Targeted Cancer Immune Therapy

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Editors

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Preface

Stimulation of the immune system's ability to control and destroy tumors continues to be the goal of cancer immune therapy; but the scope has rapidly expanded; approaches are constantly updated; new molecules are continually introduced; and immune mechanisms are becoming better understood. This book has no intention of covering every aspect of immune therapy but rather focuses on the novelty of cancer immune therapy in an attempt to give readers an opportunity to absorb the new aspects of immune therapy from a single source. In this regard, three areas were selected: cytokine immune therapy, cell-based immune therapy, and targeted immune therapy. In each of these three sections, only the novel aspects of immune therapy were described instead of attempting to cover any historical achievement. In the first section, Cytokine Immune Therapy, the IL12 family, IL18, IL21, IL24, IL28, and IL29 were emphasized in regard to the antitumor function and application in treating tumors. Most of these selected cytokines were discovered in last 10 years. In the second section, Cell-based Immune Therapy, the focus was engineering potent immune regulatory or effector cells such as dendritic cells, T cells, and stem cells. Cell engineering design is primarily based on the increased understanding of the interaction of tumor antigen-presenting cells, antigen- specific effector cells, and the tumor microenvironment. Rapidly evolving stem cell research presents us with additional promising measures to incorporate engineered stem cells in order to augment the immune function of T cells and DCs for long-term therapeutic efficacy. In the third section, Targeted Immune Therapy, the focus was rearticulating the antibody therapy for boosting immune response, which includes immunocytokines, "T-body," and tumor targeted CpG ODN. Immunocytokines represent a new class of biopharmaceuticals composed of two well known immune components–antibodies and cytokines – with the unique ability to target cytokines to the tumor microenvironment and thereby activate antitumor responses. The "T-body" approach uses the antitumor antibodies and the efficient tissue rejection of T- cells for adoptive cancer therapy. Tumor-targeted CpG ODN targets Toll-like receptors within the tumor using

hybrid molecules of antibody conjugated CpG-ODN for the induction of antitumor responses. Some or all of these innovative approaches may ultimately become effective future immune therapies for treating malignancy.

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Part I
Cytokine Immune Therapy

Role of IL12 Family in Regulation of Antitumor Immune Response

Denada Dibra and Shulin Li

Abstract The efficacy of immune therapy is partially dependent upon the tumor microenvironment. This microenvironment could either promote or demote tumor growth. Cytokines, which are secreted either by tumor, immune, or stromal cells, are key players that govern the outcome of this microenvironment. One such cytokine family is interleukin (IL)12 and the members of this family include IL12, IL27, IL23, and IL35. In this review, the expression and function of these family members and the cognate receptors in tumor microenvironment and other tissues are summarized and discussed. Our review indicates that, although these heterodimeric cytokines share subunits p35, p40, p19, EBI3, and p28 among these family members, each of them have distinct function. The same gene may also play different function when the expression is localized in different tissues.

Introduction

Tumor eradication or progression is dependent on interaction and communication with immune cells. Such crosstalk between tumors and immune cells is partially conducted through cytokines. In reality, the tumor microenvironment is frequently immunosuppressive and contributes to a state of immune tolerance [1]. As such, delivery of potent immune enhancer cytokines such as Interleukin (IL)12 may reverse immune tolerance because IL12 is a potent proinflammatory and immunoregulatory cytokine that plays a central role in tumor eradication via induction of IFNγ and cytotoxic T lymphocytes [2]. IL12 therapy for treating tumors was successful in a variety of murine models [3–5]. Although it is a promising immunotherapeutic agent, excess toxicity in preclinical trials is associated with systemic delivery of IL12 [6]. Decreasing the amount of IL12 administration reduces its therapeutic

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efficiency. Only immunogenic cancers are susceptible to this cytokine; therefore, local expression of IL12 or alternative cytokines in the tumor microenvironment is needed in cancer therapy.

The IL12 family is composed of IL12, IL23, IL27, and IL35, and although they are in the same family, these cytokines have different functions. IL12 is the key cytokine that promotes Th1 differentiation, while IL23 promotes Th17 differentiation [2, 7]. IL35 enhances the function of T regulatory cells (Tregs), while IL27 exerts pro and antiinflammatory functions [8]. Interestingly, while these family members perform such diverse functions they share many receptors and subunits. As such, IL12 and IL23 share subunit p40 and receptor IL12Rβ1, IL27, and IL35 share subunit EBI3 and receptor gp130, while IL12 and IL35 share subunit p35 and receptor IL12Rβ2 [9]. Many reviews have heavily described how these cytokines affect autoimmune diseases [10–12] as well as the behavior of immune cells [13, 14]. But the literature lacks reviews that focus on how IL12 family members signal in tumors and how they affect the crosstalk between tumors and immune cells. In summary, this review focuses on the expression and function of IL12 family members and their cognate receptors in tumors and how these cytokines affect Tregs.

