

REVIEW ARTICLE

Whole Tumor Cell Vaccine Adjuvants: Comparing IL-12 to IL-2 and IL-15

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ABSTRACT

Cancer immunotherapy (passive or active) involves treatments which promote the ability of the immune system to fight tumor cells. Several types of immunotherapeutic agents, such as monoclonal antibodies, immune checkpoint inhibitors, non-specific immunomodulatory agents, and cancer vaccines are currently under intensive investigation in preclinical and clinical trials. Cancer vaccines induce permanent activation of the immune system and may be considered the most promising method for cancer treatment, especially in combination with other agents of passive immunotherapy. Among various approaches to cancer vaccines, whole tumor cell vaccines have been attracting attention for several years. Despite their low to moderate clinical effects, these vaccines have numerous advantages. Their ability to generate immune responses against tumor-associated antigens reduces the possibility for tumor cells to escape and facilitates the development of “off-the-shelf” allogeneic tumor vaccines. Understanding the reciprocal interactions between tumor cells and leukocytes is a key to harness the full potential of whole cell vaccination. Cytokines are considered as potent immunomodulatory molecules which behave as adjuvants in whole tumor cell vaccines. Improved mechanistic understanding of key cytokines in tumor immunity will serve as a resource for rational design of whole cell cancer vaccines. Although there are several reports about the use of different immunostimulatory cytokines as adjuvants, interleukin (IL)-12 appears to have superior effects compared to other cytokines. This review describes the effects of IL-12 compared to other immunomodulatory cytokines, such as IL-2 and IL-15, and highlights its application in whole cell tumor vaccination.

Sheikhi A, et al. Iran J Immunol. 2016; 13(3):148-166.

Keywords: Cancer, Cytokines, Immunotherapy, Tumor Vaccines

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INTRODUCTION

Despite the outstanding progress in the field of cancer therapy, cancer remains the second cause of death worldwide after cardiovascular disorders. While cancer affects people of all ages, the risk increases by age (1). Current cancer treatment modalities, including surgery, radiotherapy, and chemotherapy, are associated with various adverse effects. In fact, due to their lack of specificity for tumors, these treatments may fail to eliminate residual and micro-metastatic tumor cells (e.g. cancer stem cells) and exert severe negative effects on normal cells (2).

During the last decades, several attempts have been made to develop different therapeutic strategies, such as immunotherapy, to induce potent immune responses against cancer cells (3-8). At least two different approaches, including passive and active immunity, fall into the definition of immunotherapy. Passive immunotherapy, involving the adoptive transfer of immune effectors, is rapid but does not induce long-lived immunity (9). Monoclonal antibodies (mAbs) that disrupt tumor cells by different mechanisms are the most widely applied passive immunotherapies. The mAbs are associated with different success rates and some have been approved by the US Food and Drug Administration (FDA) for cancer treatment (4). Cetuximab and trastuzumab (anti-epidermal growth factor receptors HER-1 and HER-2, respectively), bevacizumab (anti-vascular endothelial growth factor), tremelimumab and ipilimumab (anti-cytotoxic T-lymphocyte-associated protein 4), and rituximab and ofatumumab (anti-CD20) are among the several therapeutic mAbs for targeting tumor cells in different cancers (10-14).

Table 1. Current methods for cancer immunotherapy using cytokines.

Type of adjuvant	Examples	Malignancy	Clinical stage	Clinical trial no:
IL-2	Aldesleukin (IL-2)	Renal cancer and melanoma	2	NCT01160445
	Allogeneic Large Multivalent Immunogen (LMI) Vaccine and IL-2	Metastatic Breast Cancer	2	NCT00784524
	Autologous Tumor-Infiltrating Lymphocytes (TILs) and IL-2	primary peritoneal cancer	1	NCT01883297
	B7-1 Gene-Modified Autologous Tumor Cell Vaccine and Systemic IL-2	Renal Cell Carcinoma	2	NCT00031564
	Natural Killer Cells Plus IL-2	Melanoma or Kidney Cancer	2	NCT00328861
	IL-2	Kidney Cancer	2	NCT01702909
	Aldesleukin (IL-2)	Breast Cancer	1	NCT00027807
	IL-2 and CD40 Ligand and Plasmid Gene Modified Autologous Tumor Cells	B-CLL	1	NCT00078520
	CD40 Ligand &IL-2 Gene Modified Tumor Vaccine Modified Autologous Skin Fibroblasts and Tumor Cells	Acute Leukemia	1	NCT00058799
	Aerosol IL-2	Pulmonary Metastases	1/2	NCT01590069

