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REVIEW

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IL-12: a promising adjuvant for cancer vaccination

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Abstract The clinical development of interleukin 12 (IL-12) as a single agent for systemic cancer therapy has been hindered by its significant toxicity and disappointing anti-tumor effects. The lack of efficacy was accompanied by, and probably related to, the declining biological effects of IL-12 in the course of repeated administrations at doses approaching the maximum tolerated dose (MTD). Nevertheless, IL-12 remains a very promising immunotherapeutic agent because recent cancer vaccination studies in animal models and humans have demonstrated its powerful adjuvant properties. Therefore, IL-12 may re-enter the arena of cancer therapy. Here, we review the immune modulating characteristics of IL-12 considered responsible for the adjuvant effects, as well as the results of animal and human cancer vaccination studies with IL-12 applied as an adjuvant. In addition, we discuss how studies with systemic IL-12 in cancer patients, and several other lines of evidence, indicate that IL-12 may exert optimal adjuvant effects only at low dose levels. Therefore, the MTD may not constitute the maximum effective dose of IL-12 for adjuvant application.

Keywords Interleukin 12 · Adjuvant · Cancer · Vaccination · Inflammatory responses · T-helper type-1 promotion

Introduction

Specific immunity against cancer, if present, is usually not effective, as shown by the course of most human cancers. Therefore, the discovery of tumor-associated antigens for an increasing number of human malignancies [15, 132] has raised expectations of effective vaccination therapy of cancer, with the goal to induce immunity against cancer. Apparently, an effective immune response is not elicited by the tumor antigens expressed by cancers that have become clinically manifest. Indeed, in animal models, antigenic tumor cells have been shown to grow in immune-competent hosts without stimulating an acute or memory T-cell response [166, 147]. An important role in the ineffective immune responses to cancer is thought to be played by the mechanism of immune tolerance. Some tumors are capable of in vitro tolerance induction in T lymphocytes that are specific for their tumor antigens [148]. The reversal of immune tolerance into immune activation may be one of the mechanisms by which cancer vaccination can become an effective treatment modality.

Among the strategies to stimulate an effective immune response against tumor antigens is the presentation of antigens together with an appropriate immune adjuvant. Recently, IL-12 has been identified as a powerful adjuvant substance in a variety of vaccination models of infectious disease. Promising results have also been obtained in animal cancer vaccination studies using either local or systemic co-administration of IL-12 or IL-12 gene-transduced cellular vaccines. The first results in humans clearly demonstrate that IL-12 enhances tumor-specific cellular responses [85, 51]. IL-12 has several characteristics that seem essential for its adjuvant effects. In the vaccination area, IL-12 activates innate immune cells and promotes production of cytokines and chemokines, thereby mediating the attraction of other innate as well as specific immune cells to this region. We hypothesize that the co-administration of tumor antigens together with the strong pro-inflammatory cytokine IL-12 provides the environment with inflammatory danger signals required to activate antigen-presenting dendritic cells (DC) and prevents tolerance induction towards the tumor antigens. In addition, IL-12 directs the development of T-helper lymphocytes towards the

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type 1 (Th1) functional profile that promotes cellular immune responses and stimulates the proliferation of antigen-specific cytotoxic T lymphocytes (CTL) and thereby the establishment of immune memory.

The beneficial adjuvant properties of IL-12 that were demonstrated in infectious disease models [56] may not apply to tumor immunology. Various theoretical models of immune activation share the view that tumor cells and infectious pathogens are differently recognized by the immune system. The ''danger model'' hypothesizes that specific immune activation, as opposed to tolerance, is initiated when innate immune cells recognize danger signals [91]. Invasion of pathogens is usually accompanied by local inflammation and tissue destruction, resulting in danger signals and activation of antigen-presenting cells (APC), which then provide co-stimulatory signals to initiate specific immune responses. In contrast, when tumor antigens arise during malignant transformation, tissue destruction is initially minimal. The consequent absence of adequate danger signals is thought to result in immune tolerance towards the antigen. The ''innate immune recognition model'' assumes that specific immune responses are only activated when innate immune cells recognize conserved microbial structures with their pattern recognition receptors (PRR) [92]. Once the PRR identify a pathogen-associated molecular pattern, the innate immune cells are triggered to perform their effector functions and activate specific immune cells. Tumor cells are unable to activate PRR, and hence do not trigger innate immune cells to activate specific immune cells. Because of these essential differences between the immune recognition of tumor cells and infectious pathogens, the present discussion is restricted to results obtained in experimental tumor models and cancer patients.