Aberrant Expression of IL12 Family Member Receptors and Subunits

The IL12 cytokine family is composed of IL12, IL27, IL23, and IL35. IL27 is a heterodimeric cytokine that consists of EBI3, an IL12p40-related protein, and p28, a newly discovered IL12p35-related polypeptide [15]. IL-27 is produced by dendritic cells, monocytes, and endothelial cells [16, 17]. This cytokine exerts its biological functions through the heterodimeric receptor WSX1/TCCR and gp130 [18]. gp130 is ubiquitously expressed and is a receptor of other cytokines such as IL6, while WSX1 is specific for IL27 [19]. WSX1 is expressed mainly in monocytes, dendritic cells, T and B lymphocytes, NK cells, mast cells, and endothelial cells [18].

Recent discoveries have found that subunits or receptors of the IL12 family members are expressed not only in immune cells but also in tumor cells. Tumors have many ways to outfox the immune system such as retention of certain receptors while eliminating others or modulation of the downstream signaling of a receptor. IL23 subunits p19 and p40 are upregulated in multiple human cancers such as colon, ovarian, head and neck, lung, breast, stomach, and melanoma cancers [20]. IL12 is not upregulated in these tumors, as IL12p35 expression in tumors was similar to adjacent tissues. We have shown that WSX1 is expressed and functional in human breast cancer cells [21], while others later have confirmed its expression in human melanoma cells [22] and leukemia cells [23]. In addition to the IL27 receptor WSX1, the EBI3 subunit of IL27 is expressed in a variety of blood-related tumors [24–26]. We will carefully examine the role that each subunit/receptor plays.

While overexpression of WSX1 in epithelial cells delayed IL27-mediated tumor cell proliferation [22], WSX1 expression in leukemia cells transformed two leukemia

cell lines, 32D and BaF3, by eliciting antiapoptotic and mitogenic signals [23]. Overexpression of WSX1 not only induces cytokine (IL3)-independent growth, but also activates Jak2, ERK1/2, and STAT5, which are all markers of acute myeloid leukemia (AML) transformation. However, the activation of these genes via WSX1 is not the determining factor to induce cell transformation. The key factor is the presence of a point-mutation of Jak2 at V167F. [27–29]. WSX1-dependent transformation of the leukemia cells is dependent upon activation of Jak2-V617F. The coexpression of WSX1 and mutated Jak2, but not wildtype Jak2, results in phosporylation of STAT3 and Jak2. Therefore, overexpression of WSX1 does not transform cells per se but instead acts as a scaffold receptor to activate tumor cells with already mutated JAKs. On the contrary to the leukemia cells lines, overexpression of WSX1 in melanomas enhances IL27-mediated antiproliferative activities [22]. Enhanced signaling of IL27/WSX1 signaling is dependent on the presence of STAT1 and upregulation of MHC class I. IL27 signaling also enhances transcription factor IRF1 and IRF8 expression. IL27-mediated delayed tumor growth is partially dependent on IRF1, as downregulation of IRF1 with siRNA partially reversed the aforementioned process [22]. As a synopsis, human and mouse melanoma cells downregulate WSX1 as a mechanism to enhance cell survival.

The Pradhan group also showed that WSX1-dependent transformation of the leukemia cells is independent of gp130 and IL27, as gp130 is not expressed in the tested leukemia cells [30]. In accordance with other publications, they also show the IL27 downstream signaling is inhibited, which demonstrates that IL27 needs a heterodimeric receptor to signal [31]. As the study in the melanoma cells showed the protective role of IL27/WSX1 signaling, the study in leukemia cells did not evaluate the role of IL27 signaling by overexpression of the missing receptor gp130 on these WSX1-transformed leukemia cells. One possibility is that leukemia cells downregulate gp130 while maintaining WSX1 therefore inhibiting IL27 signaling while preserving scaffold receptor WSX1.

Not only IL27 receptor WSX1 is expressed in dissociation to gp130 in cancers, but also its subunit EBI3 is selectively expressed in dissociation to p28 in a series of Epstein-Barr virus (EBV) and human T cell leukemia virus (HTLV) type associated lymphomas [26]. EBV associated lymphomas are associated with several human malignancies such as Burkitt lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma [32]. Although these tumors express antigen-presenting molecules such as HLA-1 and immune costimulatory molecules, such as CD80 and CD86, are susceptible to CTL in vitro, CTL specific against EBV are rarely found in patients' lymph nodes [33]. One question is how these tumors downregulate immune surveillance. IL10 is one of the cytokines associated with the immunosuppressive environment in EBV-positive tumor cells [34]. Another possible factor associated with EBV-derived tumors is the IL27-EBI3 subunit. EBI3 is a downstream factor of NFkB activation, and its expression is associated with other oncogenes responsible for T cell transformation such as LMP1 and Tax [26]. Nearly 90% of tumor cells in each case tested from Hodgkin's lymphoma patients were positive for EBI3, but only 5% were positive for p28 subunits. Similarly, in EBV-associated lymphoproliferative disorders (EBV-LPDs), EBI3 was expressed at high levels, whereas p28

or IL27 were not detected. In addition, EBI3 levels were detected in follicular lymphomas and in diffuse large B cell lymphomas of both germinal centre and nongerminal B cell like types [25]. Also, EBI3 was overexpressed in a subset of adult T cell leukemias that are dependent on IL2. These lymphomas upregulate EBI3 and express significant levels of the WSX1 receptor but lack p28, a necessary subunit to form a bioactive IL27 [26]. Although normal T cells express EBI3 after activation, these levels are 16 times lower than HTLV-positive T cells. Interestingly, the EBI3 expression level in EBV-LPDs was correlated with LMP1, an oncogene that plays a role in EBV-mediated growth transformation. EBI3 induction in HTLV positive T cells is dependent on NFkB activation via Tax protein, which plays an important role in T cell transformation. The inhibition of NFkB signaling reduces EBI3 expression only in the presence of wildtype Tax, but not mutated Tax, which is defective in NFkB activation [26]. These studies suggest that EBI3 is a downstream factor of oncogenes that are associated with lymphoma transformation and might play a role in tumor progression and immune evasion.