Type of adjuvant	Examples	Malignancy	Clinical stage	Clinical trial no:
IL-12	DC/Tumor Fusions and IL-12	Breast Cancer	1/2	NCT00622401
	PSMA Peptide-Pulsed Autologous PBMC Plus rhIL-12	Metastatic Prostate Cancer	2	NCT00015977
	rIL-12	Ovarian Cancer	2	NCT00016289
	rhIL-12 in combination with rhIL-2	Breast, Lung and kidney Cancer	1	NCT00005655
	IL-12 in Combination With Paclitaxel Plus Herceptin	Breast Cancer	1	NCT00028535
	rhIL-12	Solid Tumors	1	NCT00003107
	Intratumoral Delivery of Plasmid IL-12 Electroporation	Breast Cancer	1	NCT02531425
	rIL-12	Breast Cancer	2	NCT00004893
	rIL-12	Cervixcancer	2	NCT00003017
	rIL-12	Multiple Myeloma	2	NCT00003149
	Adenoviral Vector Delivery of The IL-12 Gene	Prostate Cancer	1	NCT00406939
	Adenovirus- Mediated IL-12 Gene Transduction	Prostate Cancer	1	NCT00110526
	Adenoviral Vector Delivery Of The Human IL-12	Liver Metastases	1	NCT00072098
	IL-12 gene	Skin Cancer	1	NCT00028652
Plasmid IL-12 Electroporation	Melanoma	2	NCT01502293	

Type of adjuvant	Examples	Malignancy	Clinical stage	Clinical trial no:
IL-15	Recombinant Human IL-15 (rhIL-15)	Melanoma, Kidney Cancer, Non-small Cell Lung Cancer, or Squamous Cell Head and Neck Cancer	1	NCT01727076
	Recombinant Human IL-15 (rhIL-15)	Melanoma and Metastatic Renal Cell Cancer	1	NCT01021059
	Recombinant Human hetIL-15 (IL15/sIL-15Ra)	Metastatic Cancers	1	NCT02452268
	ALT-803 (a Novel Recombinant IL-15)	solid tumors: melanoma, renal cell, non-small cell lung	1	NCT01946789
	HaploidenticalIL-15 stimulated NK cells	Solid Tumours	1/2	NCT01337544
	NK Cell Infusion With IL-15	Acute Myelogenous Leukemia (AML)	1	NCT01385423
	MT2014-25: Haplo NK With IL-15	Acute Myelogenous Leukemia (AML)	2	NCT02395822
	IL15-DC Vaccine	Melanoma	1/2	NCT01189383
	ALT-803 (a Novel Recombinant IL-15)	B Cell Non-Hodgkin Lymphoma	1/2	NCT02384954
ALT-803 (a Novel Recombinant IL-15)	Multiple Myeloma	1/2	NCT02099539	

*clinical trials.gov identifier

Active immunotherapy (tumor vaccines), on the other hand, provides long-term immunity and memory against tumor cells (Table 1)(15).

Vaccination is one of the most investigated topics in cancer immunotherapy. Several preclinical and clinical trials have investigated a variety of vaccines, such as purified antigens, immunodominant peptides, naked DNA encoding tumor-specific antigens (TSAs) or tumor-associated antigens (TAAs), recombinant viruses encoding tumor antigens, and whole tumor cells, as candidates in cancer immunotherapy (7, 16, 17).

Whole tumor cells usually express all relevant TSAs and TAAs, including those identified and unidentified for simultaneous priming of CD8⁺ and CD4⁺ T cells, and can stimulate specific immune responses. However, most tumors have no naturally potent immunogenicity due to immune-editing, a process that allows tumor cells to evolve during continuous interactions with the host immune system and eventually results in tumor escape from the immune surveillance phenomenon (18). Therefore, improving the immunogenicity of tumor cells is very important. Several studies have reported genetically modified or transduced tumor cells, that express costimulatory molecules or secrete activating cytokines, to enhance tumor immunogenicity and induce anti-tumor immune responses (19).

Moreover, in addition to poor immunogenicity, tumor vaccines may suppress immune responses and prevent appropriate immune responses through the secretion of immunosuppressive cytokines such as interleukin-10 (IL-10) and transforming growth factor- β (TGF- β) which induce differentiation of regulatory T cells (Tregs) and inhibit the maturation of dendritic cells (DCs). Thus, the use of proper immune costimulatory agents as adjuvants would be necessary in overcoming the immune evasion mechanisms (20, 21).

Despite their potential toxicities, cytokines, which are potent immunomodulatory agents, can be used as adjuvants with whole tumor cell vaccines. Among various immunostimulatory cytokines administered as adjuvants, IL-12 has been shown to have more potent effects when used with whole tumor cell vaccines (22). This manuscript reviews recent findings regarding the superiority of IL-12 over IL-2 and IL-15 and highlights its application in whole tumor cell vaccination.

Adjuvant Armed or Naked Whole Tumor Cell Vaccines

It is known that cell-mediated cytotoxicity, particularly by cytotoxic T lymphocytes (CTL), plays a major role in controlling tumor cell growth. The T cell receptor (TCR) on T lymphocytes recognizes peptides (linear epitopes) derived from tumor antigens on the surface of tumor cells or on the professional antigen-presenting cells (APCs) (23-26). A benefit of vaccination with whole tumor cells is that several TAAs or TSAs are simultaneously delivered. As a result, the need to predetermine which antigens are the most immunogenic to the host immune system is eliminated. Furthermore, this kind of vaccination can stimulate the immune response through both direct tumor-antigen presentation by tumor cells through major histocompatibility complex (MHC) class I molecules to CD8⁺ T cells as well as prolonged release of tumor antigens by APCs on MHC class II molecules to CD4⁺ T cells (7, 27-30).