Molecular structure, production and cellular receptors

Interleukin-12 is composed of two disulfide-linked subunits with molecular weights of 40 kDa (p40) and 35 kDa (p35) [77, 115]. The human p35 and p40 subunits are structurally unrelated and have been mapped to chromosomes 3p12–3q13.2 and 5q31–q33, respectively [141]. Cells require co-expression of both genes to secrete biologically active IL-12 [167]. IL-12 is primarily produced by phagocytic cells and antigen-presenting cells (APC) such as monocytes, DC and activated B lymphocytes [156, 64, 138], and production is strongly stimulated by infectious pathogens and their products [37, 134]. The other important stimuli for IL-12 synthesis are interactions between CD40 and its ligand (CD154), on APC and B cells or T cells, respectively [140].

Natural killer (NK) and T cells were first shown to express high affinity receptors for IL-12 composed of two sub-units, designated $\beta 1$ and $\beta 2$ [121]. Subsequently, several other cell types, such as neutrophils, DCs, B lymphocytes and eosinophils, were shown to respond to IL-12 in vitro [38, 109, 104, 160, 22, 19, 2]. Signal transduction through the high affinity receptors on lymphocytes involves tyrosine phosphorylation of the Tyk2 and Jak2 kinases and of the transcription factors STAT3 and STAT4 [11, 12, 69].

Clinical studies of systemic IL-12 as a single therapeutic anti-tumor agent

Recombinant human IL-12 has been studied as a single agent for systemic treatment in patients with various types of cancer. The development of IL-12 has proceeded along usual, FDA-required lines, with initial phase I studies to determine tolerability and safety [9, 117, 57, 111] and subsequent efficacy studies [86, 100, 68]. The maximum tolerated dose (MTD) of IL-12, i.e., one dose level below the dose that caused dose-limiting toxicity, was defined between 200 and 500 ng/kg in several intravenous (i.v.) and subcutaneous (s.c.) schedules consisting of three to six injections per 3 weeks. Common side effects consisted of fever and flu-like symptoms, nausea, fatigue, mucositis and elevation of liver enzymes. IL-12 appeared to have an exceptionally long elimination half life, estimated to be between 9 to 25 h, in comparison with other cytokines, [117, 9, 99, 13, 111]. A remarkable decrease of the area under the plasma concentration time curve (AUC) occurred after repeated injections of IL-12 [117, 124, 13]. This reduction in AUC was possibly due to up-regulation of IL-12 receptors on lymphocytes in the course of treatment, in accordance with results obtained in a mouse model, and unrelated to anti-IL-12 antibody production [154].

The first phase II study unexpectedly resulted in severe toxicity and deaths. IL-12 was administered at the MTD defined in a previous phase I study [9], and the schedule was identical except for the omission of a treatment-free period after the first dose [86, 31]. Subsequent animal studies revealed that insertion of a treatment-free period of a week after the first administration of IL-12, conforming with most phase-I studies, reduced the toxicity of subsequent injections [86, 133]. Moreover, the phase-I studies also revealed that the reduction of side effects that occurred upon repeated injections [117, 99] was accompanied by reduced IFN-γ induction [133, 86, 13, 119, 124, 57, 9]. In vitro, decreased IFN-γ secretion by T cells was related to cellular depletion of the signaling component STAT4 after prolonged IL-12 stimulation [161]. Since IFN-γ is considered to be the key regulator of IL-12-mediated antitumor effects, the down-regulation of its induction in the course of IL-12 treatment raised concerns [18, 105, 50]. In addition, our group showed that the down-regulation of biological effects also comprises the induction of TNF-α, IL-8 and IL-6 and the effect on leukocyte subset counts in the circulation. The concentrations of IL-10 remained elevated upon repeated IL-12 administrations [119, 13, 111, 100]. It has been hypothesized that IL-10, as an endogenous counter regulator of many IL-12-mediated effects, is produced during IL-12 therapy to

