-12 microbeads (41-43). IL-12 therapy was found to mediate regression of tumors, which was dependent on the reactivation of CD4+ T cells within the tumor. These cells were stimulated to proliferate, secrete IFNγ, and mediate complete eradication of the tumor. Additionally, anergy induced in murine CD4+ T cells by regulatory T cells can be overcome with the addition of IL-12 (44). The authors demonstrate that IL-12 mediates effects on the CD4+ effector T cell allowing proliferation and IFNγ secretion despite the presence of suppressive Tregs.

IL-12 has further been demonstrated to impact regulatory cell populations present in the tumor microenvironment. Using a murine model of lung carcinoma, it was demonstrated
that IL-12 microsphere therapy resulted in a reduction of CD4+ CD25+ suppressor T cells (38). This study reported an IFNγ dependent induction of apoptosis in the suppressive T cell subset. Relieving tumor resident T cells of suppressive factors may allow the generation of effective endogenous anti-tumor responses. Recent reports have demonstrated that IL-12 can also inhibit the expansion of regulatory T cells (45). IL-12 was found to inhibit the expansion of Tregs in vitro and in vivo in a murine model of lymphoma. Inhibition of Treg expansion was shown to occur in an IFNγ dependent fashion as IL-12 did not effect Treg expansion in IFNγ receptor deficient mice.

Tumor-associated macrophages (TAMs) play a major role in promoting tumor growth and metastasis and in suppressing the antitumor immune response. Such local immune dysfunction is recognized as one of the major barriers to cancer immunotherapy (46). Macrophages are also functionally plastic, meaning that they can convert between functional states (47). In particular, it has been reported that TAMs can be converted from a M2 suppressive phenotype to an M1 inflammatory phenotype following treatment of tumor-bearing mice with IL-12 containing microspheres (48, 49). Moreover, tumor lesions treated with tumor targeted T cells engineered to secrete IL-12 were infiltrated with activated M1 type of macrophages that were not found in tumors upon T cell therapy without IL-12. The accumulation of activated macrophages was critical to the antitumor immune response as depletion of these macrophages abolished the anti-tumor response (50).

4. Preclinical studies investigating the anti-tumor therapeutic potential of IL-12

Early studies investigating the utilization of IL-12 as an anti-cancer therapeutic agent provided encouraging results, conveying the anti-tumor potential of this powerful cytokine. Intra-tumoral and systemic administration of this cytokine demonstrated marked tumor regression in several murine models of cancer. These include a poorly immunogenic mammary adenocarcinoma, lung alveolar carcinoma, fibrosarcoma, spontaneous breast cancer, ovarian carcinoma, lymphoma, renal cell carcinoma, as well as a model of pulmonary metastasis of melanoma (1-5, 38, 39, 51). IL-12 was shown to mediate disease eradication in primary tumors, as well as eradication of metastasis following surgical removal of primary tumors (2). Additionally, using a murine model of minimal residual lymphoma disease following transplant, IL-12 was shown to eradicate disease following transplant without affecting lympho-hematopoietic recovery (39). Significantly, IL-12 exerts anti-tumor effects and mediates tumor regression in models of early, intermediate and late stage disease, illustrating the efficacy of IL-12 anti-cancer therapy even in the setting of advanced disease (51). These studies provided rational for the use of this cytokine in cancer therapy and several clinical approaches to utilize IL-12 in cancer therapy have been developed as discussed below.

Other therapeutic strategies to deliver IL-12 to the tumor microenvironment include the sustained release of cytokine using nanoparticles. Anti-tumor efficacy has been demonstrated in a murine model of mammary carcinoma, wherein IL-12 microsphere treatment was found to result in NK cell mediated anti-tumor effects (52). Combination therapy involving IL-12 and TNFα receptor microspheres was found to lead to superior anti-tumor function with the recruitment of CD8+ T cells. Additional studies of IL-12 microsphere treatment utilized a combination of IL-12 and GM-CSF, which mediated
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regression of lung alveolar carcinomas in a murine model (38, 53). This study reported IL-12 mediated effects including reactivation of tumor resident T cells as well as predicted apoptosis of regulatory T cells. The anti-tumor efficacy of polymer-mediated IL-12 delivery has also been tested in murine models of malignant glioma and disseminated ovarian cancer (54, 55). These studies validate the nanoparticle mediated delivery approach, demonstrating a sustained release and therapeutic efficacy, whilst contributing to the understanding of the mechanisms of IL-12 mediated anti-tumor efficacy.