Several mechanisms are involved in the immunosuppression in cancer and the degree of immunosuppression depends on several parameters such as cancer type, tumor stage, and dose of immunosuppressive chemotherapies. Unresponsiveness to tumor treatments

may be specifically limited to tumor antigens or may be due to a more generalized immune defect. Several tumor cell types are known to down-regulate or mutate their MHC molecules and/or TAAs/TSAs either initially or during clonal evolution. This process impairs their ability to efficiently present tumor-derived peptides to tumor-infiltrating T lymphocytes (31-34).

When antigen-specific stimuli are delivered to the TCR, a second set of costimulatory signals is necessary to induce an effective immune response. Optimally, these antigen nonspecific costimulatory signals are delivered by professional APCs. B7 family members, such as B7.1 (CD80) and B7.2 (CD86) molecules, on the APCs bind to their cognate ligand, CD28, on the responding T cell. Some adhesion molecules such as intracellular adhesion molecules (ICAMs) and leukocyte function-associated antigens (LFAs) may also contribute to T cell activation. The main function of costimulatory and adhesion molecules is to stabilize the relationship between the TCR and the MHC-peptide complex to produce a tight immune contact and thus provide necessary signals for T cells activation. Failure to deliver costimulatory signals in a timely fashion after TCR engagement renders the T cells anergic rather than activated (35-37).

Moreover, growing evidence, suggest that CD8⁺ lymphocytes in patients with cancer (such as hematologic malignancies) are unresponsive to relevant tumor antigens because tumor cells express MHC class I molecules without the expression of costimulatory molecules such as B7 family (38-40). Therefore, although whole tumor cell vaccines can simultaneously deliver many TAAs/TSAs (known and unknown), the direct presentation pathways of tumor antigens to T lymphocytes may be inaccessible due to the mentioned reasons. The effective way for the immune system to deal with this problem is hence to use indirect antigen presentation through professional APCs pathway. In fact, unless APCs, such as DCs, are properly licensed to initiate effector T cell responses, whole tumor cell vaccination may have a tolerization effect (41).

Tumor cells can secrete inhibitors, such as TGF- β , prostaglandins (PGs), IL-10, and vascular endothelial growth factor, which can inhibit DCs differentiation, maturation, trafficking, and antigen presentation (42-44). Therefore, since whole tumor cell vaccines may not have stimulatory effects, other adjuvants should be used for immunostimulation. Preclinical studies have shown that T cells from mice vaccinated with unmodified tumor cells were able to recognize and lyse tumor cells that were used to vaccinate the animal. In contrast, T cells from mice vaccinated with granulocyte-macrophage colony-stimulating factor (GM-CSF) plus modified tumor cells were only able to recognize tumor cells (45).

Among various adjuvants, cytokines are biologic immunomodulators that are naturally produced by numerous cell types. Cytokines are small proteins with several activities. They can both enhance and attenuate the immune response. The balance of their immune stimulatory and inhibitory properties is critical for host immunity against malignant cells. Nevertheless, cytokine networks are complex and many cytokines are currently being investigated for cancer immunotherapy. IL-2, IL-15, and IL-12 have been well characterized and are still under evaluation by several preclinical and clinical trials (46, 47).

IL-2 and IL-15 as T Cell Growth Factors

Since its first characterization as a T cell growth factor in the 1970s, the immunomodulatory effects of IL-2 have been widely studied. All members of IL-

2cytokine family, i.e. IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, consist of four alpha helix bundles and share a common receptor subunit, called the common gamma chain (γ_c). As the first approved cytokine in the treatment of cancer, IL-2 is known to be involved in numerous immune activities (Figure 1) ranging from the activation and promotion of CTL growth and generation of lymphokine-activated killer (LAK) cells to promoting the growth and proliferation of regulatory T (Treg) cells that inhibit the immune response (48, 49).