protect the body from the resultant ongoing and damaging inflammatory activity [96]. This hypothesis was supported by in vitro results, demonstrating that IL-12 induces high levels of inhibitory IL-10 production by lymphocytes [55, 166]. The results of phase-II studies, performed in patients with advanced renal cell and ovarian cancer, were disappointing, with overall response rates of only 7% and 4%, respectively [100, 68]. The lack of efficacy in these studies may be due to down-regulation of biological effects, including potential anti-tumor effects, due to endogenous IL-10 induction that occurs at relatively high dose levels of IL-12.

In the development of IL-12 as a vaccine adjuvant, strategies that do not result in long-term systemic exposure to high concentrations of IL-12, such as administration of low doses or infrequent administrations, may therefore be necessary to prevent down-regulation of effects.

Properties of IL-12 that can mediate adjuvant effects

Effects on the innate immune system

Inflammation

As a strong pro-inflammatory cytokine, IL-12 induces the production of multiple other cytokines. Although the induction of IFN-γ predominates, it also enhances production of other pro-inflammatory cytokines such as GM-CSF, TNF-α, IL-8, IL-6, IL-15 and IL-18 in humans [119, 111, 57]. Importantly, IFN-γ operates in a positive feedback mechanism, as IFN-γ in turn stimulates IL-12 synthesis by phagocytic cells [25]. In accordance with previous results in non-human primates [83], we have shown activation of multiple inflammatory mediator systems in patients with advanced renal cell cancer after s.c. IL-12 [120].

In vitro, binding of IL-12 to receptors on neutrophils results in activation of Ca2+ and tyrosine-signaling pathways [33]. We also observed activation and degranulation of neutrophils in humans. The activation of neutrophils might be a prominent adjuvant property of IL-12, as neutrophils can operate as intermediates between innate and adaptive immunity, not only responding to cytokines, but also producing cytokines and chemokines that enable the attraction of other immune effector cells [34, 43]. For example, platelet-activating factor, released from human neutrophils in response to IL-12, attracts other neutrophils and NK cells via chemotaxis [22]. The potentially important role played by neutrophils in cancer immune surveillance was demonstrated with tumor cells engineered to produce pro-inflammatory cytokines, such as IFN-γ and TNF-α. Vaccination with these cells resulted in anti-tumor immunity against wild-type parental tumors depending on neutrophils and CTL [101].

Another inflammatory effect observed in humans after IL-12 administration, was an increase in serum concentrations of secretory phospholipase A_2 ($sPLA_2$) [120]. Secretory phospholipase A_2 may be released from endothelial cells in response to IL-12-mediated TNF-α and IL-6 synthesis, both known promotors of $sPLA_2$ production. This lipolytic enzyme releases fatty acids, often arachidonic acid, from membrane phospholipids [35]. These are considered strong mediators of the inflammatory response.

Fibrinolysis and coagulation were also activated by IL-12 in humans [118]. The coagulation system is integrally related to the innate immune response and its activation promotes other inflammatory responses [113]. Thrombin formation occurred in 50% of patients after IL-12. Thrombin induces up-regulation of P-selectin and E-selectin; as a result aggregation of platelets with neutrophils, and interactions of neutrophils and monocytes with endothelial cells are promoted [39, 6].

In conclusion, the pro-inflammatory properties of IL-12 can mediate activation and attraction of innate immune cells, resulting in the recruitment of specific immune cells.

