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Review

New insights into IL-12-mediated tumor suppression

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During the past two decades, interleukin-12 (IL-12) has emerged as one of the most potent cytokines in mediating antitumor activity in a variety of preclinical models. Through pleiotropic effects on different immune cells that form the tumor microenvironment, IL-12 establishes a link between innate and adaptive immunity that involves different immune effector cells and cytokines depending on the type of tumor or the affected tissue. The robust antitumor response exerted by IL-12, however, has not yet been successfully translated into the clinics. The majority of clinical trials involving treatment with IL-12 failed to show sustained antitumor responses and were associated to toxic side effects. Here we discuss the therapeutic effects of IL-12 from preclinical to clinical studies, and will highlight promising strategies to take advantage of the antitumor activity of IL-12 while limiting adverse effects.
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Facts

- Interleukin-12 (IL-12) regulates inflammation by linking innate and adaptive immune responses. Most of the IL-12-induced effects are mediated by the secretion of interferon -γ.
- IL-12 is a potent inducer of antitumor immunity in preclinical models.
- The mechanisms by which IL-12 induces antitumor immune responses involve distinct effector cell types and cytokines depending on the tumor type and/or tumor location.
- The delivery of IL-12 for therapeutic purposes focuses on novel methods to deliver this cytokine directly to the tumor site.
- The robust antitumor response exerted by IL-12 in preclinical models has not yet been successfully translated into the clinics.

Open Questions

- A better understanding of the multiple mechanisms by which IL-12 mediates tumor protection in different types of tumors or affected tissues.
- Optimization of therapeutic schedules to locally deliver IL-12. Does it require a detailed knowledge of the molecular pathology of each individual tumor at a particular time?
- Revisiting the use of IL-12 as an adjuvant in combinatorial treatments. Do we need to inhibit particular immunosuppressive mechanisms to improve clinical benefits?
- How can we achieve durable, local, non-toxic antitumor responses with IL-12 in cancer patients? What is the best strategy to deliver this cytokine into the tumor microenvironment in a controlled manner?

The Biology of IL-12

Cytokines are among the chief players in controlling immune responses, and cytokine-based approaches for cancer therapy have been pursued in a number of ways. In that respect, the immunomodulatory cytokine IL-12, a key member of the IL-12 family of cytokines, emerged as a potent inducer of antitumor immunity. IL-12 was originally identified in 1989 as a natural killer (NK) cell-stimulatory factor with multiple biologic effects on peripheral blood lymphocytes.[1] It is mainly produced by antigen-presenting cells (APCs) such as dendritic cells (DCs), monocytes, macrophages and B cells upon Toll-like receptor engagement.[2] Thus, IL-12 is secreted as an early pro-inflammatory cytokine in response to infections.[3] Additional amplifying signals such as interferon-γ (IFN-γ),[4] IL-15[5] or cluster of differentiation (CD)40L–CD40 cell–cell interactions[6] are necessary for the optimal production of biologically active IL-12. Conversely, IL-12 is negatively regulated through cytokines such as IL-10 and transforming growth factor-β1 (TGF-β1).[7,8] IL-12 is a heterodimer with a molecular weight of 70 kDa consisting of a heavy (p40) and a

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Abbreviations: APCs, antigen-presenting cells; CAR, chimeric antigen receptor; CXCL9, chemokine (C-X-C motif) ligand 9; CXCL10, chemokine (C-X-C motif) ligand 10; CD, cluster of differentiation; DCs, dendritic cells; DNA, deoxyribonucleic acid; FDA, Food and Drug Administration; GM-CSF, granulocyte-macrophage colony stimulating factor; HER-2, human epidermal growth factor receptor 2; IL-2, interleukin-2; IL-10, interleukin-10; IL-12, interleukin-12; IL-12R, interleukin-12 receptor; IL-15, interleukin-15; IL-18, interleukin-18; IFN-γ, interferon-γ; IFN-α, interferon-α; i.p., intraperitoneal; i.t., intratumoral; i.v., intravenous; NK, natural killer; MCA, methylcholanthrene; PEI, polyethylenimine; STAT, signal transducer and activator of transcription; s.c., subcutaneous; Th17, T helper 17 cell; TSA, thricostatin-A; Treg, regulatory T cell; TGF-β1, transforming growth factor-β1; Th1, type 1 helper T cell; VEGF, vascular endothelial growth factor
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light (p35) chain subunit, which are covalently linked by disulfide bonds.[9–11] While p40 is produced in abundance by phagocytic cells, p35 is ubiquitously and constitutively expressed only at low levels and is thought to require p40 co-expression for secretion of the biologically active cytokine.[12]