As an alternative approach to systemic infusion of IL-12, specific delivery of IL-12 directly into the tumor site may be achieved through gene therapy strategies. One such approach utilizes adenoviral vector mediated delivery of the IL-12 gene with subsequent expression and secretion of IL-12 by the infected cell. In this manner, direct intra-tumoral injection of adenovirus encoding the IL-12 gene resulted in anti-tumor responses in murine models of melanoma, laryngeal squamous cell carcinoma, glioma, renal cell carcinoma and bladder cancer (40, 56-59). Other models utilizing combinations of adenovirus and other chemotherapies have also demonstrated encouraging results. It was shown that a combination of cyclophosphamide and intra-tumoral injection of adenoviral gene transfer of IL-12 resulted in tumor eradication in a murine model of colorectal cancer (60). Additional strategies to further improve the safety of this response involve the utilization of organ specific, drug inducible adenoviral vectors. One study reported the use of a liver-specific, mifepristone-inducible adenoviral vector encoding IL-12 for the treatment of colorectal cancer liver metastasis (61). This system allowed for controlled and long term expression of the vector following systemic infusion, and was enhanced by additional treatment with the chemotherapeutic agent oxaliplatin (62). Other strategies testing drug inducible IL-12 expression have yielded similar results, confirming the ability to tightly control IL-12 production in vivo (63).

Additional viral vector based strategies include the utilization of other oncolytic viruses, which preferentially infect and lyse tumor cells. Intra-tumoral injection of a vesicular stomatitis virus carrying an IL-12 transgene was demonstrated to reduce tumor volume in a murine model of squamous cell carcinoma (64). This reduction in tumor volume correlated to an increased survival of treated tumor bearing mice.

Other delivery strategies include electroporation, where a voltage is applied to a cell membrane, allowing entry of plasmid DNA into a cell. To achieve this in vivo, studies have injected plasmids encoding the IL-12 gene intra-tumorally, followed by in vivo electroporation to allow for the introduction and expression of the gene into and by the regional cells (65). Utilizing a murine model of melanoma, it was found that electroporation of the IL-12 gene into the tumor resulted in tumor eradication in 47% of mice (65). This study also demonstrated the ability of IL-12 to stimulate an endogenous anti-tumor immune response as surviving mice were resistant to tumor re-challenge. Therapy resulted in increased levels of IL-12 and IFNγ in the tumor, increased lymphocyte infiltration and reduction in vascularity. These findings are consistent with previously documented effects of IL-12. This approach has similarly been successfully applied to a murine model of fibrosarcoma (66).

Other novel strategies to target IL-12 to the tumor site include anchoring IL-12 to a tumor-specific protein. One group utilized a single chain variable fragment (scFv) from an
antibody specific to erbB2 anchored to IL-12 to specifically deliver IL-12 to erbB2+ murine bladder cancers (67). This approach resulted in increased survival but failed to completely eradicate established disease. Similarly, investigators linked a IL-13Ra3 protein to IL-12 as a means of targeting murine melanoma (68). This group chose IL-13Ra3 as it can be a negative regulator of tumors that utilize IL-13 as a pro-tumorigenic factor. This therapy led to a significant NK T cell mediated inhibition of tumor growth in vivo.