Another independent study revealed that EBI3 is expressed in Hodgkin's lymphoma and nasopharyngeal carcinoma [24]. In addition to EBI3, IL12p35 was also expressed, but IL12p40 subunit was not expressed. The copresence of IL12p35 and EBI3 and the absence of IL12p40 could result in the production of immunosuppressive cytokine IL35. It seems logical that immune evasion of these lymphoma cells may be attributed to IL35 function. In accordance with these conclusions, others have indicated that nasopharyngeal carcinoma cells are not capable of inducing IL12p70 [35]. These findings suggest that EBV HTLV-type associated lymphomas selectively modulate IL12 family members by enhancing EBI3 and/or p35 while downregulating p40 and/or p28 to attain a favorable tumor microenvironment.

The dissociated expression of EBI3 and p35 expression is observed not only in a pathogenic scenario but also in normal settings such as the intestinal tract [36]. The intestinal tract is the initial contact site between host and pathogens. In a balanced system, proinflammatory signals are cancelled with antiinflammatory signals. Overexpression of proinflammatory cytokines, such as IL12, in this environment would result in an autoimmune disease. Defining the mechanism on how the intestinal tract differentiates between pathogenic and commensial bacteria is of crucial importance. It would not only provide insight into the control processes in the peripheral tolerance but also indicate several potentially important therapeutic targets. One potential use of these targets would be cancer therapy since immune cells develop tolerance toward tumor cells. Human mucosal epithelial cells produce EBI3, IL12p35, and IL23p19 but not their counterpart subunits, such as p28 and IL12p40, which are necessary to form bioactive and functional IL12, IL27, and IL23. Proinflammatory mediators such as IL1α and TNFα induce EBI3 and p19 but not IL12p35 [36]. p35 is induced after IFNγ responses and its expression was delayed when compared with EBI3 and p19. This model suggests that in a balanced system such as the intestinal tract induction of p35 to make a functional IL35 is pushed to a later time point; therefore, only after a prominent cell-mediated immune response are both subunits of IL35 induced.

IL27 signaling in tumors is inhibited by dissociated and/or aberrant expression of its receptor WSX1 and gp130 [21–23]. As mentioned earlier, one possibility is that certain tumors preferentially lower one or the other receptor as a mechanism to enhance cell survival. Although modulation of IL27 receptors in tumors might be useful therapeutically, clinical translation as a therapy would require further investigation of (I) the pathways that are activated by WSX1 receptor and (II) the critical pathways activated by this receptor that are malfunctioning in tumor-bearing patients. Further mechanisms are needed to establish how this receptor activates downstream pathways in either epithelial or blood-related tumors; nonetheless, its therapeutic potential is promising.

Role of IL23 in Tumor

IL23 is another member of the IL12 family. This cytokine is composed of two subunits: p19 and p40 [9]. Although IL23 shares subunit p40 and receptor IL12Rβ1 with IL12, they drive quite different immune pathways. IL12 drives the classical IFNγ pathway, and IL23 is an essential factors required for the expansion of already committed Th17 cells into pathogenic cells [37]. Although many reviews and research articles have focused on the role of IL23 in autoimmune diseases, only a few articles have focused on the role of IL23 in cancers [38–40].

The role of IL23 in tumor biology is double-faced because the lack of IL23 shows protection against tumor initiation while IL23 used as a therapeutic or vaccine adjuvant reduces tumor growth [20, 41, 42]. To study the role of IL23 expression in epithelial tumorigenesis, the authors tested susceptibility of IL12p35$^{-/-}$, IL12/23p40$^{-/-}$, and IL23p19$^{-/-}$ mice to tumor formation during cancer progression [20]. Mice lacking IL23 subunits p19 and p40 but not p35 were resistant to tumor initiation and papilloma formation. Reduced tumor initiation in IL23 deficient mice was consistent with reduction of inflammatory markers, which are essential for tumor promotion such as IL17, GCSF, MMP9, and CD31. Another interesting factor is that the lack of IL23 in the tumor microenvironment enhanced CD8 infiltration in vivo. Lack of CD8 T cell infiltration was dependent on IL23, as intradermal injection of IL23 reduced CD8 T cell infiltration while IL12 enhanced CD8 infiltration as previously observed [43].