IL-12 may also promote immune activation against tumor antigens because the activation of multiple inflammatory systems results in danger signals [91]. IL-12 production by phagocytic cells and APC is an early event shared by a variety of pathological states that evoke activation of the innate and, subsequently, antigen-specific immune responses [146]. During sepsis and endotoxemia, IL-12 is produced [63, 70, 62], and the inflammatory response in these situations seems dependent on IL-12, as neutralizing antibodies against IL-12 can arrest the inflammatory cascade following bacterial lipopolysacharide administration [168]. Therefore, we propose, that co-administration of IL-12 and tumor antigens results in a local inflammatory response, with release of neutrophil elastase and other proteases, and synthesis of thrombin and lipid mediators, resulting in micro environmental damage [163, 112, 81]. This provides danger signals required for immune activation. As a result, local APC are activated and can provide co-stimulatory signals and activate T cells.

Notably, stimulation of IL-12 production is also considered an important working mechanism in vaccination, whereby classical adjuvant substances exert their effects. This was shown, for example, for immune-stimulating complexes containing saponin Quil A and nucleic acid vaccines containing unmethylated CpG tracts [142, 134, 28].

Natural killer cells

NK lymphocytes are also activated by IL-12, in fact, it was initially discovered in 1989 as NK cell stimulatory factor [77]. IL-12 promotes NK cell cytotoxicity, cytokine production, in particular high levels of IFN-γ [8, 107, 127] and mediates NK cell chemotaxis [4, 47]. In cancer patients, IL-12 indeed enhances the cytolytic activity of NK cells and increases the expression of CD2, LFA-1 and

CD56 molecules that mediate NK cell migration [128]. In a mice Leishmania infection model, NK cells were shown to exert an intermediate function between the innate and specific immune responses. The strong Th1 response, obtained after administration of leishmanial antigens in combination with IL-12 to mice, depended on NK cells and could be completely abrogated by in vivo depletion of NK cells [3]. NK cells also have direct cytotoxic effects against MHC class-I deficient tumors [155]. Tumor eradication after vaccinations supported by adjuvant IL-12 is dependent on NK cells in several animal models [74, 144, 79]. In this context it is of interest that IL-12 deficient mice are more sensitive to chemical carcinogens and develop increased numbers of metastases following injection of transplantable tumor as compared to wild-type controls and that this immune surveillance defect is related to sub-optimal NK-cell function [143].

Dendritic cells

Moreover, IL-12 enhances the function of DC, which are professional APC capable of processing antigen in the setting of vaccination, as they provide high concentrations of peptide/MHC ligands for T-cell receptor engagement required to activate specific immunity. DCs express IL-12 receptors, and their occupation initiates nuclear localization of members of the NF-κB family of transcription factors [59]. They are supposed to increase the maturation of DC and enhance their capability to present antigen [114], e.g., by up-regulation of class II MHC expression [60]. Furthermore, IL-12 promotes the differentiation and maturation of DC indirectly, via the induction of pro-inflammatory cytokines. The pro-inflammatory cytokines TNF-α, IL-6 and GM-CSF have been shown to mediate migration of DC to T-cell-rich areas of lymphoid organs in order to form clusters with antigen-specific T cells, creating the appropriate environment for T-helper-cell differentiation [10, 73]. In addition, IFN-γ enhances antigen processing by DC and their MHC class I-presentation of antigen [17, 48].

In recent years, DC based vaccines have received intense interest [126]. DC can be generated in vitro [169] and loaded with tumor cell lysates or tumor peptides before administration to the patient. In this way the physiological process that recruits antigen-specific T cells is mimicked to some degree. Although mature DC themselves are potent producers of IL-12, co-administration of IL-12 improves the results of DC-based vaccines. In vitro, CTL responses, triggered by autologous human monocyte-derived DC that were modified to express melanoma antigens, could be enhanced by co-transfecting these DC with IL-12 genes [158]. In situations where antigen presentation by DC without IL-12 co-administration induced T-cell anergy, IL-12 could reverse or prevent development of tolerance in favor of immune activation [58]. Similarly, IL-12 was shown to be able to convert DC from a state of tolerance to activity. In patients that presented simultaneously with progressing and regressing metastases, tissue DC from progressing metastases appeared unable to induce T-cell proliferation and did not produce Th1 cytokines, in contrast to DC from regressing metastases, and this defect could be overcome by IL-12 addition [42].

In conclusion, IL-12 plays an important role in the activation of innate immunity and potentially provides tumor antigens with a background of inflammatory effects with resultant "danger" signals that can promote activation of specific immunity.