Indeed, combinations of these treatment modalities have also yielded encouraging results in preclinical testing. Intra-tumoral injection of adenovirus encoding IL-12 and 4-1BBL combined with DC injection was found to mediate marked inhibition of tumor growth in a murine model of melanoma (69). Another study demonstrated the intra-tumoral injection of IL-12 encoding plasmid followed by DC vaccination led to the suppression of primary hepatocellular carcinoma and metastases (70). Significantly, utilization of this combined immune based therapy approach to augment surgical resection of primary tumor yielded superior results, leading to long term survival and resistance to tumor re-challenge in murine models of ovarian cancer, prostate cancer and hepatocellular carcinoma (51); (71); (72). Combination of IL-12 gene therapy with IL-27 gene therapy, or retinoic acid based therapies have also been described, with encouraging responses against systemic tumors (73, 74).

5. Clinical trials: Toxicity tempers the potential of IL-12 as an anti-cancer agent

Previous pre-clinical studies demonstrating the anti-tumor efficacy of IL-12 warranted the translation of this therapeutic agent to the clinical setting. A number of tumors were targeted in these trials, with modest, mixed responses. The first published trial of systemically administered IL-12 was a phase I dose escalation trial of intravenous (i.v.) administered recombinant human interleukin 12 (rhIL-12) (75). Cohorts of four to six patients with advanced solid tumor malignancies received escalating doses (3-1000 ng/kg/day) of rhIL-12 by bolus i.v. injection once and then, after a 2-week rest period, once daily for five days every 3 weeks. Forty patients were enrolled on this study including 20 with renal cell carcinomas, 12 with melanoma, and 5 with colon cancer. One melanoma patient experienced a complete regression of metastatic disease for a period of four weeks, while a second patient with renal cell carcinoma experienced a partial response that was ongoing at 22 months. Toxicities observed in this trial were fever, chills, fatigue, nausea, vomiting, and headache. Routine laboratory findings reported abnormalities including anemia, neutropenia, lymphopenia, hyperglycemia, thrombocytopenia, and hypoalbuminemia. Dose limiting toxicities included oral stomatitis and elevated transaminases. The maximum tolerated dose (MTD) (500 ng/kg) was associated with asymptomatic hepatic function test abnormalities in three patients and one on study death due to Clostridia perfringens septicaemia. Lymphopenia was observed at all dose levels, with recovery occurring within several days of completing treatment without rebound lymphocytosis (75). These adverse events were hypothesized to be related to administration of recombinant human IL-12 and so the immune effects of this therapy were interrogated. Consistent with pre-clinical data, IL-12 was shown to up-regulate IFN-γ, in a dose-dependent fashion. Additionally, a single 500 ng/kg dose of rhIL-12 was shown to increase NK cell
cytolytic activity and T cell proliferation, as determined by studies on peripheral blood samples collected pre and post treatment (76). In a subsequent Phase II study of 17 patients investigators observed unexpected toxicities related to the dosing schedule of IL-12 administration (77). On this study, 12 out of 17 patients required hospitalization and two patients died. Two patients deaths occurred during the phase II study were determined to be related to IL-12 administration. Postmortem examination of these two patients showed hemorrhagic ulceration in the large intestine (patient 1) and necrotizing aspiration pneumonia and diffuse hemorrhagic colitis (patient 2). The constitutional, cardiac, renal, hematopoietic, hepatic and neurologic toxicities observed in the phase II were similar to those dose-limiting toxicities observed on phase I studies with IL-12. These toxicities resulted in the suspension of IL-12 trials by the Food and Drug Administration (FDA). Significantly, investigators subsequently determined that a single IL-12 infused loading dose given two weeks prior to consecutive treatments (as done in the initial trial) abrogated these observed toxicities.

A subsequent study by Gollob and colleagues (78), the authors describe two patients with renal cell carcinoma treated with twice-weekly intravenous rhIL-12 during a phase I trial. A cycle of therapy lasted 6 weeks. The patients had grade 4 neutropenia and grade 3 hemolytic anemias. The severe neutropenia was associated with bone marrow agranulocytosis and a preponderance of large granular lymphocytes in the peripheral blood, whereas the hemolytic anemia was associated with splenomegaly. Both patients had stable disease 4 months after the IL-12 treatment was stopped with persisted agranulocytosis and hemolytic anemia.