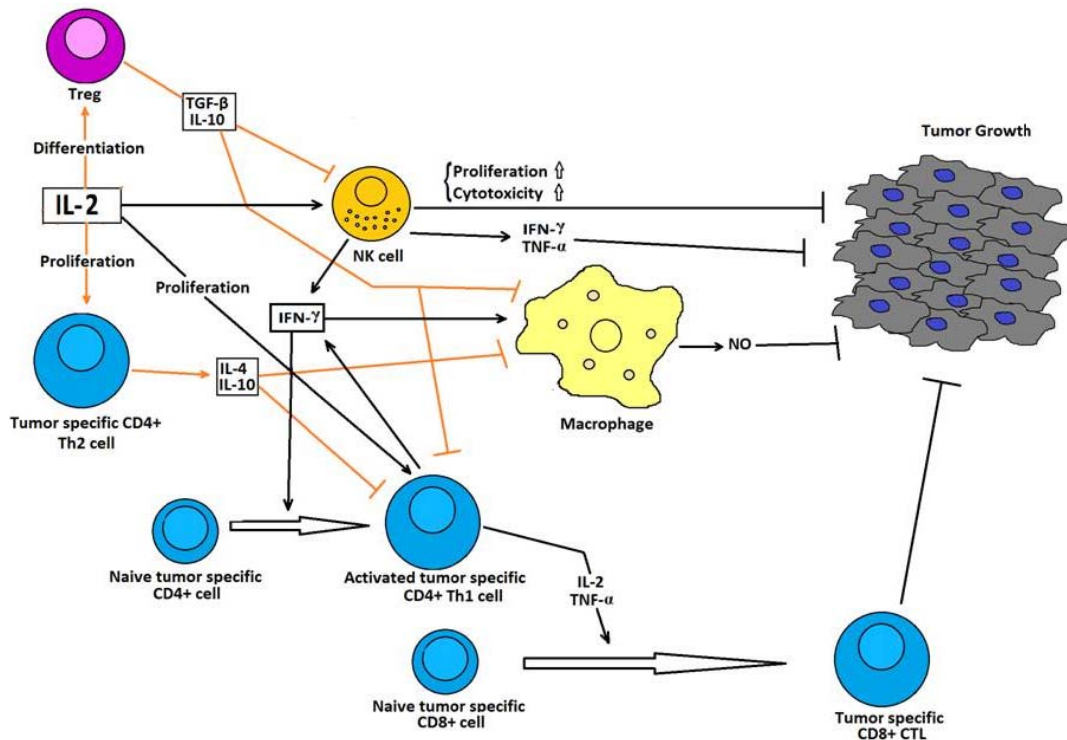


Figure 1. Immunomodulatory effects of IL-2.

Because of the broad activities of IL-2, a recombinant form of this cytokine (rIL-2) has been developed. This recombinant form activates the immune system, promotes the proliferation and differentiation of T cells, B cells, and natural killer (NK) cells, facilitates the cytolytic activity of subsets of lymphocytes, and improves the interaction between the immune system and malignant cells. Furthermore, IL-2 stimulates the principal cells that mediate tumor cell killing, specifically tumor-infiltrating lymphocytes (TILs) and LAK cells (50, 51).

Six years after its initial approval for the treatment of metastatic renal cell carcinoma (RCC) by the Food and Drug Administration (FDA) in 1992, rIL-2 therapy was approved as a procedure for treatment of metastatic melanoma (52, 53). However, a main drawback of IL-2 therapy is severe toxicity caused by its potent stimulation of pro-inflammatory cytokines. This may lead to vascular leak and a sepsis-like syndrome. The side effects include hypotension, cardiac arrhythmia, metabolic acidosis, fever, chills, lethargy, diarrhea, nausea, anemia, thrombocytopenia, eosinophilia, confusion, and

organ failure (e.g. liver and kidney failure). Considering such serious toxicities caused by the systemic administration of IL-2, novel treatments tend to supersede recombinant cytokines with genetically modified tumors and immune cells. In fact, vaccination with genetically modified tumor cells which secrete IL-2 has been found to not only inhibit tumor growth, but also encourage the formation of immunological memory (54). Unfortunately, in addition to its immunostimulatory effects, IL-2 can also suppress the immune system by promoting activation-induced T cell death and Tregs expansion. Research on IL-2- or IL-2R α -deficient mice has confirmed the non-redundant immunoregulatory (rather than immunostimulatory) role of IL-2. Due to such evidence, it is essential to evaluate the therapeutic potential of other interleukins in the IL-2 family, e.g. IL-15 (55).

IL-15 as an Effective Adjuvant for Immunotherapy

Despite their common receptor subunits and functions, particularly in the innate immunity, IL-2 and IL-15 may sometimes play different and even contrasting roles in T cell-mediated immune responses. The capability of IL-15 to promote anti-tumor immunity through the activation of NK and CD8⁺ T cells and also inducing a long-lasting immune response by the activation of memory T cells turns it into a favorable interleukin for tumor immunotherapy (Figure 2). In comparison to IL-2, IL-15 has lower toxicity and is less likely to induce Treg activity. In fact, under particular conditions, IL-15 has been reported to protect effectors T cells against the immunosuppressive effects of Tregs (56-61).