The sensing of IL-12 is mediated through the heterodimeric IL-12 receptor (IL-12R) composed of IL-12Rβ1 and IL-12Rβ2.[13] Co-expression of both receptor subunits is required for the generation of high-affinity binding sites for IL-12. The IL-12R complex is found on NK cells, NK T and activated T cells[14] but has also been detected on cell types of myeloid origin[15] and tonsillar B cells.[16] Naive T cells express IL-12Rβ1 but not IL-12Rβ2, which is critical for the signal transduction downstream of the receptor complex.[17] Upon activation of T cells via the T-cell receptor, both IL-12 receptor chains are induced, which is additionally enhanced by IL-12 itself, IFN-γ, tumor necrosis factor-α (TNF-α) and anti-CD28 costimulation.[18,19] Successful triggering of the receptor activates the Janus kinase–STAT (signal transducer and activator of transcription) signaling pathway, predominantly leading to STAT4 phosphorylation, which mediates subsequent cellular responses.[20,21]

IL-12 Sensing by Innate and Adaptive Lymphocytes

IL-12 has a key role in the regulation of inflammation by linking innate and adaptive immune responses. IL-12 release by microbe-sensing APCs results in subsequent activation and proliferation of NK and T cells and promotes their effector functions by inducing the transcription of cytokines and cytolytic factors such as perforin and granzyme B.[22–24] Moreover, IL-12 polarizes T cells into a type 1 helper T (Th1) effector cell phenotype.[25–27] Th1 polarization is further pronounced by IL-12 inhibiting the developmental program of type 2 helper T cells[28] and interference with the differentiation of regulatory T cells (Tregs) and Th17 cells induced by TGF-β.[29] Additionally, IL-12 programs effector T cells for optimal generation of effector memory T cells and T follicular helper cells.[30,31] Direct effects of IL-12 on APCs have also been reported. Even though the activation of IL-12R in these cells did not involve the canonical STAT pathway, it increased their ability to present poorly immunogenic tumor peptides.[32,33]

A central mediator of IL-12-induced responses is IFN-γ, which is secreted upon IL-12 stimulation alone or with synergizing factors such as IL-2 and IL-18.[34,35] IFN-γ, in turn, acts on APCs to initiate or increase IL-12 secretion in a positive feedback loop.[36] Apart from IFN-γ release, IL-12 triggers the secretion of a number of other factors, including TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2.[37]

Mechanisms of Tumor Protection through IL-12

Whereas the role of IL-12 was primarily studied in the context of infection and autoimmunity, mice lacking the IL-12 specific subunit p35 developed significantly increased numbers of chemically induced papillomas[38] and were more susceptible to *N*-methyl-*N*-nitrosourea-induced T-cell lymphomas[39] compared to wild-type mice. In addition, IL-12p40 deficiency resulted in earlier appearance of 3-methylcholanthrene (MCA)-induced sarcomas, compared to wild-type mice.[40] The observation that animals deficient for IL12Rβ2 developed spontaneous tumors at higher frequencies and showed enhanced growth of transplantable tumors confirmed the importance of IL-12 signaling in tumor protection.[41] In humans, polymorphisms in the 3′-untranslated region of *IL12A* lead to decreased IL-12 production, which coincides with an increased susceptibility to develop glioblastomas.[42] Despite the remarkable anticancer activity exerted by IL-12, other members of the IL-12 family play critical roles in the regulation of tumor development (see Box 1).

To determine the mechanisms by which IL-12 induces antitumor immune responses, cancer cells have been engineered to continuously release this cytokine. The overexpression of IL-12 in B16 melanoma, thricostatin-A (TSA) mammary adenocarcinoma and C26 colon carcinoma cells induced tumor suppression upon subcutaneous (s.c.) inoculation.[43–46] Whereas in B16 melanoma this effect was mediated by a subset of innate lymphoid cells (ILCs), the

Box 1. The IL-12 family of cytokines: role in tumor growth

- The IL-12 family of cytokines includes IL-12, IL-23, IL-27 and IL-35.[141]
- By using mice lacking IL-23, IL-23 receptor or anti-IL-23p19 blocking antibodies, endogenous IL-23 was shown to promote tumor growth in different tumor models.[142,143]
- In colorectal carcinoma, the effects of IL-23 were mimicked after blocking IL-17A.[144] In DMBA/TPA-induced skin papillomas and MCA-induced fibrosarcomas, the protumorigenic effects of IL-23 were independent of the main Th17 cytokine.[145]
- In contrast to the protumoral role of endogenous IL-23, the administration of this cytokine by different delivery methods induced potent antitumor responses.[146–148]
- Similarly to IL-12, IL-27 has been shown to inhibit tumor growth and metastasis.[149]
- The mechanisms that drive IL-27 antitumor effects include inhibition of angiogenesis[150–152] and tumor cell proliferation,[153,154] and the activation of $CD8^+$ T and NK cells.[155,156]
- As IL-27 acts only on naive T cells, the concentrations of IL-27-induced pro-inflammatory cytokines are presumably not high enough to induce the appearance of adverse side effects.[157]
- IL-35 was initially described as a Treg-specific cytokine with potent immunosuppressive properties.[158]
- Studies conducted with IL-35-overexpressing cancer cells reported a role for IL-35 in promoting tumor growth through the suppression of T-cell responses.[159]