Additionally, thirty-four patients with measurable metastatic, recurrent or inoperable cervical carcinoma were enrolled on phase I clinical trial to investigate the anti-tumor effect of i.v administrated IL-12 at 250 ng/kg daily up to 21 days. Over half of these patients had received prior cisplatin-based chemotherapy. The most common serious toxicities were hematologic or hepatic, and all were reversible. The median survival was 6.5 months. This was the first clinical trial to demonstrate induction of cell-mediated immune (CMI) responses to specific antigens (HPV16 E4, E6, and E7 peptides) following treatment with IL-12 in women with cervical cancer. However, this improvement in immune response was not associated with enhanced objective response or survival (79).

Pharmacokinetic advantages of intraperitoneal (i.p.) rhIL-12 infusion, tumor response to i.p. delivery of cytokines, as well as its potential anti-angiogenic effect provided the rationale for further evaluation of rhIL-12 in patients with refractory or relapsed ovarian or peritoneal carcinoma. In this study (80) rhIL-12 was administered to 29 previously treated patients with peritoneal carcinomatosis from Müllerian carcinomas, gastrointestinal tract carcinomas and peritoneal mesothelioma in a phase I trial. rhIL-12 doses were dose escalated between patients from 3 to 600 ng/kg weekly up to 6 months. Three or more patients at each level received weekly i.p. injections of rhIL-12. Dose-limiting toxicity (grade 3 elevated transaminase levels) occurred in 50% of treated patients at the 600 ng/kg dose. More frequent, but less severe, toxicities included fever, fatigue, abdominal pain, and nausea. Ten patients received 300 ng/kg with acceptable frequency and severity of side effects. Two patients (one with ovarian cancer and one with mesothelioma) had no remaining disease at laparoscopy. Eight patients had stable disease
and 19 patients had progressive disease. Cytokines including IL-1α, IL-2, IL-10, TNFα, and IFNγ were determined in serum and peritoneal fluid samples during therapy. Immunobiological effects included peritoneal tumor cell apoptosis, decreased tumor cell expression of BFGF and VEGF, elevated IFNγ levels and IP-10 transcripts in peritoneal exudate, and increased proportions of peritoneal CD3+ T cells relative to CD14+ monocytes (80). In a subsequent phase II trial thirty-four patients with ovarian carcinoma or primary peritoneal carcinoma were treated i.p. with rIL-12 (300 ng/kg weekly) (81). 12 patients completed this second phase were evaluated for response. There were no treatment related deaths, peritonitis or significant catheter related complications. Toxicities included grade 4 neutropenia (1), grade 3 fatigue (4), headache (2), myalgia (2), non-neutropenic fever (1), drug fever (1), back pain (1), and dizziness (1). Two patients had stable disease (SD) and 9 had progressive disease (PD). The authors concluded that rIL-12 can safely be administered by i.p. scheduled to patients after first line chemotherapy for ovarian/peritoneal carcinoma. Future i.p. therapies with rhIL-12 will require better understanding and control of pleiotropic effects of IL-12 since proteins with potential for both anti-tumor (IFNγ, IP-10) and pro-tumor growth effects (VEGF, IL-8) were detected in this study (81).

To avoid toxicities associated with systemic infusion of rIL-12, others have investigated subcutaneous administration of IL-12. Rook and colleagues initiated a phase I dose escalation trial of rhIL-12 treating 10 patients with cutaneous T-cell lymphoma (CTCL) with dose escalating regimens of subcutaneous (s.c.) 50, 100, or 300 ng/kg rhIL-12 twice weekly or intralesional injections for up to 24 weeks (82). Histological analysis of regressing skin lesions revealed increased numbers of CD8+ T cells. In contrast to systemic rIL-12 infusion, sq or intralesional rIL-12 regimens were well tolerated with adverse effects limited to low-grade fevers and headaches.