Contrary to the discovery described above, local and systemic administration of IL23 reduces tumor growth. Local overexpression of single chain IL23 in cell lines such as immunogenic CT26 grew in Balb/c mice but then spontaneously regressed in a CD8 T cell-dependent matter [41]. This same phenomena was also observed using a poorly immunogenic melanoma cell line such as B16F10 [42]. IL23-mediated tumor growth inhibition was dependent on CD8 T cell and IFNγ production. In another study by Overwijk, overexpression of IL23 in nonimmunogenic B16 tumors did not show tumor growth inhibition. Nonetheless, this group studied how IL23 could be used as an adjuvant to vaccination of already established nonimmunogenic

melanoma tumors [44]. They used a gp100 peptide vaccination after adoptively transferring antigen-specific pmel CD8 T cells toward this peptide. IL23 aided tumor suppression by vaccine-induced T cells and enhanced function of intratumoral T cells. The enhanced T cell effector functions were characterized by high ability of antigen specific CD8 T cells to produce IFNγ without need of in vitro stimulation with the peptide. Although IL23 enhances IFNγ production by tumor-specific T cells, they conclude that IFNγ production by CD8 T cells does not have a major role in enhancing the role of IL23 as an adjuvant; however, adoptive transfer of IFNγ$^{-/-}$ pmel T cells still remains responsive to IL23 therapy. Contrary to the statement that IFNγ is dispensable, others have shown that IFNγ is absolutely necessary for IL23 antitumor activity [45]. In IFNγ knockout (KO) mice, IL23 antitumor effects were nonexistent, and in IL12 KO mice, the effects were partially abrogated. In addition, this study shows that IL23 administered systemically reduces tumor growth and is dependent on CD4, CD8, and partially on NK cells. The authors suggest that once a Th1 response is fully established, IL23 does exert its potent antitumor activity. This claim is not in disagreement with Overwijk study since their argument solely depends on IFNγ$^{-/-}$ CD8 transfer, but not on the endogenous IFNγ. In summary, IFNγ is primarily involved in IL23-mediated antitumor activities while IFNγ production from CD8 is dispensable in this process. Further conclusive studies are needed to elucidate whether prevalence of Th1 response is necessary to mediate IL23 antitumor response and molecular mechanism associated with it.

IL23-mediated antitumor effects are observed not only in mouse tumor models but also in human pancreatic cancer cell lines such as AsPC [46]. Interestingly, AsPC overexpressing IL23 showed retarded tumor growth in nude mice but not in SCID mice. In addition, depletion with anti-asialo GM1 antibody did not affect tumor growth inhibition in nude mice. In this particular tumor model, IL23 mediates its antitumor effect mainly through γδ T and/or NKT cells.

One of the downfalls of using IL23 systemically is weight loss, which is toxicity dependent on the expression of TNFα [44]. Depletion of TNFα reduces side effects; nonetheless, it is not feasible as it mediates not only weight loss but also antitumor activity. Therefore, local rather than systemic administration of this cytokine would improve its antitumor activities as a direct immune stimulator or as a vaccine adjuvant. Indeed, local expression of IL23 augmented vaccine-induced antitumor activity without weight loss.

Contradictory to the aforementioned role of IL23 as an anticancer therapeutic agent/adjuvant, IL23 expression in the microenvironment enhances tumor growth partially by activating a tumor-promoting inflammation and angiogenesis. It is well known that IL23 promotes pathogenic Th17 lineage. It also promotes tissue restructuring and neovascularization and all tumor-adopted strategies to thrive and grow. To explain the contradictory role of IL23, these authors suggest that high expression of IL23 in the tumor microenvironment induces an overwhelming myeloid infiltration of DC, macrophages, and granulocytes that destroy tumors [40]. Others indicate that a Th1 priming microenvironment in the host is necessary for systemic delivery of IL23 to eradicate tumors as IL23 was noneffective to eradicate tumors in INFγ$^{-/-}$ mice [45]. IFNγ also has been shown to downregulate Th17 while promoting Th1

induction [47, 48]. On the one hand, it is also reasonable to assume that exogenous IL23 can serve as a potent adjuvant/therapeutic anticancer agent to enhance an already established but probably weak Th1/IFNγ immune response. On the other hand, endogenous IL23 produced at the local microenvironment together with other inflammation-promoting agents from tumors, such as TGFβ, might reroute the immune response toward more of wound-healing tumor-promoting Th17.

Role of IL27 in Tumor

One of the earlier functions attributed to IL27 is its ability to synergize with IL12 to induce IFNγ and proliferation of naive CD4 T cells [15]. IL27 also induces T-bet expression and IL12Rβ expression, key components to Th1 commitment, through STAT1 [49]. Given its role in initiation of Th1 response and induction of IL12 receptor, several researchers evaluated the role of IL27 in cancer immunotherapy. The function of IL27 was examined in different tumor models such as colon cancer 26 (CT26), neuroblastoma (TBJ), and aggressive melanoma (B16F10) [50–54].

IL27 possesses T cell and NK cell-mediated antitumor activities. In an immunogenic colon cancer system, IL27 mediates antitumor activities mainly though CD8 T cells and epitope-specific CTL. Immunogenic CT26 overexpressing IL27 showed reduced tumor growth in vivo. IL27 expression induced IFNγ and increased CTL against CT26 cells. The mechanism was dependent on CD8 and IFNγ, since antitumor activity was abolished in nude mice or mice depleted of IFNγ and CD8. Interestingly, in T-bet$^{-/-}$ mice, IL27 did not display any antitumor activities [55]. Not only CD8 T cells but also other subsets of T and NK cells account for IL27 antitumor properties. In nude mice, CT26-IL27 tumor growth was retarded when compared with parent CT26. These phenomena were partially reversed upon administration of anti-asialo GM1 depletion antibody [50]. This result suggests that γδ T cells or NKT are the possible effector cells that are mediated by IL27 and contribute to antitumor effect. Others show that in highly aggressive melanoma B16F10, NK cells were mainly involved [53].