rejection of breast cancer TSA-IL-12 cells was dependent on CD8 cytotoxic T cells, secreting IFN-γ.[43] However, tumor rejection in IL-12-transduced C26 colon carcinoma was partially dependent on GM-CSF-producing CD4 T cells or NK cells, but independent of IFN-γ.[46] IL-12-responsive T cells were also ascribed a major role in rejection of tumors of the central nervous system.[47,48] These results indicate that differences in tumor cell type and tumor location determine the quality of the tumor immune response, involving distinct effector cell types and cytokines.

Although tumor-specific immune responses are pivotal for cancer control, changes in the vasculature status are also relevant for tumor development. Hence, overexpression of IL-12 in B16 melanoma tumors was shown to regulate the tumor vasculature, either by upregulating of adhesion molecules that may facilitate leukocyte recruitment[44] or by inhibiting angiogenesis in an IFNγ-dependent manner.[49,50] The inhibitory effects of IL-12 on the tumor vessels have been associated with increased levels of the IFNγ-inducible chemokine (C-X-C motif) ligand (CXCL) 9 and CXCL10, and a decreased production of vascular endothelial growth factor (VEGF) and metalloproteinase-9.[51–54] In general, these studies highlight the importance of IL-12-induced IFN-γ in the control of tumor growth.

The main effects of IL-12 on the tumor microenvironment have been summarized in Figure 1.

Therapeutic Effects of IL-12 in Preclinical Models

The therapeutic potential of IL-12 has been extensively investigated in various preclinical models of cancer. Especially systemic intravenous (i.v.), intraperitoneal (i.p.), subcutaneous but also intratumoral (i.t.) delivery of recombinant IL-12 leads to reduced or delayed tumor growth and increased survival in transplantable, carcinogen-induced and spontaneous tumors arising in genetically modified mice.[55–57] It is by now clear that the antitumor effectiveness of IL-12 is dose and context dependent.[40,55,58] Delivery of IL-12 for therapeutic purposes has thus far been accomplished through direct infusion of the recombinant protein, by gene therapy using viral and non-viral vectors, electroporation, by IL-12-containing microspheres and nanoparticles or by the transfer of IL-12-overexpressing stromal and immune cell types (Figure 2).

Already in 1993, Brunda *et al*.[59] reported an antitumor response of recombinant IL-12 in B16 melanoma, M5975 sarcoma and RENCA renal cell carcinoma when administered intraperitoneally. The antitumor effects of IL-12 were even observed when treatment was started at later stages after tumor inoculation and were found to be partially dependent on CD8 T cells. In a later study, however, both NK and NK T cells were reported to be the cell types responsible for IL-12-induced antitumor responses in a variety of transplantable tumor models including B16 melanoma.[40] Interestingly,