Effects on specific immune cells

Cellular immune response

The cytokines present in the micro-environment at the time of initial antigen stimulation direct the differentiation of naive T cells into effector T-cell subsets. MHC-restricted, Ag-specific T lymphocytes are considered to be an important effector mechanism against cancer.

In the presence of IL-12, naive T cells differentiate into the functionally defined Th1 subset [66] that is involved in cell-mediated immunity. Subsequently, IL-12 is an important co-stimulus for proliferation and further activation of fully differentiated Th1 cells and their secretion of IFN-γ [53, 88].

T lymphocytes respond to IL-12 through high affinity receptors, which are composed of two sub-units, termed β1 and β2 [121]. Th1 cells express both sub-units. However, if T helper cells differentiate along the Th2 pathway, supporting humoral immune responses, they selectively lose IL-12Rβ2 and thereby become unresponsive to IL-12 [149]. Th1 commitment is enhanced by IFN-γ, which further up-regulates the IL-12 receptor [110, 156]. Once a Th1 response is induced in vivo, IL-12 is in most cases not necessary for maintaining this response [52]. This observation is important for vaccination strategies, as it implies that the addition of IL-12 to the vaccine only would be sufficient to induce and maintain the desired response.

In addition, IL-12 modulates a number of genes involved in Th1 trafficking and regulates the migration and homing of these cells. IL-12 can attract and maintain Th1 cells to the site of administration by the up-regulation of the Th1-specific adhesion molecule and their ligands. For example, IL-12 selectively increases the expression of integrin-α6/β1 and chemokine receptor CCR1 on Th1 cells in vitro [32]. Also, IL-12 upregulates the expression of glucosyltransferase enzymes that increase the expression of P-selectin and E-selectin ligand on Th1 cells, which enables their recruitment to inflamed tissues [164]. Finally, IL-12 strongly induces the expression of IP-10 in various cell types in vitro [139]. IP-10 is the ligand of chemokine receptor CXCR3, selectively expressed on Th1 cells [89]. In accordance, peripheral blood mononuclear cells (PBMC) and tumor biopsies from cancer patients showed increased expression of IP-10 after IL-12 treatment [61, 21].

DCs play an essential intermediate function in the facilitating interaction between T helper cells and antigen-specific cytotoxic CD8+ T lymphocytes (CTL). Priming of CTL is enabled by the ligation of CD40 on DC and its ligand CD154 on activated CD4+ cells [137, 14]. The strong induction of IL-12 synthesis that occurs as a result of CD40 ligation suggests an important role for IL-12 in the molecular mechanisms responsible for the CTL priming. This contention is further supported by studies using latex microspheres coated with various combinations of class I MHC-peptide complexes and co-stimulatory molecules, thus avoiding the use of APCs whose function may be affected by cytokines. It was then shown that IL-12, in the presence of antigen, acts directly on the naive CD8+ CTL to promote clonal expansion and differentiation [36].

That IL-12 plays an important role in the establishment of immunological memory was demonstrated in an experimental system in which a small number of antigen-specific CD8+ CTL were adoptively transferred into naive, syngeneic mice, in order to monitor responses to peptide immunization in the absence or presence of IL-12. Peptide immunization without simultaneous IL-12 administration induced a weak and transient expansion of CD8+ CTL, whereas in the presence of IL-12, a large clonal expansion of CD8+ T cells was induced in the draining lymph nodes. These cells were capable of antigen-specific killing in in vitro assays. Additionally, a stable memory T-cell population was generated that responded to a second challenge with IL-12 and peptide [135]. A strong specific CTL response was observed in patients with advanced melanoma after administration of IL-12. The numbers of tumor-specific CTL increased in the circulation and influx of specific-memory CD8+ T cells into metastasized lesions was documented [98].