Similarly, in another phase I trial, 28 patients with advanced renal cell carcinoma were treated s.c. with rhIL-12 that was administered on day 1 and followed on day 8 with repeated s.c. injections 3 times a week for 2 weeks. The MTD of the initial injection was evaluated at dose levels of 0.1, 0.5, and 1.0 µg/kg. A dose limiting toxicity (DLT) was observed at 1.0 µg/kg consisting of fever, perivasculitis of the skin, and leukopenia. Other notable toxicities were oral mucositis and transaminitis. These toxicities were more severe after the initial injection than after repeated injections at the same dose level. In this study, one patient had a partial response and seven patients had stable disease (83).

The efficacy of s.c. rhIL-12 for the treatment of patients with early mycosis fungoides (MF; stage IA-IIB) has similarly been tested in a phase I clinical trial. In this study rhIL-12 was administered subcutaneously biweekly (100 ng/kg for 2 weeks; 300 ng/kg thereafter). Ten of 23 patients (43%) achieved partial responses (PR); 7 (30%) achieved minor responses; and 5 (22%) had stable disease. The duration of PRs ranged from 3 to more than 45 weeks. Twelve patients (52%) ultimately progressed with a mean time to progression of 57 days (range, 28-805). Seventeen patients had treatment-related adverse events that were generally mild to moderate in severity including asthenia, headache, chills, fever, injection site reaction, pain, myalgia, arthralgia, transaminitis, anorexia, and sweating. One patient in PR died of hemolytic anemia, possibly exacerbated by rhIL-12 treatment (84).
Little and colleagues conducted phase II clinical trial wherein 36 patients with AIDS-associated Kaposi sarcoma requiring chemotherapy were treated with six 3-week cycles of pegylated liposomal doxorubicin (20 mg/m$^2$) plus interleukin-12 (300 ng/kg subcutaneously twice weekly), followed by 500 ng/kg subcutaneous IL-12 twice weekly for up to 3 years (85). Thirty patients had a major response, including 9 with a complete response, with an 83% overall response rate. Patients had elevated levels of IFN$\gamma$ and IP-10 in their serum, indicative of an rIL-12 mediated immune response.

Finally, 42 previously treated patients (32 patients with relapsed or refractory non-Hodgkin's lymphoma (NHL) and 10 patients with relapsed Hodgkin's disease (HD)) were enrolled in a phase II clinical trial to evaluate the clinical activity and toxicity of rIL-12. Patients were treated with either intravenous (n = 11) or subcutaneous (n = 31) rIL-12. The patients had received a median of three prior treatment regimens, and 16 patients had undergone prior autologous stem cell transplantation. All patients were assessable for toxicity, and 39 of 42 (93%) patients were assessable for response. Six of 29 (21%) patients with NHL had a partial or complete response, whereas none of the 10 patients with HD responded to rIL-12 therapy. Furthermore, 15 patients had stable disease that lasted for up to 54 months. The most common toxicity was flu-like symptoms. Reversible grade 3 hepatic toxicity was observed in three patients requiring dose reduction (86). This study demonstrated increased numbers of peripheral blood CD8$^+$ T cells as well as decreased VEGF and BFGF in 37% of the treated patients indicative of a rIL-12 mediated immune response.

Additional methods of delivering IL-12 to the tumor site have been investigated. Ten previously untreated patients with head and neck squamous cell carcinomas (HNSCC) received direct injection of rhIL-12 in the primary tumor weekly, at two dose levels of 100 or 300 ng/kg, as neoadjuvant therapy prior to surgical resection. In this trial the histologic and immunohistopathologic effects of intratumorally (i.t) infused rhIL-12 were evaluated in the primary tumors and regional lymph nodes. In the primary tumor, the number of CD56$^+$ NK cells was increased in rhIL-12-treated patients compared with control non-rhIL-12 treated patients. After i.t. rhIL-12 treatment of HNSCC patients, significant effects were noted on B cells, with altered lymph node architecture in every IL-12-treated patient and excessive peritumoral infiltration of B cells in some patients (87, 88).