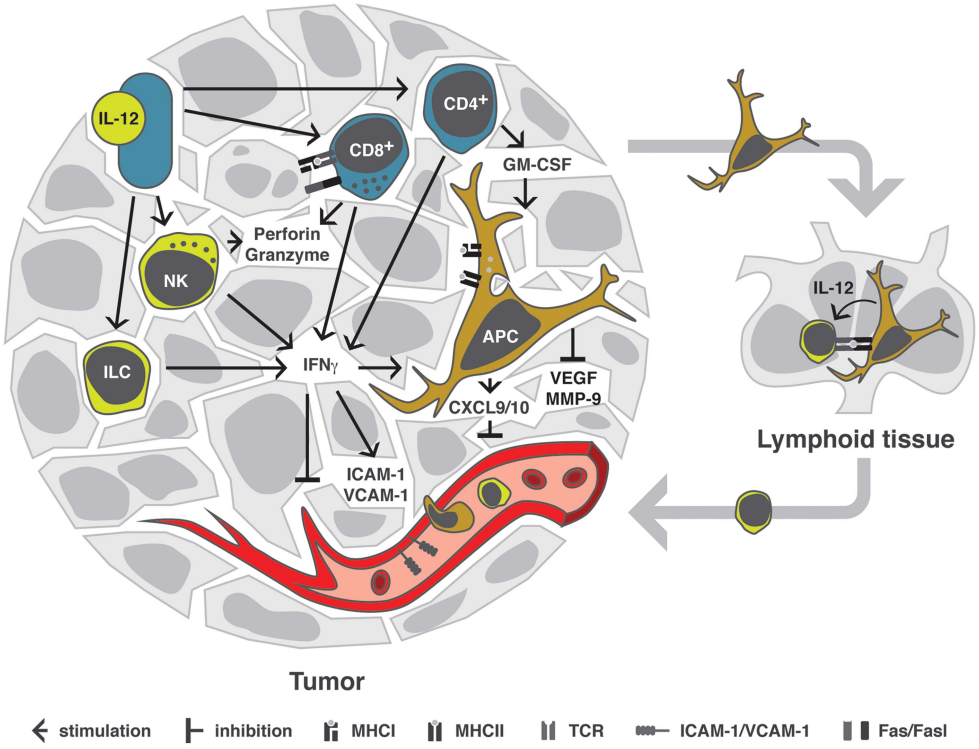

Figure 1 Cellular responses to IL-12 stimulation in the tumor tissue. IL-12 acts mainly on lymphoid cells such as NK cells, T cells and ILCs. All of these subsets increase their IFN-γ secretion upon stimulation and thereby induce most of the tumor-suppressing pathways observed upon IL-12 treatment. Furthermore, IL-12 and IFN-γ potentiate cytotoxic responses by NK cells and CD8 T cells (CD8$^+$), involving the production of perforin, granzyme and Fas ligand (FasL). The secreted IFN-γ is involved in direct tumor vascular responses such as ICAM-1 and VCAM-1 upregulation and inhibition of angiogenesis. The adhesion molecule upregulation is thought to facilitate leukocyte recruitment to the tumor tissue. Moreover, IFN-γ stimulates myeloid cells, which induce the secretion of CXCL9 and CXCL10 and suppress the production of VEGF and MMP-9, yielding in the inhibition of angiogenesis. Moreover, IL-12 stimulates antigen presentation and cross-presentation by APCs and thereby further promotes the cytotoxic activity of CD8 T cells and cytokine response of CD4 T cells. Helper T cell-derived GM-CSF upon IL-12 stimulation has also been shown to mediate the tumor suppressive effect. DC, dendritic cell; MØ, macrophage; NP, neutrophil granulocyte

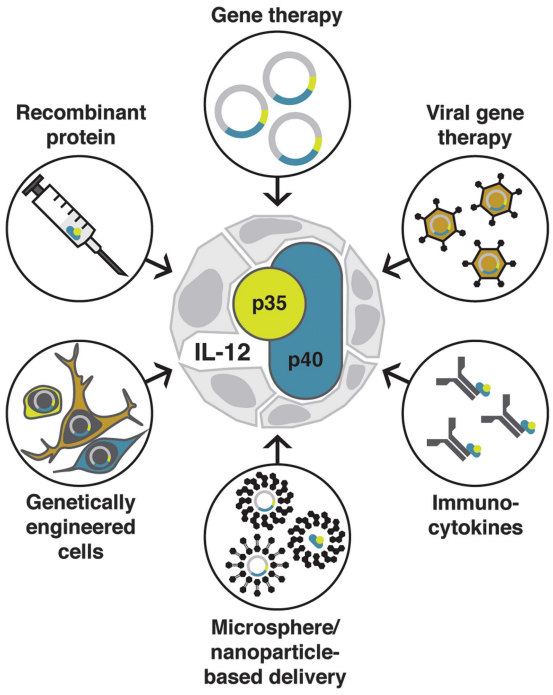

Figure 2 Different strategies used for IL-12 administration to tumors. The injection of recombinant protein, both systemically and locally, was shown to induce tumor suppression. To more specifically target the tumor tissue, recombinant IL-12 is fused to tumor cell-specific antibodies, so called immunocytokines. Furthermore, various gene therapy approaches such as electroporation and hydrodynamic dynamic injection have been used to deliver an IL-12-encoding plasmid to the tumor site. The IL-12 gene has also been transferred by viral vectors, mainly using engineered adenoviruses. Microspheres and nanoparticles have been utilized for both gene therapy and delivery of the recombinant IL-12 molecule. Moreover, stromal cells, tumor-specific T cells and DCs have been engineered to release IL-12 and transferred into tumors

discrepancies between the relative roles of each cell population in suppressing tumor growth could be attributed to the dose and time of IL-12 administration.[40] I.p. administration of IL-12 also induced effective antitumor immune responses against malignant glioma[60] and 4T1 mammary carcinoma.[61] Apart from direct effects on primary tumors, IL-12 was also able to eradicate lung metastasis when mammary tumors were surgically removed.[61]