IL27 antitumor activity does not depend on either STAT4 or IL12 [56]. Since IL27 exerted antitumor effects in either IL12p40 or STAT4 KO mice, IL12 is not necessary for IL27-mediated antitumor activity. In contrast to IL27, IL23 is partially dependent upon IL12 to exert antitumor activity [45]. The mechanism associated with IL27 as an anticancer agent depends upon CTL induction and enhancement of cytolytic molecules such as granzyme B and perforin [56]. STAT1 was the important transcription factor necessary for induction of T-bet, IL12Rβ2, perforin, granzyme B, and synergistic induction of IFNγ with IL12 [56]. T-bet was important for induction of IL12Rβ2, perforin, granzyme B, and synergistic induction of IFNγ with IL12. IL27 is known to enhance T-bet expression, suggesting an important role of IL27 in regulating the expression of these genes. However, IL27 could enhance allogeneic CTL activity independent of T-bet [56]. Although IL27 increased CTL activity in a T-bet independent matter, this transcription factor is important for IL27-mediated antitumor

activities in vivo, as tumors grew much faster in T-bet$^{-/-}$ mice but not in wildtype mice. These phenomena could be explained by a later study, which emphasizes that T-bet and WSX1 expression in CD8 T cells are indispensable for IFNγ production in CD8 T cells in vivo but not in vitro [57].

In the neuroblastoma TBJ cell line, overexpression of IL27 completely eradicated more than 90% of tumors and rendered these mice resistant to tumor challenge [58]. IL27 producing tumors, TBJ27, but not control TBJ tumors reduced adjacent parental tumor cells. IL27 overexpression conferred tumor memory not only on neuroblastoma but also in another independent tumor model such as CT26 [55]. In addition to inducing tumor memory, overexpression of this cytokine in the tumor microenvironment also reduced metastasis of primary tumors. TBJ27 reduced the number of metastatic tumors in the liver, and 40% of the mice were free of their metastatic tumors. The mechanism responsible for IL27-mediated tumor regression was dependent on CD8 T cells but not on NK or CD4 T cells. IL27 also enhanced IFNγ and MHC class I expression in the tumor microenvironment [58]. Such great antitumor effect associated with IL27 production in the tumor could be attributed to IL27 signaling in the host as well as in the tumor itself. Although this study did not determine WSX1 levels on the TBJ cell line, enhanced MHC class I induction in the tumor environment suggests the presence of a functional WSX1.

IL27 antitumor effect has been shown not only in immunogenic models such as CT26 and neuroblastoma but also in B16F10, a mouse melanoma that is a model of poor immunogenicity characterized by low MHC class I expression. B16F10 that overexpress single chain IL27 show reduced tumor growth of primary tumors and pulmonary metastases [59]. Interestingly, IL27-mediated antimetastatic activities were not dependent on the host as T, B, and NK deficient mice still retained tumor growth inhibition. The authors also showed that IL27 enhanced expression of anti-angiogenic markers such IP10 and MIG while it reduced in vivo angiogenesis. What seems to be quite impressive is that IL27 acts independently of IFNγ to induce anti-angiogenesis markers. Even in IFNγ$^{-/-}$ mice, IL27 expressing B16F10 tumors, B16F10-IL27, showed reduced tumor growth and metastasis. Similarly, another group using the same tumor model, B16F10, showed that IL27 exerts anti-tumor activities in the absence of IFNγ [42]. This phenomenon is quite different from IL12 and IL23 as both these cytokines depend on IFNγ to induce antitumor activity [45]. It also seems that IL27, a downstream molecule of IFNγ and IL12, might act synergistically or independently of IL12 to suppress tumor growth.

As we have previously seen, IL27 antitumor effects are not only attributed to signaling in tumors or immune cells but also to vascular endothelial cells that surround the tumor microenvironment [59, 60]. During tumor progression, endothelial cells can have two roles: promoting or inhibiting tumor growth. On the one hand, endothelial cells act as a support matrix in tumors and provide many growth factors to tumors through enhancing angiogenesis. On the other hand, endothelial cells can function as antigen-presenting cells and can upregulate MHC class I and II to aid CTL activity [61]. In addition, they can upregulate certain receptors to recruit innate immune cells [62]. The balance between anti and protumor environments depends on the cytokine

profiles and the level of each cytokine expressed in these cells. The IL27 receptor WSX1 was present in endothelial cells, and IL27 signaling directly on endothelial cells has increased antiangiogenic molecules such as IP10 and MIG [59]. IL27 also upregulated MHC class II and MHC class I together with microglobulin and *Tap* genes [60]. It also increases fractaline expression in endothelial cells, a chemokine that attracts and activates CX3CR1 NK positive cells and DC cells [63, 64]. Activation of NK and maturation of DC cells in the local microenvironment leads to an enhanced expression of IL12 and IFNγ, both factors necessary to tip the microenvironment balance toward an antitumor response.