Humoral immune response

With respect to humoral immunity, the addition of IL-12 to protein and hapten vaccinations strongly up-regulates the synthesis of Ag-specific, complement-fixing IgG2a, IgG2b and IgG3 antibody subclasses [54, 20]. Further experiments in mice revealed that the elevation of these antibody isotypes is dependent on IFN-γ induction [95]. However, in IFN-γ knock-out mice, IL-12 still significantly enhances the synthesis of specific IgG1 and IgG2b. Therefore, a two-step model of humoral immune enhancement by IL-12 was proposed [95]. Initially, the IL-12 induced production of IFN-γ by Th1 and NK cells would mediate early switching of B cells towards IgG2 immunoglobulin secretion with temporal suppression of IgG1 production. Subsequently, IL-12 would stimulate the switched B cells to secrete increased amounts of antibody, regardless of their isotype [94].

IL-12 was also identified as a pivotal molecule secreted by activated human DC that promote the differentiation of naive B cells into IgM-secreting plasma cells and hence plays an important role in the generation of primary antibody responses that are initiated by DC [40]. Finally, IL-12 may exert indirect effects on B cells via the induction of other cytokines than IFN-γ. We have shown in patients with renal cell cancer that IL-12 induces the elevation of serum levels of IL-6 [119], which is a prominent stimulator of B-cell differentiation and immunoglobulin synthesis [159].

Adjuvant effects of IL-12 in animal studies

The addition of IL-12 to different types of cancer vaccines has been extensively studied in animal (mostly murine-) models. The first vaccination protocol addressed the co-administration of IL-12 with tumor-derived peptide and resulted in the induction of peptide-specific CTL in naive, tumor-bearing mice and the eradication of established tumors [108]. Several studies used cancer cells as vaccines that had been transduced to express IL-12 [151, 152, 129, 44, 45, 136, 26, 30, 116, 65, 87, 102, 49, 41, 23, 1, 84, 103]. Alternative approaches also resulted in the presence of IL-12 at the site of tumor antigen. Recombinant viral vectors, encoding IL-12 [16, 24, 12, 125, 46] or fibroblasts, transfected for IL-12 production, were injected near the tumor [150, 90]. More recently, studies have applied the co-administration of genes encoding for IL-12 and various tumor antigens [153, 145, 5, 76]. The addition of IL-12 to these vaccines clearly enhanced the anti-tumor effects, with resultant inhibition of tumor growth and eradication of established tumors. Additionally, immune memory was established with the rejection of tumor cells at a subsequent challenge. In several studies, separate analyses have demonstrated that IL-12 plus vaccine was more effective than either component alone [123, 129, 84, 49, 65, 71, 1, 26, 41, 44, 45]. In vivo depletion of cellular subsets [1, 41, 87, 101, 123, 162, 122] and knockout mice [122, 67, 145] have been used to investigate the anti-tumor mechanism of IL-12. Additionally, the cellular infiltrate in tumor metastases after vaccination has been characterized [67, 46, 106]. In most studies, lymphocytes were pivotal effector cells. The lymphocyte subsets involved, such as CD 8+ T cells, CD4+ T cells, NK cells or a combination of these, varied with the specific vaccine and the tumor model studied. Infrequently, other immune effector cells such as macrophages have been implicated in the anti-tumor effects of IL-12 [157]. Additionally, IL-12 was shown to stimulate humoral immunity. In a model of colon carcinoma, vaccination with IL-12 transduced tumor cells cured 40% of tumor-bearing mice. Favorable anti-tumor responses were related to the synthesis of antibodies against tumor-associated antigens that induced tumor cell lysis in a complement dependent cytotoxicity assay [129]. Moreover, IL-12 increased anti-neu antibody synthesis in a model in HER-2/neu transgenic mice. Although antibody levels were not correlated with anti-tumor

protection, vaccination with a combination of plasmids encoding the neu oncogene and IL-12 resulted in protection against mammary tumors that normally develop spontaneously in these mice [5].