The potent antitumor activity exerted by IL-12 made it an ideal candidate for combination with other therapy modalities directed to increase the immunogenicity of the tumor. In this respect, the combination of IL-12 with cytokines, chemotherapeutic agents, multipeptide vaccines and monoclonal antibodies potentiated the therapeutic activity of this cytokine in a variety of tumor models such as melanoma, bladder carcinoma and mammary carcinoma.[62–67] Even though the rationale for combining IL-12 with other cytokines was to achieve complementary and more durable immune-stimulating responses, most of these treatments resulted in high levels of systemic IFNγ production and therefore a potential degree of toxicity after translating them into the clinics.[65] In combination with chemotherapy, an improved antitumor activity could only be observed in immunogenic tumors when IL-12 was administered early after chemotherapy, thus highlighting the importance of the timing for immune intervention in chemotherapy-induced antitumor responses.[68] An example of a successful combinatorial treatment with IL-12 was reported in human epidermal growth factor receptor (HER)-2/neu transgenic mice, where the treatment with IL-12 together with tamoxifen or HER-2/neu multipeptide vaccines resulted in an effective prevention of tumor growth.[66,67] An enhanced tumor regression was also achieved upon co-administration of IL-12 and the anti-HER-2 antibody trastuzumab in colon adenocarcinoma.[69]

Even though systemic delivery of IL-12 showed great potential as an experimental anticancer agent, the instability and short half-life of this cytokine after bolus administration led to focus on novel methods to deliver it directly to the tumor site. Following this strategy, vom Berg *et al*.[48] implanted osmotic minipumps to locally deliver IL-12 into the brain of GL-261 glioma-bearing mice. Notably, the combined treatment of IL-12 with systemic blockade of the co-inhibitory receptor cytotoxic T-lymphocyte antigen 4 (CTLA-4) eradicated even very advanced tumors at late disease stages in a T cell-dependent manner. On the basis of this evidence, the rationale of combining IL-12 with the targeting of regulatory pathways holds a great potential to overcome tumor-associated immune suppression.

Specific delivery of IL-12 to the tumor site was also achieved through gene therapy. Thus, delivery of IL-12 by electroporation or viral-based strategies reduced growth of established colon carcinoma, melanoma and brain tumors.[70–74] In the case of breast cancer, the success of IL-12 gene therapy was dependent on the immunogenicity of the tumor. Whereas IL-12 led to regression of highly immunogenic TS/A tumors, the growth of 4T1 tumors, which are considered less immunogenic, was not affected.[75] In the 4T1 model, however, the treatment resulted in a marked reduction of lung metastasis, an effect that was partially dependent on IFNγ-producing NK cells.[75,76] Despite most of these strategies provided a continuous release of IL-12 and IFNγ within the tumor, just a few of them reported that IL-12 was acting locally by showing low serum levels of these cytokines.[71,74] Nevertheless, synergistic antitumor responses in breast cancer have been achieved by combining local adenovirus-mediated gene transfer of IL-12 with T-cell chemoattractants (lymphotactin, CXCL10), costimulatory molecules (B7.1, glucocorticoid induced TNF receptor ligand, 4-1BB ligands), GM-CSF, radiotherapy or antiangiogenic therapy.[77–83] Furthermore, the combination with chemotherapeutic or antiangiogenic agents in lung, skin and colorectal cancer, or with antiangiogenic agents in prostate cancer proved to be more efficient than viral-mediated IL-12 gene therapy alone in different types of tumors.[84–89] Even though local delivery of IL-12 by gene therapy resulted in a more sustained expression of IL-12 in comparison with the levels obtained by injecting the recombinant protein, the lack of selectivity and the occurrence of non-specific immune responses associated with the use of viral vectors remain a major concern when using this strategy to deliver IL-12.

In order to overcome these limitations, IL-12 gene was delivered to the tumor site embedded in biodegradable polymeric microspheres and nanoparticles. This method results in enhanced cellular uptake, tissue penetrability and escape from endolysosomal compartments. Tumor regression

was observed in transplantable tumor models upon i.t. injection of biodegradable polylactic miscrospheres loaded with IL-12 alone[90] or in combination with TNF-α, IL-18 or GM-CSF.[91–93] An effective strategy for treating malignant glioma was described by Sonabend *et al.*,[94] using modified polyethylenimine complexes as vehicles for IL-12 gene therapy. Of note, the synergy resulting from combining this treatment with chemotherapy resulted in 100% survival of treated mice.[94]