IL27 overexpression exerts antitumor effects not only in mouse carcinoma but also in human oesophageal carcinoma Eca cell line [65]. When injected into nude mice, Eca cells overexpressing IL27 showed a retarded tumor growth and enhanced survival. Similar to previous tumor models, NK cells from mice overexpressing IL27 showed increased IFNγ production and cytolytic activities when compared with splenocytes from control mice. The retarded tumor growth could not be due to direct effect of IL27 signaling into the tumor cells as IL27 did not increase MHC class I or reduced cell proliferation. While IL27 showed an increase in NK cell function, IL27 did not increase NK cell infiltration or NK cell activation marker CD69 [65]. Although IL27 has been shown to increase the antiangiogenic markers MIG and IP10 in other reports [59], this study showed no change in IP10, MIG, or vessel number. One possibility could be that IFNγ induction in nude mice is limited, although other reports show that IL27 can have anticancer properties independent of IFNγ [42, 59]. Once again, this suggests that IL27 employs different antitumor pathways depending on the tumor microenvironment that particular tumors create.

Contradictory to the aforementioned role of IL27 in reducing tumor growth, this cytokine has been shown to have an antiinflammatory role. Other groups have shown that IL27 receptor knockout mice have a prolonged cytokine expression, while DC cells have a prolonged expression of activation markers CD80/86 after LPS stimulation [66]. In addition, IL27 directly downregulates these activation markers in LPS-stimulated DC. The IL27 receptor WSX1 was upregulated in DC cells after LPS stimulation and IL27 also downregulates IL2 production in activated T cells. These authors propose a model that IL27/WSX1 delivers little inhibitory signals at the initial immune response, while at later phases upregulation of IL27/WSX1 promotes more profound inhibitory functions. In vivo this model does not hold true as constant IL27 expression enhanced NK and T cells functions. Immature dendritic cells reside primarily in peripheral tissues where they uptake antigens and process it, while mature DC reside in the lymphoid tissue to interact with antigen-specific T cells. One of the problems associated with tumor microenvironment is lack of DC maturation such as in human breast, ovarian, and prostate cancers [67]. These immature DC rarely leave the tumor environment to mature and travel to the lymphoid organs as tumor-associated factors such as IL-10, TGFβ, and VEGF inhibit DC cell differentiation [68, 69]. Therefore, the role of IL27 to downregulate activation markers would mean that these DC need to be matured in the first place.

Role of IL12 Family in Treg Cells

T regulatory cells are part of the T cell repertoire that keep the immune system in check by inhibiting proliferation and function of T cells and attenuating responses against self and non-self. There are two types of Treg cells: naturally occurring Treg cells that are generated in the thymus and inducible Treg, which are generated in the periphery from naïve T cells via TGFβ [70–72]. Treg cells express Foxp3, a transcription factor that controls both development and function of these cells [73]. Besides Foxp3 Tregs, there are other types of regulatory T cells, such as Tr1 and Th3, which also contribute to suppression in the periphery. Tr1 and Th3 are characterized by secretion of immunosuppressive IL10 and TGFβ, respectively [74, 75]. In a clinical setting, high numbers of Treg is an indication of poor prognosis for cancer patients [76–78]. Many tumors enhance number of Treg as a mechanism to evade tumor recognition. A high accumulation of Treg in the tumor microenvironment, lymph nodes, or blood is not a result of high trafficking into these areas but rather a result of proliferation and de novo induction [79]. TGFβ is a key player in this process, as it increases the proliferation as well as the induction of de novo Treg. Tumors release TGFβ or induce immature DCs to release TGFβ that can enhance the number of Tregs [80]. Tregs not only suppress CD8 T cell proliferation but also inhibit NK cell function [79]. Tregs and TGFβ inhibit NK cell cytolysis, IL12-mediated IFNγ secretion, and NKG2D expression.

The IL12-associated cytokines modulate induction and function of Treg. Induction of Foxp3^{+} Treg via TGFβ is completely inhibited by IL6 [81, 82]. The combination of IL6 and TGFβ diverts induction of Foxp3 Treg into Th17 cells. Not only IL6, but also IL27 has a prominent role in induction of Treg. Recent reports have shown that IL27 not only suppressed Th17 induction via TGFβ and IL6 but also suppressed the number of inducible Treg in vitro [83, 84]. IL27 suppresses number of TGFβ-induced Treg in a dose and time-dependent manner. This suppression was not dependent on either IL2 or STAT1 as high doses of recombinant IL2 did not rescue IL27-mediated suppression of Treg while IL27 retained Treg suppression in STAT1$^{-/-}$ splenocytes [83].

Although IL27 suppresses induction of Treg, the lack of IL27 does not attenuate naturally occurring Treg as WSX1$^{-/-}$ mice and wt mice have a similar number of Treg [85, 86]. In addition, EBI3 deficiency does not affect the population of Foxp3 CD25+ T cells. Moreover, IL27 itself does not aid Treg in suppression of T cell proliferation. In a classical Treg functional assay, WSX1$^{-/-}$ Treg suppressed T cell proliferation in the same manner as wt cells in the presence or absence of IL27 [81]. Thus, IL27 seems to not play a role in T cell development or function, but rather plays a role during Treg induction.