In several recent studies IL-12 has been combined with other strategies aimed at promoting effective immune responses against tumor antigens. The administration was systemic, together with tumor cells transduced to express co-stimulatory molecules such as B7–1 or MHC class II [72, 123]. A important and promising vaccination strategy consists of the addition of IL-12 to DC-based vaccines [93, 170, 45, 80]. In a mouse model of chemically induced fibrosarcoma, DC were pulsed with tumor peptides, that had been eluted with acid from autologous tumor [170]. These DC were combined with intra-peritoneal administration of IL-12. Alternatively, antigen-loaded DC were transfected with a retroviral vector or a pro-viral construct encoding murine rIL-12. Both strategies augmented the anti-tumor effect of the vaccine, enhanced the growth arrest of established tumors and increased specific cytotoxicity of splenic T cells, as compared to treatment with non-transfected, peptide pulsed DC or IL-12 alone. A recent experiment with DC in MHC-1 transgenic mice demonstrated that IL-12 can even reverse tolerance in vivo. MUC1 is over-expressed in human breast and other cancers. Administration of MUC-1 expressing DC to the MUC-1-transgenic mice only elicited a specific anti-MUC immune response, if IL-12 was co-administered along with the DC [80]. MUC-1-specific CTL were also induced when antigen pulsed PBMC, instead of DC, served as APC. Because peptide-loaded autologous human PBMC can be obtained relatively easy, in contrast to DC, this is an attractive approach to translate for clinical use and indeed, similar studies are now performed in humans.

Adjuvant effects of IL-12 in human studies

Clinical experience in humans is still limited. Results of two studies with tumor peptide vaccination and IL-12 co-administration in patients with malignant melanoma were recently published [51, 85]. One study was performed in patients with metastasized melanoma using a vaccine consisting of autologous PBMC pulsed with MAGE-3 or MelanA peptides and co-administration of recombinant IL-12 [51]. Fifteen HLA-A2 positive patients with metastases expressing MAGE-3 or Melan-A were vaccinated with these tumor peptides at least three times at 3-week intervals. Different doses of IL-12 were used (0, 30, 100 or 300 ng/kg) and IL-12 was admnistered s.c., adjacent to the vaccination site, on days 1, 3 and 5. Only one out of four patients treated with pulsed PBMC without IL-12, but all patients treated with 30 or 100 ng/kg of IL-12, developed a specific CD8+ T-cells response after three immunizations. Remarkably, only one out of three patients treated at the highest dose level of 300 ng/kg of IL-12 did so. Furthermore, grade 2 or 3 toxicity (fatigue, depression and decreased numbers of peripheral blood cells) only occurred with the highest dose of IL-12. Most importantly, six of eight patients with tumor-specific CD8+ T cells showed regression of all or part of their metastases. In the second study, patients with stage III or IV malignant melanoma who had undergone complete resection of macroscopic tumor were vaccinated with peptides derived from the tumor antigens gp100 and tyrosinase, emulsified with incomplete Freund's adjuvant [85]. Patients received for 26 weeks a total of eight vaccinations, with or without 30 ng/kg IL-12. The combination augmented gp 100-specific DTH reactivity and boosted the gp100- and tyrosinase-specific production of IFN-γ by peripheral blood T cells after repeated vaccinations. The number of gp100 specific CTL as measured by tetramer flow cytometry was also augmented by IL-12. Of note, the generation of specific CTL responses took several vaccinations over multiple months. This observation confirmed the clinical impression that patients with rapidly progressive disease may not benefit from therapeutic vaccination. Time to relapse was not influenced by the addition of IL-12 to the regimen and did not correlate with any of the immunological results. In a third study, the treatment of six patients with advanced melanoma with weekly vaccinations using IL-12 gene-transfected autologous irradiated tumor cells resulted in one mixed response (disappearance of part of the metastases). Two of six patients had an increased specific CTL response, as measured 2 weeks after the third vaccination, one of whom had a mixed response, the other stable disease [97]. In this study, lymphokine-activated killer cell activity was induced in the majority of patients, but was not related to the clinical outcome. In another clinical protocol, peritumoral injection of IL-12 transfected fibroblasts was shown feasible, and reduction of tumor masses near the injection site were observed [75].