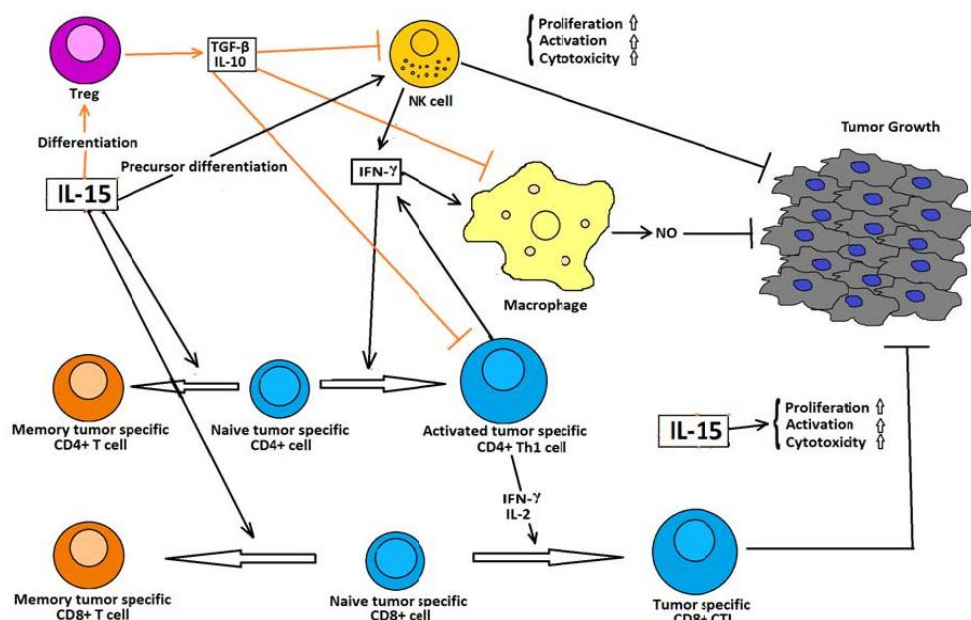


Figure 2. Immunomodulatory effects of IL-15.

As mentioned earlier, IL-2 has been approved for the treatment of both metastatic RCC and malignant melanoma. It inhibits tumor cell activity through the expansion of lymphocytes and increases the effector functions of lymphocytes. Nevertheless, this interleukin cannot effectively inhibit tumor cell growth for two possible reasons. First, the CTLs produced in the presence of IL-2 might confuse tumor cells with other CTLs and initiate activation-induced cell death (AICD). On the other hand, IL-2-dependent Tregs may also inhibit the required immune response. In contrast, IL-15 may be a more effective alternative for cancer treatment as it activates T and NK cells, inhibits AICD, and promotes the activation of memory CD8⁺ T cells (62). IL-15 was found to prevent tumor growth and eliminate syngeneic MC38 colon carcinoma cells in transgenic mice. However, it failed to produce similar effects in the wild type mice which received intravenous infusion of carcinoma cells, i.e. the animals died due to pulmonary metastases after 40 days (97).

Previous studies have also assessed the role of IL-15 trans-presentation to IL-15R α in the therapeutic effects of IL-15. While the wild type mice administered with unmodified MC38 cells died in 40 days, animals which received IL-15R α -transfected tumor cells had normal endogenous IL-15 levels and did not develop the tumor. It is believed that the trans-presentation of IL-15 to NK cells through the binding of IL-15R α (existing on tumor cells) to circulating IL-15 promoted tumor cell lysis in the second group of mice (63, 64). However, a number of studies have rejected the anti-tumor effects of IL-15 (65, 66). Differences in the studied tumor types and applied experimental designs might have been responsible for such inconsistencies.

Various tumor types adopt different evasion strategies including Treg induction, T-cell anergy induction, blockage of the immune response to TAAs through the use of immunosuppressive factors, and immune tolerance to TAAs. Consequently, a major challenge in the development of efficient tumor immunotherapy approaches is to overcome the unresponsiveness of lymphocytes of cancer patients to TAAs. Fortunately, in the presence of adequate levels of IL-15, T effector cells develop resistance against the immunosuppressive effects of Tregs. As a result, CD8⁺ T cells will regain their responsiveness to tumor cells and tolerance to TAAs will be eliminated. Such effects have not been reported following the administration of IL-2 (67, 68).

Several strategies have been employed to increase the anti-tumor properties of IL-15. Simultaneous application of IL-15 and IL-21 has been suggested to have synergistic effects on memory CD8⁺ T cells and Tregs expansion. This combination can boost the level of interferon gamma (IFN- γ) produced by NK and T cells and enhance the cytotoxicity of these lymphocytes. The majority (80%) of mice injected with plasmid-encoded IL-15 and IL-21 showed an enhanced anti-tumor immune response. Moreover, lymphomas were thoroughly regressed in the mentioned group of mice. Combinations of IL-15 and other cytokines, such as IL-7 and IL-12, have also been found to have desirable anti-tumor properties (69, 70).

On the contrary, the role of IL-15 in the development of some types of leukemia and solid tumors, inhibition of tumor cell apoptosis, promotion of tumor cell migration, survival, and proliferation has been reported. Furthermore, the role of IL-15 in enhancement of epithelial-mesenchymal transition (EMT) and tumor invasion, metastasis, and angiogenesis has also been noted (71-73). Therefore, before the administration of any tumor immunotherapy method with IL-15, thorough in-vitro examinations have to be performed to determine the exact effects of the proposed

method on target tumor cells. Moreover, due to their genetic instability, tumor cells may show higher rates of mutation, stop the expression of particular genes, or start to express different genes. Hence, cytokines (e.g. IL-2 and IL-15) which are expected to stimulate the growth, migration, or survival of normal cells, including immune cells, may mistakenly produce similar effects on specific tumor cells. This can be the case even when the original normal cells do not respond to the administered cytokine. IL-15 acts as a T cell proliferation cytokine as well as an adjuvant therapeutic agent in B cell lymphoma to potentiate antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by Rituximab activity (74).