In addition to Foxp3 inducible Treg, other regulatory T cells such as Tr1 contribute to active suppression in the periphery. Tr1 cells express IL10 and exert their immune suppression mainly through this cytokine [87]. IL27 not only suppresses the induction TGFβ-mediated Treg, but also enhances generation of Tr1-like cells to produce IL10 [88]. The presence of TGFβ produced from Treg converts immature

DC into tolerogenic ones. These modified DC produce IL27 and TGFβ. Production of IL27 and TGFβ by these tolerogenic DC in turn induces T effector cells to produce IL10. Other groups also confirmed that IL27 upregulated IL10 expression in CD4 and CD8 effector cells [89]. Interestingly, IL27 enhances production of IL10 in these cells only once activation of T cells has occurred (Fig. 1).

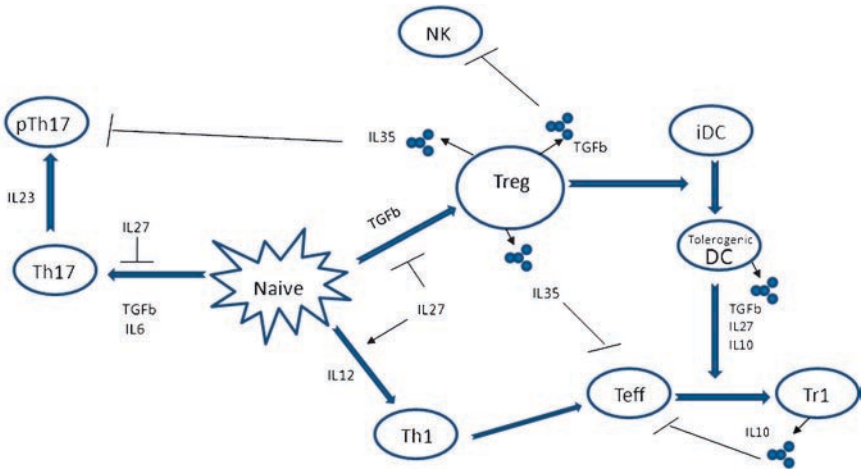

Fig. 1 IL12 family affects different pathways of immune system

Not only does IL27 neutralize the TGFβ effect on Treg but it also neutralizes IL6-induced T cell hyperproliferation [81]. IL6 renders effector T cell refractory to Treg cell-mediated suppression while inducing hyperproliferation of these cells [90]. Therefore, although IL6 and IL27 share the same receptor, these cytokines exert different functions.

The mechanistic cues on how IL27 in some instances promotes a proinflammatory environment by reducing Treg numbers [83, 84] while on others promotes an antiinflammatory by neutralizing IL6-induced T cell proliferation and inducing Tr1 cells still needs to be clarified [81, 88, 89]. Probably timing and state of T cells dictates the role of IL27. IL27 priming of naïve CD4T cells inhibits Treg induction, resulting in a proinflammatory environment. Addition of this cytokine at a later time when the Treg cells have already acquired Foxp3 enhances Treg function indirectly by converting IL6 mediated refractory T cells into T cells susceptible to Treg cytolysis. In addition, production of IL27 in the presence of TGFβ gears effector cells toward production of IL10. Therefore, IL27 has opposing roles during different stages of an immune response.

Many questions need to be answered on the role of IL27 in Treg. First, Villarino et al. show that WSX1 expression is modulated during an immune response. WSX1 is increased not only in activated T cells and memory T cells but also in Treg $CD62L^{high}CD25^{high}$ when compared with naïve $CD62L^{high}CD25^{low}$ [85]. Although others have shown that IL27 does not affect Treg suppression, why is there a higher

expression in Treg rather than in naïve T cells? Next, there is no evidence whether IL27 suppresses inducible Treg in vivo or whether IL27 exerts antitumor effects partially by effecting Treg. Although many authors and studies have described IL27 as a future candidate for cancer therapies, caution should be made when interpreting these studies. The timing of expressing IL27 will determine whether IL27 suppresses or enhances Treg. Primarily, most studies use tumors transduced with single chain overexpressing IL27, thus expressing this cytokine at the initial stage of mounting an immune response. Presence of IL27 at the initial stage establishes a Th1 proinflammatory environment. There are no studies showing the prophylactic properties of IL27 administration after tumor establishment. Systemic delivery of IL27 has little to no effect on already established tumors (our unpublished data). In such, the tumors that are clinically detectable have already established an immunosuppressive environment characterized by the presence of Treg. In this perspective, IL27 might promote an immunosuppressive environment; therefore, the use of IL27 as a therapeutic agent alone might not be effective. Administration of IL27 in addition with Treg suppressors such as cyclophosphamide might provide a synergistic effect. The timing and sequence of administration between these two agents should be considered as well. The first phase should consist of cyclophosphamide, followed by IL27 after a lag period. In the presence of lower numbers of Treg, IL27 might stimulate T cells and NK to produce cytotoxic molecules against tumors. The lag period should provide enough stimuli to revert the immune-suppressive tumor environment at least for a short time. In addition, the lag period should be in reverse proportion to tumor size and aggressiveness. In other words, the lag period in aggressive tumors models should be shorter than immunogenic tumors. Also, lower number of Treg would translate into fewer tolerogenic DC; therefore, there is less chance that IL27 might enhance induction of Tr1 cells. In light of new antiinflammatory properties, further in-depth studies are needed to explore the anticancer role of IL27.