The transfer of cells engineered to produce IL-12 also proved to be successful in inducing long-term antitumor immunity. Early studies in this field reported the capacity of IL-12-secreting fibroblasts to delay tumor growth and eradicate established sarcomas.[95–97] In the case of CT26 colon carcinoma, however, the inhibition of tumor growth by unpulsed IL-12-transduced DCs was significantly better than the one achieved by using IL-12-transduced fibroblasts or the IL-12 gene-encoding adenovirus itself.[98] These approaches were followed by the development of therapeutic vaccines based on IL-12-expressing DCs that were additionally pulsed with tumor cell lysates or peptides. This method, used for colon carcinoma and melanoma, induced more potent tumor-specific T-cell responses than vaccination in the absence of IL-12.[99,100]

Alternative ways of cell-mediated delivery of IL-12 have taken advantage of the tumor-homing capacity of mesenchymal stem cells[101] or transfer of tumor-infiltrating lymphocytes. The latter approach has been used to deliver $CD8^+$ T cells specific for melanoma antigens[102–104] or engineered to express a chimeric antigen receptor (CAR) against CD19 in B-cell lymphomas.[105] The administered IL-12 was found to activate myeloid cells by increasing the expression of Fas and cross-presentation, leading to the stimulation of tumor antigen-specific CD8 T cells and regression of established tumors[102,106] (Figure 1). More recently, the development of novel approaches that direct IL-12 activity to the tumor site focus on immunocytokines, for example, the fusion of the cytokine to an antibody that binds specifically to the tumor vasculature,[107,108] or to exposed deoxyribonucleic acid (DNA) in the necrotic core of a tumor.[109] The targeting of necrotic areas within the tumor is of special interest due to the lack of perfusion of solid tumors and subsequent cell death.[109] The use of antibody-targeted cytokines, however, needs to be carefully evaluated for each specific tumor context, since a high avidity and retention to the targeted tissue are essential for efficient therapeutic effects. In a combinatorial approach, a dual cytokine–antibody fusion protein that simultaneously targeted IL-12 and IL-2 to $CD30^+$ lymphoma cells suppressed tumor growth more efficiently than by just targeting IL-12 or IL-2 alone.[110]

IL-12 to Treat Human Cancer

The potent antitumor effects of IL-12 in preclinical models justified the translation of this approach to a clinical setting. Unfortunately, systemic i.v. administration of recombinant IL-12 not only demonstrated poor efficacy but also caused severe adverse effects. Early studies evaluated the safety of i.v. or s.c. injected IL-12 in patients with metastatic renal carcinoma, melanoma, colon carcinoma, recurrent ovarian cancer, and neck and head carcinoma.[111–118] The goal was to administer IL-12 in a schedule that minimized common toxicities associated with cytokine therapy such as fever, fatigue, hematological changes or hyperglycemia. In general, the best way to administer IL-12 appeared to be in cycles consisting of either i.v. boluses for five consecutive days or s.c. injections for two consecutive weeks. Even though these trials established maximum tolerated doses for IL-12 for the different schedules, treatment response rates were not very promising, with only few cases of partial or complete responses (Table 1).[111,113–115,118] Moreover, the systemic administration of IL-12 displayed schedule-dependent toxicity, which appeared to be reduced when a single test dose of IL-12 was administered i.v. 2 weeks before initiation of the scheduled daily treatment cycle.[111,115] The combination of IL-12 therapy with active vaccination against tumor-associated antigens[119] or IFN-α[120] did not further improve clinical responses in malignant melanoma or renal cell carcinoma. Even in patients with metastatic $HER2^+$ breast cancer, where the combination of IL-12 with paclitaxel and trastuzumab resulted in a 52% rate of clinical benefit in a phase I trial, the combinatorial strategy was not further pursued.[121] More encouraging results, however, were obtained when treating hematological cancers (Table 1). A phase I dose escalation trial with s.c. delivery of IL-12 twice a week for up to 24 weeks resulted in an overall response rate of 56% in cutaneous T-cell lymphoma patients.[122] When the s.c. treatment was applied to patients with mycosis fungoide who had failed previous treatments, 43% of partial response to the treatment was observed.[123] Promising results were also obtained for refractory non-Hodgkin's B-cell lymphoma. Here, s.c. treatment with IL-12 led to partial or complete response in 21% of the patients, and almost 50% showed stable disease.[124] This clinical response was further improved when the therapy was combined with the anti-CD20 antibody rituximab, achieving partial response in 25% and 42% of the patients, respectively.[125] Even though the treatment with IL-12 in most of these patients was well tolerated, a repeated administration evoked increased levels of IL-10, which presumably neutralized the antitumor effects of IL-12.[125,126]