In a phase I/II clinical trial (89), plasmid DNA encoding human IL-12 was produced under good manufacturing practice (GMP) conditions and injected into lesions of nine patients with stage IV malignant melanoma previously treated with both standard and salvage chemotherapy regimens. Plasmid DNA was injected in cycles, three injections per cycle, for up to seven cycles. One cycle consisted of three injections at weekly interval, that is, on day 1, 8 and 15, followed by a resting period of about 8 days (89). Local injection site anti-tumor responses were seen in a majority of patients, with four patients exhibiting responses at distant metastases and a complete remission was achieved in one patient. Biopsies of lesions from responding patients demonstrated a predicted increase in IL-12, IFN$\gamma$ and IP-10 expression analyzed by real-time polymerase chain reaction.

In a similar study, nine patients with metastatic melanoma were treated by intra-tumoral injection of a recombinant viral vector expressing human IL-12 derived from the canarypox virus (ALVAC-IL-12). Increases in IL-12 and IFN$\gamma$ mRNA, were observed in ALVAC-IL-12-
injected tumors compared with saline-injected control tumors in four of the nine patients. ALVAC-IL-12-injected tumors were also characterized by increased T cell infiltration of the tumor (90). This therapy was well tolerated with no reported dose limiting toxicities. One patient achieved a complete response in the injected subcutaneous metastasis, but all patients developed neutralizing IgG antibodies to the viral vector, demonstrating a limitation to this viral delivery strategy.

Viral vectors, probably the most commonly used for gene delivery, often result in host immune response, systemic toxicity and integration into host genome. Plasmid DNA-based vectors avoid these problems but are lacking in efficient gene transfer efficiency. In vivo electroporation, which utilizes an electric charge to facilitate entry of macromolecules into the cell, can be a reproducible and highly efficient method to deliver plasmid DNA. A phase I dose escalation trial of plasmid IL-12 gene electroporation was studied in patients with metastatic melanoma. Patients received electroporation treatments on days 1, 5, and 8 during a single 39-day cycle, into metastatic melanoma lesions through a penetrating six-electrode array immediately after DNA injection. A sterile applicator containing six needle electrodes arranged in the circle was inserted into the tumor and six pulses at field strength of 1,300 Volts/cm and pulse duration of 100 µs were applied using a Medpulser DNA EPT System Generator. Twenty-four patients were treated at seven dose levels, with minimal systemic toxicity. Transient pain after electroporation was the primary adverse effect. Post-treatment biopsies showed plasmid dose proportional increases in IL-12 levels as well as marked tumor necrosis and increased lymphocytic infiltrate. Two of 19 patients with nonelectroporated distant lesions and no other systemic therapy showed complete regression of all metastases, whereas eight additional patients (42%) showed disease stabilization or partial responses (91).

A phase I trial to assess the safety and tolerability of i.p. injected human IL-12 plasmid (phIL-12) formulated with a synthetic lipopolymer, polyethyleneglycol-polyethyleneimine-cholesterol (PPC), was conducted in women with chemotherapy-resistant recurrent ovarian cancer. A total of 13 patients were enrolled in four dose-escalating cohorts and treated with 0.6, 3, 12 or 24 mg/m2 of the formulated plasmid once every week for 4 weeks (92). This approach is attractive because of the ability of nanoparticles to transport larger amounts of genetic material than viral vectors, as well as the ability of this approach to bypass the induction of an endogenous immune response as is the case with viral vectors (93). However, nanoparticles lack the specificity required to home to sites of tumor (92). Intraperitoneal administration of this IL-12 gene bearing nanoparticle was well-tolerated, with mild to moderate fevers and abdominal pain reported for each patient. Treatment was associated with stable disease and decrease in serum cancer antigen (CA)-125 by 3% in one of the three patients in cohort-1; 36 and 86% in two of three patients in cohort-2; and 2, 11 and 16% in three of four patients in cohort-4 at the 5-week follow-up visit. There was an overall clinical response of 31% stable disease and 69% progressive disease at the 5±1 week post-treatment follow-up visits.