IL-12 Directs the Immune Response to Th1

As a heterodimeric cytokine, IL-12 contains two disulfide-linked subunits, namely p35 and p40, which form a bioactive protein called IL-12 p70 (75). This protein is produced by activated antigen-presenting cells including DCs, monocytes, neutrophils, and macrophages (5, 76, 77). In contrast, the IL-12-p40/p40 homodimer is mainly involved in the competitive suppression of IL-12-p70 (78). IL-12 receptor comprises two amino acid chains, known as IL-12R β 1 and IL-12R β 2. Constitutive or inducible expression of this receptor occurs in various immune cells, such as NK, T, and B cells (79). One of the most important functions of IL-12 is to enhance IFN- γ secretion (as the most potent mediator of IL-12 activities) by NK and T cells. Other major activities of this cytokine include the activation and proliferation of NK, CD8⁺T, and CD4⁺ T cells, enhancement of CD4⁺Th0 cells differentiation into Th1 cells, and promotion of ADCC against tumor cells (80-82). Moreover, IL-12 increases the secretion of specific antibodies which are assumed to activate the complement system and initiate tumor cell opsonization. Such a mechanism will sensitize tumor cells to the cytotoxic activity of myeloid and NK cells. IL-12 particularly boosts the production of immunoglobulin G (IgG) antibodies. These antibodies, which largely contribute to opsonization and complement fixation, have been confirmed to possess strong anti-tumor properties *in vivo*. Furthermore, IL-12 uses pro-inflammatory cytokines such as IFN- γ to promote the production of oxygen and nitrogen metabolites which are toxic to particular tumor cells (Figure 3) (83, 84).

Following their activation by IL-12, NK cells can either directly kill tumor cells or damage the vascular endothelial integrity of the tumor. The secretion of chemokine receptor CXCR3 ligands (CXCL10 and CXCL9) due to the IL-12-induced production of tumor necrosis factor alpha (TNF- α), IFN- γ , and other pro-inflammatory cytokines interfere with angiogenesis process or cause endothelial cell injury by attracting activated NK and T cells (85). In other words, the induction of pro-inflammatory cytokines such as IFN- γ by IL-12 will enable them to exert direct toxicity on tumor cells or to initiate anti-angiogenic processes (86-88).

Type I IFNs, IL-10, TGF- β , prostaglandin E2 (secreted by a variety of cancer cells), along with direct cell-to-cell contact have been reported to suppress the production of IL-12. For example, tumor-derived CD4⁺ CD25⁺T regs have been found to inhibit IL-12 production through signals mediated by cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) as well as CD200-CD200L interactions. While IL-12 is not generally able to directly prevent cancer growth (exceptions to this general rule are possible), it is well capable of managing the Th1 cells-dependent anti-tumor immunity (89-91).

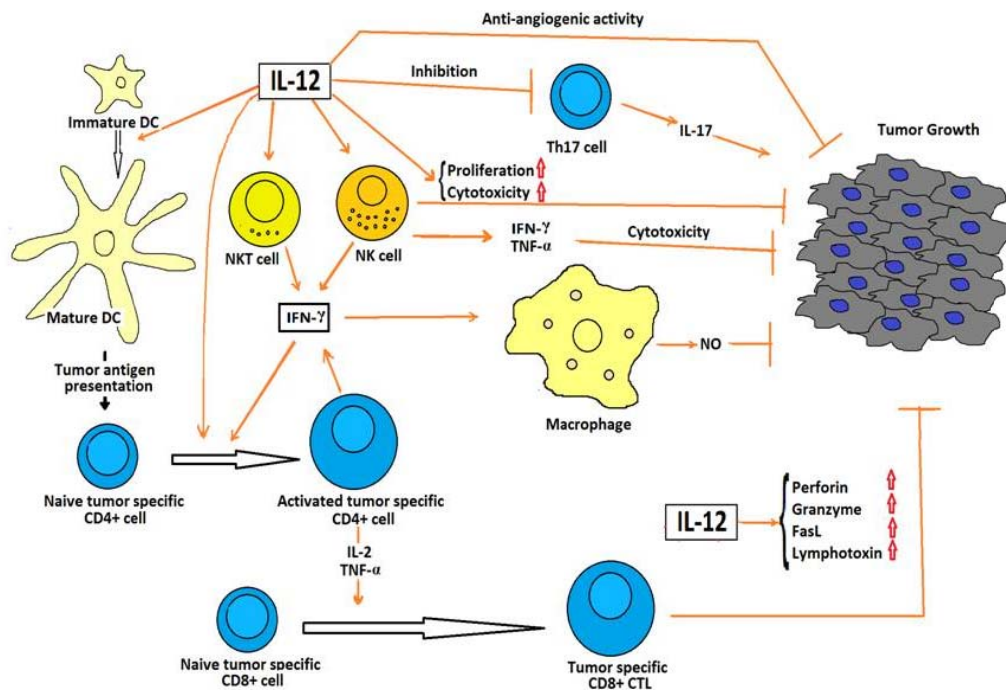


Figure 3. Anti-tumor effects of IL-15.