I.t. delivery of IL-12 to cancer patients was pursued by gene therapy approaches (Table 1). In patients with malignant melanoma, i.t. delivery of a plasmid encoding IL-12 led to some beneficial clinical effect at the tumor site and even in non-treated lesions.[127–129] The treatment seemed however less efficient when IL-12 was delivered encoded by a vector derived from a highly attenuated strain of the canarypox virus.[130] Viral delivery of IL-12 was also used in patients with advanced digestive cancer, but only led to mild antitumor effects.[131] Even though the treatment was well tolerated, adverse reactions were associated to vector injection.[131] Hence, a conscious manipulation of the balance between antivirus and antitumor responses is of special importance when using oncolytic viruses for IL-12-based immunotherapy.[132] In order to minimize toxicity, Rudman *et al.*[133] delivered IL-12 to the tumor site by fusing it to the humanized antibody targeting the ED-B variant of fibronectin (AS1409). Despite the authors not confirming the targeting of this immunocytokine to the tumor, the treatment led to stable disease associated to moderate toxicity in 46% of patients

Table 1 Summary of clinical trials with IL-12 alone or in combination therapies

Tumor type	Number of patients	Treatment	Clinical response	Reference
Renal cancer Melanoma Colon cancer	Renal cancer (20) Melanoma (12) Colon cancer (5)	i.v. injection rIL-12	1 PR (renal) 1 transient CR (melanoma)	Atkins *et al.*[111]
Metastatic melanoma	10	s.c. injection rIL-12	4 tumor shrinkage not reaching PR or CR	Bajetta *et al.*[112]
Recurrent or refractory ovarian cancer	26	i.v. injection rIL-12	1 PR 13 SD	Hurteau *et al.*[113]
Plateau phase Multiple Myeloma	48	i.v. injection rIL-12	4 CR	Lacy *et al.*[114]
Renal cancer Melanoma Colon cancer	12	i.v. injection rIL-12	-	Leonard *et al.*[115]
Advanced renal cell carcinoma	51	s.c. injection rIL-12	1 CR 34 SD 14 PD	Motzer *et al.*[116]
Metastatic renal cancer Malignant melanoma	28	i.v. injection rIL-12	1 PR (renal) 2 SD (renal)	Gollob *et al.*[118]
Malignant melanoma	16	Melan-A and influenza matrix peptides with s.c. rIL-12	1 PR 5 SD 7 PD	Cebon *et al.*[119]
Renal cell carcinoma Malignant melanoma	Renal cell carcinoma (19) Malignant melanoma (7)	s.c. injection rIL-12 and IFN-alpha-2b	2 PR (renal) 1 PR (melanoma)	Alatrash *et al.*[120]
Metastatic Her2$^+$-breast carcinoma	21	i.v. and s.c. injection rIL-12 Trastuzumab Paclitaxel	1 CR 3 PR 6 SD	Bekaii-Saab *et al.*[121]
Cutaneous T-cell lymphoma	9	s.c. or intralesional injection rIL-12	2 CR 3 PR	Rook *et al.*[122]
Mycosis fungoides (IA, IB, IIA)	23	s.c. injection rIL-12	10 PR 5 SD	Duvic *et al.*[123]
Relapsed and refractory non-Hodgkin's lymphoma	29	s.c. injection rIL-12	6 PR or CR 15 SD	Younes *et al.*[124]
Relapsed and refractory non-Hodgkin's lymphoma	43	s.c. injection rIL-12 Rituximab	11 CR 18 PR	Ansell *et al.*[125]
Metastatic melanoma	24	plasmid IL-12 electroporation	2 CR (distant lesions) 8 PD or PR	Daud *et al.*[127]
Metastatic melanoma	9	i.t. injection plasmid IL-12	1 CR 2 SD	Heinzerling *et al.*[128]
Metastatic melanoma	12	i.t. injection plasmid IL-12	5 PR (treated lesions)	Mahvi *et al.*[129]
Malignant melanoma	9	i.t. injection plasmid IL-12 in canarypox viral vector	1 CR (treated and non-treated lesions)	Triozzi *et al.*[130]
Advanced digestive tumor	21	i.t. injection plasmid IL-12 in adenoviral viral vector	1 PR 6 SD	Sangro *et al.*[131]
Malignant melanoma	11	IL-12 linked to antibody ED-B variant of fibronectin	1 PR 6 SD	Rudman *et al.*[133]
Recurrent ovarian cancer	20–22	IL-12 plasmid with PEG-PEI-cholesterol lipopolymer	7 SD 9 PD	Alvarez *et al.*[135]

Abbreviations: CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease

with malignant melanoma.[133] Low levels of toxicity were also achieved when delivering an IL-12 plasmid formulated with a synthetic lipopolymer (EGEN-001) to patients with ovarian cancer.[134] The evaluation of EGEN-001 in a subsequent Phase II trial, however, only showed limited activity as well as increased toxicity in patients resistant to platinum.[135]