At present, several additional trials utilizing IL-12 as an anti-cancer therapy are currently enrolling patients. Avigan and colleagues are recruiting patients for a phase I/II trials to evaluate co-administration of a dendritic cell/tumor fusion vaccine with subcutaneously administered IL-12 to patients with stage IV breast cancer (Avigan D., Vaccination of patients with breast cancer with dendritic cell/tumor fusions and IL-12, NCT00622401).
Gajewski and colleagues are investigating the role of multipeptide vaccination with or without an admixture of intradermally or subcutaneously delivered IL-12, with subsequent daclizumab therapy in patients with metastatic melanoma (Gajewski T.F., A randomized phase II study of multipeptide vaccination with or without IL-12, then combined with regulatory T cell depletion using daclizumab in patients with metastatic melanoma, NCT01307618). A group at the National Cancer Institute is conducting a clinical trial using a novel IL-12 agent in patients with treatment-refractory solid tumors. This trial is designed to test the safety and effectiveness of experimental drug NHS-IL12 as a treatment for solid tumors that have not responded to standard treatments. The NHS-IL12 immunocytokine is composed of 2 IL-12 heterodimers, each fused to one of the V_H-chains of the NHS76 antibody, which has affinity for both single- and double-stranded DNA. Thus, NHS-IL12 targets delivery to regions of tumor necrosis where DNA has become exposed (NCT01417546). Other phase I/II study of metastatic melanoma will be conducted by Rosenberg group at NCI using lymphodepleting conditioning followed by infusion of tumor infiltrating lymphocytes genetically modified to express IL-12 (NCT01236573) (see below). These ongoing clinical trials convey the potential of this powerful immune stimulatory cytokine, while highlighting the necessity for careful dosing and more importantly, targeted delivery to reduce the risks of toxicity.

6. The promise of adoptive cell immunotherapy

Adoptive cell therapy involves the isolation, modification and expansion of endogenous immune cells, followed by the "ex vivo" expansion of and re-infusion of these cells into a tumor-bearing host. Indeed, the use of cells to deliver IL-12 to a tumor is attractive as natural immune cell features can be exploited whilst delivering IL-12 to the tumor microenvironment. One such example of this approach is the utilization of mesenchymal stem cells (MSCs) to deliver IL-12. MSCs have a widely reported ability to traffic to sites of tumor growth making them ideal delivery for IL-12 (94). Several groups have reported the use of MSCs to deliver IL-12 to tumors in murine models of glioma, renal cell carcinoma, breast cancer, melanoma, Ewing sarcoma and prostate cancer (95-99). These studies demonstrate that MSCs are successful delivery vehicles of IL-12 and the IL-12 delivered mediates anti-tumor responses in preclinical murine models involving increased IFN_γ, increased infiltration of T cells and anti-angiogenic effects (95).

Antigen presenting cells (APCs) can also be utilized in cell transfer therapy. Although APCs have the endogenous capacity to produce IL-12, the hostile tumor microenvironment often suppresses this immune stimulatory response. Adoptive transfer of APCs genetically modified to continually produce IL-12 is aimed at initiating an endogenous anti-tumor immune response. The most commonly utilized APCs for IL-12 delivery are DCs. Intratumoral injection of DCs modified to express IL-12 mediated complete regression of neuroblastoma tumor in a mouse model (100). This effect was shown to correlate with increased tumor-specific splenocyte cytotoxic capacity. Other groups have tested this approach in murine liver tumor models with similar encouraging responses (101). This latter study specifically demonstrated the generation of a protective immune response, which was dependent on T and NK cells. One alteration of this approach involves the pulsing of the IL-12 modified DCs with tumor lysates to increase the immune-stimulatory capacity of the injected cells. Using a model of colon cancer, mice were treated with tumor lysate pulsed,
IL-12 gene modified DCs which was found to dramatically inhibit tumor growth (102). This therapy resulted in an increase of endogenous immune tumor specific cytotoxicity and increased IFNγ levels, consistent with previously reported effects of IL-12. Injection of IL-12 gene modified macrophages was also found to inhibit tumor growth and spontaneous metastasis following prostatectomy in a murine model of prostate cancer (71). These studies support the utilization of APC mediated delivery of IL-12 to the tumor microenvironment as a method to initiate an effective endogenous anti-tumor response.