As mentioned earlier, IL-2 inhibits tumor growth in cases of RCC and melanoma. While such a promising effect increased interest in the use of cytokine therapy in the treatment of cancer, the application of this method was gradually hindered due to the severe toxicity reported in early trials. In 1989, however, a novel NK cell stimulatory factor (NKSF) and cytotoxic lymphocyte maturation factor (i.e. IL-12) was purified from the cell-free supernatant fluid of the phorbol-diester-induced Epstein-Barr virus (EBV)-transformed human B-lymphoblastoid cell line RPMI 8866 (75). Being among the first cytokines produced in the presence of an antigen, IL-12 is critical to IFN- γ secretion and promoting Th1-cells dependent immunity. Studies on mice have confirmed the effects of IL-12 on the regression of tumor growth and metastasis. As a pleiotropic cytokine, IL-12 can affect the activity of different cell types and act as a link between innate and adaptive immunity (75).

Despite its several reported side effects (92), IL-12 is considered as an ideal candidate for tumor immunotherapy in humans. Recent investigations continue to explore novel ways to decrease its toxicity and boost its anti-tumor properties in different malignancies.

In 1996, Rodolfo and colleagues assessed the anti-tumor potential of IL-12. They used transduced tumor cells as a vaccine for treating metastatic disease. In this study, vaccinations were conducted with irradiated tumor cells that shared cross-reacting antigens with the metastatic tumor to be cured. The immunogenic and therapeutic potential of vaccination with IL-12- or IL-2-transduced or parental non-transduced cells were compared in this study. They showed that vaccination with IL-12-secreting tumor cells resulted in a better therapeutic effect as compared with non-transduced or to IL-2-

transduced counterparts (93). In an in vitro study, the same researchers found that sera from mice treated with the IL-12 (but not IL-4) can induce complement-mediated tumor cell lysis, compared to sera from non-responder mice (94).

Lasek *et al.* reported that subcutaneous injection of genetically modified B78/IL-12 cells triggered highly potent anti-tumor mechanisms which thoroughly inhibited tumorigenicity. Moreover, intratumoral injection of irradiated B78/IL-12 cells could decelerate tumor growth and thus regress melanoma. The researchers concluded that the use of IL-12 gene-modified tumor cell vaccines resulted in more favorable outcomes in comparison with the administration of unmodified tumor cell vaccines (95).

In 1999, Dunussi-Joannopoulos *et al.* discovered that the systemic and local release of IL-12 had dissimilar clinical effects in SJL (a mice model of experimental autoimmune encephalomyelitis for multiple sclerosis) mice with transplantable leukemia. More precisely speaking; they found that although the systemic administration of rIL-12 could significantly decelerate leukemia growth and cause longer survival, it failed to completely inhibit disease progression. On the contrary, vaccines containing IL-12-expressing acute myeloid leukemia (AML) cells induced strong prophylactic and therapeutic immunity mechanisms against leukemia (96-98). In a study by Weiss *et al.*, an electroporation-based gene transfer technique was adopted for the transfection of IL-12 into two poorly immunogenic B16-F10 melanoma and RCC cell lines. After the subcutaneous injection of transfected tumor cells into the mice, IL-12 could significantly decelerate tumor formation and result in efficient and constant effects (99).

In 2007, Tatsumi *et al.* isolated IL-12 gene-transfected DCs from subcutaneous CMS4 tumor-bearing mice. They then evaluated the effects of the isolated DCs on the treatment of intrahepatic tumors. They found lower number of antigen-presenting cells along with lower allostimulatory capacity in the endogenous DCs isolated from the mentioned mice. Moreover, lower levels of IL-12p70 were produced by these DCs than by those obtained from normal mice. Antigen-presenting cells activity and allostimulatory capacity of DCs from subcutaneous CMS4 tumor-bearing mice turned back to their normal levels after the adenoviral transfection of IL-12 gene. Intratumoral administration of IL-12 gene-transfected DCs caused complete rejection of intrahepatic CMS4 tumors by inducing innate and acquired immunity and ensured long-term prevention of subcutaneous rechallenge with CMS4 tumor cells in mice. The crucial role of CD4⁺ and CD8⁺ T and NK cells in the rejection of intrahepatic tumors was also highlighted by antibody depletion examinations. In conclusion, IL-12 gene transfection could restore the functions of DCs isolated from tumor-bearing mice (100).

In 2008, He *et al.* transduced tumor cell lysate-pulsed DCs with adenovirus expressing recombinant IL-12. They showed that vaccination of mice with the resultant DCs promoted their anti-tumor immunity to colon cancer (101).

Although early clinical trials on systemic rIL-12 injections in humans indicated low response rate and high toxicity in some cases, the biological effects of IL-12 suggests its potential as an effective treatment option. Therefore, research has widely focused on the development of novel methods of IL-12 delivery, e.g. local intratumoral administration of IL-12, to benefit from the immunostimulatory effects of this molecule while preventing its undesirable toxicity (102-105).