Taken together, systemic administration of IL-12 was tested extensively and failed, barring all future attempts in this direction. Local application, however, had a promising safety profile at similar or better antitumor efficacy (see all studies above) and warrants further investigation. Current efforts are evaluating some of the most successful approaches to deliver

IL-12 intratumorally in different types of solid tumors. For instance, *in situ* electroporation of a plasmid coding for IL-12 is being tested into cancerous lesions of the skin such as malignant melanomas, cutaneous T-cell lymphomas and Merkel cell carcinoma (NCT01502293, NCT01440816, NCT01579318). Furthermore, IL-12-based immunocytokines are still in clinical development. First, the immunocytokine NHS-IL-12, consisting of two molecules of IL-12 fused to an anti-DNA human IgG1 antibody that presumably targets necrotic areas of the tumor,[109] is being evaluated in a phase I trial for metastatic solid tumors (NCT01417546). Second, AS1409 is currently in clinical trials for renal cell carcinoma and malignant melanoma (NCT00625768). The biopolymer encoding for IL-12, EGEN-001, which only showed limited success when administered as monotherapy, is going to be tested in combination with chemotherapy in the context of ovarian cancer (NCT01489371) and colorectal carcinoma (NCT01300858). Finally, a novel two-component inducible gene expression system in which adenoviral IL-12 expression is controlled by an orally bioavailable small-molecule activator ligand is being currently tested. This approach is in clinical phase I trials for advanced melanoma (NCT01397708) and glioma (NCT02026271), as well as in phase II evaluation in metastatic breast cancer alone or in combination with chemotherapy (NCT01703754).

Even though some of the above-mentioned strategies to deliver IL-12 in cancer patients have shown promising preliminary results, until now IL-12-based immunotherapy has not achieved Food and Drug Administration (FDA) approval. In clear contrast, two other cytokines such as IL-2 and IFN-α were approved by the FDA as single agents for metastatic melanoma and renal cell carcinoma, and for the adjuvant treatment of high-risk melanoma, respectively.[136] The success of high-dose IL-2, for instance, was associated to durable overall clinical responses, but the administration of the cytokine has to be carefully managed to avoid toxicities such as capillary leak syndrome.[137] In the case of IFN-α, its use as an adjuvant therapy for stage III and IV melanoma still remains controversial. Even though two clinical trials launched in 1995 demonstrated longer relapse-free survival and overall survival rates when using this adjuvant in a high dose, the results obtained in subsequent trials were not so clear and failed to clarify the mechanism of action of this drug in the treatment of melanoma.[138] Given the fact that tumor-targeted IL-12 acts primarily within the tumor microenvironment, as opposed to the activating systemic immune responses induced by IFN-α or IL-2, we believe that IL-12-based therapies will emerge primarily as combination therapies. We speculate that IL-12 alters the tumor microenvironment to enhance immunogenicity, which can then be further exploited through the use of check-point blockade or other strategies to enhance antitumor responses.

Concluding Remarks

The potential of cytokines for cancer immunotherapy has been extensively investigated. In the case of IL-12, its potent antitumor properties were already observed more than 20 years ago upon systemic administration of the cytokine in various transplantable cancer models.[59] Since then, several studies aimed to evaluate the use of IL-12 for therapeutical purposes by specifically delivering this cytokine within the tumor site. Even though several of these approaches resulted in impressive antitumor responses, the translation into the clinics was sobering. The reasons for that are still being discussed in the oncology field. On the one hand, the schedule optimization for therapeutic IL-12 delivery in clinical trials has proved to be challenging. Even though the most successful way to administer IL-12 appeared to be in cycles of twice weekly injections, repeated administration of the cytokine could contribute to increase the immunosuppressive properties of the tumor by the induction of IL-10.[139,140] On the other hand, the use of IL-12 as an adjuvant in combinatorial treatments requires a detailed knowledge of the molecular pathology of each individual tumor in order to achieve clinical benefits. In this respect, the combination of IL-12 with therapies that block the type of immunosuppressive activity characteristic of the different tumor models could be of potential use. Finally, durable, non-toxic anti-cancer responses with IL-12 will likely only be achieved with a controlled and tumor-targeted delivery of the cytokine. Several of these approaches (EGEN-001, the immunocytokine NHS-IL-12, the control of IL-12 expression by an orally activator ligand or the CAR-modified IL-12-expressing T cells) are already advancing in clinical trials. Clearly, as we only now start to understand the multiple mechanisms by which IL-12 mediates tumor protection in more detail, it is time to revisit the use of IL-12 in clinical studies. Blind systemic administration of IL-12 will not be pursued in the future, but tumor-targeted IL-12 delivery combined with radiation-, chemo- and immunotherapy, respectively, holds great promise for the future of cancer immunotherapy.

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