Other immune cells employed in adoptive transfer anti-cancer therapy include adaptive immune effector cells. Cytokine induced killer (CIK) cells are generation by the *ex vivo* activation and expansion of T cells, resulting in a cell population with both T and NK cell phenotypes (103). Using a preclinical, immunocompetent murine model of breast cancer, it was shown that augmentation of CIK therapy with IL-12 resulted in enhanced anti-tumor efficacy and complete remission in 75% of mice following therapy (104). This was found to be due to IL-12-mediated increased immune mediated cytotoxicity, improved homing and persistence, as well as *in vivo* proliferation of transferred CIK cells.

A similar strategy to deliver IL-12 specifically to tumor cells involves the use of tumor-specific T cells (50, 105). In an initial report, transgenic mouse T cells specific for the gp100 melanoma antigen were genetically modified to express an IL-12 transgene (105). These cells were then infused into irradiated mice bearing subcutaneous melanoma tumors. It was found that these IL-12 producing targeted T cells mediated rejection of tumor with dose responsive toxicity. When lower numbers of T cells were transferred into these mice, toxicity was absent and anti-tumor effects still eradicated advanced tumors, though effective therapy still required prior lymphodepletion. These anti-tumor effects were dependent on the IL-12 being present in the tumor microenvironment as T cells cultured *ex vivo* in IL-12 did not have similar anti-tumor activity. Consistent with previously published results, therapy was associated with CD8+ T and NK cell infiltration and a reduction of Foxp3 expression within the tumor. To further improve the safety of this approach, an additional study reported control of IL-12 expression by a promoter containing binding sites for nuclear factor of activated T cells (NFAT) resulting in IL-12 production only upon T cell activation within the targeted tumor microenvironment (106). An additional report describes the isolation of murine T cells, genetically engineered *ex vivo* to express the IL-12 transgene and a chimeric antigen receptor (CAR) targeted to the carcinoembryonic antigen expressed on colon cancers (50). This study demonstrated the translational applicability of this approach, and a novel potentially clinically applicable approach to tumor targeted delivery of the IL-12 cytokine. This approach is currently being investigated in a clinical trial, as described to above. Rosenberg and colleagues are currently enrolling patients in a trial utilizing *ex vivo* expanded tumor-infiltrating lymphocytes modified to produce IL-12 for the treatment of metastatic melanoma (NCT1236573).

7. Conclusion

IL-12 is a potent mediator of anti-tumor immunity. The exact mechanisms of IL-12 mediated anti-tumor effects continue to warrant further investigation. While translation to the clinical setting has been hampered by toxicity and modest anti-tumor efficacy, localized delivery of IL-12 directly into the tumor may prove to be a successful approach in limited numbers of accessible tumors. However, it is perhaps in the setting of adoptive T cell immunotherapy
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utilizing IL-12 secreting, tumor specific T cells, where the full anti-tumor benefit of IL-12 therapy will be realized with tumor targeted, locally secreted cytokine. This approach will avert systemic toxicity while providing the requisite boost to the endogenous immune system to fully eradicate tumor. IL-12 remains a unique and promising cytokine with marked anti-tumor activity and warrants continued rigorous investigation in both the preclinical and clinical settings in order to realize the full anti-tumor potential of this reagent.

8. Competing interests statement

The authors have no competing professional, financial, or personal interests that may have affected the presentation of this manuscript.

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Harnessing the potential of the human body's own immune system to attack malignant tumor cells has been the goal of many scientific investigators in recent years, with advances in cancer biology and immunology enabling cancer immunotherapy to become a reality. World-class bench and clinical researchers have joined forces to collaborate and review current developments and trends in cancer immunology for the purposes of this book, and the result is a promising review of contemporary clinical treatments. In each chapter the authors present the scientific basis behind such therapeutic approaches, including cancer vaccines with special focus on prostate cancer, melanoma and novel approaches utilizing both innate and adaptive immune responses.

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