In a study in 2012, Dietrich *et al.* implanted tumors derived from autologous Lewis lung carcinoma cells in C57/BL6 mice. After seven days, a surgery was performed to not only remove the tumors, but also vaccinate the mice with IL-12-transfected Lewis lung carcinoma cells, empty plasmid, or dead cells. Analyses revealed the first group

(vaccinated with IL-12-transfected cells) to have the lowest tumor reoccurrence rate ($\approx 40\%$). The rate was estimated at 60% in the control animals. While all types of vaccination could increase survival rate in comparison with the control group, the greatest percentage of tumor-free animals was seen in the mice vaccinated with IL-12-transfected cells (73% vs. 45% in the control group). Moreover, no tumor developed in 37%-59% of all vaccinated groups (106).

As discussed, IL-12 can stimulate different types of direct and indirect anti-tumor activities including specific and non-specific immunity and non-immune mechanisms. Its efficiency has actually been well documented in tumor therapy in animals. Despite established benefits of IL-12 in treating solid tumors and hematologic malignancies (e.g. poorly immunogenic tumors) in mice, evidence of muscular, hepatic and hematologic toxicity (e.g. anemia, lymphopenia, and neutropenia) has also been reported. Studies on squirrel monkeys have reported hypoproteinemia, splenomegaly, hypophosphatemia, bone marrow hyperplasia, hypocalcemia, and enlarged lymph nodes following IL-12 administration. Researchers attribute such hematologic adverse effects to the IL-12-stimulated production of IFN- γ and TNF- α (107-110).

A variety of gene therapy protocols have been developed by experimental research to minimize the toxicity and maximize the efficacy of IL-12 in tumor therapy. These protocols include the local and prolonged release of IL-12, the application of different viral and non-viral vectors for IL-12 gene delivery, and intratumoral injection of previously grown tumors or fibroblasts. The gene has also been effectively incorporated into vaccines containing tumor antigens, tumor cells, or DCs.

The importance of IL-12 in regulating tumor-associated angiogenesis, which clearly distinguishes between the anti-tumor effects of IL-12 and IL-2, has been highlighted by a recent study suggesting that anti-angiogenic therapy with sunitinib and sorafenib, two vascular endothelial growth factor receptor inhibitors, increased hepatocellular carcinoma metastasis through the suppression of host-derived IL-12B (IL-12-p40) (111). According to research on tumor stroma, through its effects on IFN- γ secretion, IL-12 can reverse myeloid-derived suppressor cells-mediated evasion strategies adopted by tumors (112). Furthermore, Fas-mediated collapse of tumor stroma may also be expected after local IL-12 release (113). IL-12 is also believed to affect the expression of various endothelial adhesion molecules, including the vascular cell adhesion molecule 1 (VCAM-1), which is critical to controlling leukocyte recruitment to the tumor microenvironment (114). These actions clearly represent the direct effects of IL-12 on tumor growth and metastasis and distinguish this cytokine from IL-2 and IL-15.

In an experimental study on mice, we confirmed lentiviral vector-transduced leukemia cells, engineered to express IL-12, as efficient immunostimulatory agents. The studied mice rejected both the initial IL-12-secreting and non-transduced, non-IL-12-expressing leukemia cells. In fact, an adaptive immune response was observed not only to the leukemia cell line initially used for immunization, but also to other leukemia cell lines. Based on our findings, complete protection could be ensured if one out of 200 leukemia cells produced IL-12. A therapeutic, adaptive, and long-lasting immune response could hence be obtained through the described protocol (115). In order to evaluate the efficacy of the designed approach in treating solid tumors, which are definitely more common among humans, we engineered the squamous cell carcinoma cell line, SCC-VII, to express IL-12. Subcutaneous injections in mice were followed by the development of tumors presenting dense cell growth and low immune infiltration, catenation, and immunogenicity. Engineering of the cells to express IL-12 resulted in the identification

and elimination of IL-12-secreting sarcoma cells and potent immune activation. No effects were observed in non-transduced SCC-VII cells (116).

During the recent years, cancer immunotherapy techniques have significantly improved and several approaches have shown the efficiency of this therapeutic choice in cancer patients. While whole cell cancer vaccines have been revealed to be an efficient method for active immunotherapy, appropriate immunostimulatory agents such as cytokines are required for optimal activation of the immune system against tumor cells. It has been demonstrated that IL-12 and GM-CSF are proper cytokines to be used with whole tumor cell vaccines. Several studies have noted that these cytokines have potent anti-tumor activity and are involved in different activities such as activation of immune system effector cells as well as processing and presentation of antigens. IL-12 induces immune responses through a variety of mechanisms, including T, NK, and NKT cells stimulation, which are the major effector cells targeting tumor cells. Moreover, combination of IL-12 with other agents has been shown to induce synergistic therapeutic effects and serve as a promising treatment option for human cancers. While the separate application of IL-12 has yielded favorable anti-tumor effects, the complexity of the immune system signaling pathways prevents the prediction of the exact anti-tumor immune response evoked by the combination of GM-CSF and IL-12. Further investigations are required to explore the synergistic effect of the combination of IL-12 and GM-CSF on anti-cancer immune response.

ACKNOWLEDGEMENTS

Author would like to thank Semnan and Dezful Universities of Medical Sciences for financial and administrative support.

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