Inverse dose response effect

In a human study that bears similarities to the study by Lee [85], patients with advanced malignant melanoma were treated with a vaccine consisting of gp100 melanoma tumor antigen in incomplete Freund's adjuvant [131]. The vaccine elicited the generation of anti-peptide and anti-tumor T-cell precursors in the circulation, while 42% of patients exhibited objective tumor regression, but unfortunately without attaining a clinical response. In sharp contrast with results by Lee, co-administration of IL-12 reduced the number of T-cell precursors, and anti-tumor responses were no longer observed. However, IL-12 was administered i.v. at a relatively high dose level, i.e. 250 ng/kg per day, on five consecutive days after each peptide vaccination.

In analogy with clinical studies of systemic single agent IL-12 administration, the biological effects of IL-12 may be down-regulated at the higher dose levels. Additional lines of evidence indicate that IL-12 in the setting of vaccination studies does not exert optimal

immune modulation at high dose levels and that above a certain threshold level, the dose response relationship may revert [108, 71, 82]. A very low dose of 1 ng per day, eight times in 2 weeks, co-administered with a p53 tumor peptide vaccine, induced tumor rejection and CTL generation in a murine sarcoma model, whereas doses higher than 10 ng per day failed to do so [108]. In a rat glioma model, vaccination with irradiated tumor cells in combination with subcutaneous IL-12 resulted in maximal tumor eradication and optimal protective immunity against repeated tumor challenge at the lowest applied dose of 1 ng of IL-12 per day, for 28 days [71]. In contrast, treatment with high doses of 250 ng per day for 10 days prevented the generation of tumor-specific CTL induced by immunization with GM-CSF-transfected tumor cells [82]. A possible inhibitory role for IL-10 has been suggested in a model of adoptive transfer of specific CTL in immuno-deficient mice bearing autologous tumor. In these mice, tumor growth suppression by CTL increased after injection of 100 ng of IL-12 into the tumor, every 2 to 3 days, but not after higher doses of 1,000 ng. [7]. In vitro, it was then shown that high-dose IL-12 stimulated, in addition to IFN-γ, the production of high levels of IL-10 from the tumor specific CTL, and furthermore, anti-IL-10 polyclonal antibodies could abrogate the inhibition of tumor cell lysis observed after high dose IL-12. Since IL-12 and IL-10 have opposite effects on the accessory function of DC and other APC [78], dose-finding studies for IL-12 as an adjuvant in therapeutic anti-tumor vaccination should focus on those dose levels that do not induce IL-10 reduction.

Conclusions

IL-12 is a promising adjuvant for cancer vaccination and has the potential to activate an effective immunological response to cancer. Firstly, it has strong inflammatory properties and causes the induction of other pro-inflammatory cytokines, degranulation of neutrophils, the formation of lipid mediators and activation of the coagulative and fibrinolytic systems that together can provide an environment of multiple danger signals for tumor antigens, suitable for the activation of professional APC. In addition, IL-12 directly and indirectly activates innate immune effector cells such as neutrophils and NK cells and promotes their secretion of substances that alter the microenvironment and promote expression of adhesion molecules that mediate trafficking and homing of APC and specific immune cells. Moreover, IL-12 enhances the maturation and antigen-presenting efficacy of DC and promotes T helper cell differentiation towards the Th1 type necessary for cellular immune responses. Finally, it stimulates the differentiation and lytic capacity of antigen-specific CTL and promotes immune memory.

The strong adjuvant properties of IL-12 have been demonstrated in a variety of animal models using different vaccination strategies that united tumor antigens and IL-12. Sophisticated vaccines have been constructed with antigen-pulsed DC or PBMC, transduced to express increased IL-12. In these animal cancer models, IL-12 was shown to clearly enhance the eradication of established tumor and moreover was capable of inducing a specific anti-tumor immune memory. The first human studies addressing the co-administration of systemic IL-12 to cancer vaccines have shown development of tumor-specific CTL in the course of multiple vaccinations, and although clinical responses were limited, CTL responses were clearly correlated with clinical tumor regressions. Several lines of evidence indicate that the optimal immune regulatory effects of IL-12 are confined to the lower dose levels at which the induction of IL-10 does not take place. The maximally effective dose, schedule and route of administration remain to be defined.

Based on the reviewed data, we anticipate the revival of IL-12 as an adjuvant for therapeutic vaccination against cancer.

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