II35

IL35 is the newest member of IL12 family, which expands not only IL12 family but adds complexity in modulating the immune system by this family [8]. IL35 is composed of the IL27 subunit EBI3 and IL12 subunit p35, a complex already known but with no attributable function [91]. EBI3 was shown to be a downstream gene induced by Foxp3. From all the alpha chain cytokines of IL12 family that are expressed, only p35 and EBI3 are expressed by Treg. Treg upregulates this cytokine only during active suppression of T cells while in contact with T effector cells (Teff), suggesting that proximity to Teff is required for induction of this cytokine [92]. IL35 suppresses Teff proliferation and IFNγ production in response to activation in an antigen-specific and nonspecific manner [8]. Downregulation in either subunit might be necessary to reduce IL35 suppressive activity, as either subunit alone does not suppress proliferation of effector T cells.

IL35 expression may not be constricted only to Treg, as APC do induce all subunits of the IL12 family. Although both IL27 and IL35 compete for EBI3, the only known preferential expression difference between these two cytokines is location, as IL27 is mainly constrained to APC while IL35 is constrained to Treg. Moreover, since EBI3 is shared by IL27 and IL35 and TCCR$^{-/-}$ mice display a different phenotype than EBI3$^{-/-}$ mice in T cell-mediated hepatitis [93, 94], then caution should be used when interpreting results from these two different strains. Further studies are needed to establish the mechanism necessary to produce one cytokine over another in APC. In addition, future studies should elucidate whether APC produce IL35 and whether it exerts immunosuppressive role during APC-T cell interactions.

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94. Yamanaka, A., Hamano, S., Miyazaki, Y., Ishii, K., Takeda, A., Mak, T. W., Himeno, K., Yoshimura, A., and Yoshida, H. (2004) J Immunol 172, 3590Role of IL12 Family in Regulation of Antitumor Immune Response

IL-18 in Regulation of Antitumor Immune Response and Clinical Application

Chintana Chirathaworn and Yong Poovorawan

Abstract Interleukin-18 (IL-18) is a member of the IL-1 family. Since IL-18 has been identified as an IFN-γ-inducing factor, its role in the immune response related to IFN-γ function has been widely investigated. IFN-γ is one of the Th1 cytokines, which enhances cell-mediated immune response such as macrophage and cytotoxic T cell activation. In addition, NK cell activity is activated by IFN-γ. For those reasons, IL-18 has been studied as a cytokine involved in host defense especially in killing intracellular organisms. Furthermore, NK cell activity and Th1 response are crucial in tumor cell surveillance and elimination so the application of IL-18 for cancer immunotherapy has been widely investigated. Currently, IL-18 has been known to play roles not only in cancer and defense against infection but also in pathologies of various inflammatory diseases. However, this review will gather information on IL-18 functions and applications focusing only on its role in anti-cancer immune response. Studies using IL-18, alone or in combination with other cytokines or proteins, have provided promising results for cancer immunotherapy. However, various data implicating that IL-18 is involved in cancer progression have also been reported. In addition, the level of IL-18 is associated with poor prognosis in various cancers. Clinical application of IL-18 in cancer immunotherapy may not be as simple as giving IL-18 to any cancer patients and expecting enhancement of antitumor immunity.

Introduction

Interleukin-18 was initially discovered in 1995 as an interferon-gamma-inducing factor (IGIF). It was cloned from the liver of mice inoculated with *Propionibacterium acne* and challenged by lipopolysaccharide (LPS) to induce toxic shock. In humans,

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the *IL-18* gene is located on chromosome 11q22 and encodes a 193 amino acid precursor protein. It is a cytokine of the interleukin-1 cytokine superfamily and displays 12% and 19% amino acid sequence homologies with IL-1α and IL-1β, respectively [1–3].

IL-18 is expressed in biologically inactive form. Its precursor protein contains interleukin-1 beta converting enzyme (ICE-caspase-1) cleavage site. Caspase-1 processing of the IL-18 precursor is similar to that of IL-1β. Both IL-18 and IL-1β precursors are cleaved at the aspartic acid P1 position. The 24-kDa pro-IL-18 is cleaved by ICE-caspase-1 resulting in an 18-kDa functional molecule [4–6].

An extracellular serine esterase, proteinase-3 (PR-3), which is able to cleave pro-IL-18 into its active form, has been identified. This suggests that pro-IL-18 could also be processed extracellularly [7]. When epithelial cells are primed with IFN-γ and then stimulated with PR-3 in the presence of LPS, these cells release IL-18. Caspase-3 (CPP32) cleaves both precursor and mature forms of IL-18 into biologically inactive degraded products [8, 9]. This cleavage may potentially contribute to the down-regulation of IL-18. The release of IL-18 is caspase-1 independent [10].

Endotoxin, exotoxin, and a variety of microbial components induce IL-1, IL-6, and TNF-α production in macrophages. Those components can also stimulate IL-18 production. The predominant cell sources of IL-18 are macrophages and dendritic cells. However, IL-18 can be produced by other cell types such as Kupffer cells, T cells, B cells, osteoblasts, keratinocytes, and astrocytes [11]. IL-18 can also be produced in other epithelia such as intestinal epithelial cells and the stratified epithelium of the esophageal mucosa [12, 13]. Keratinocytes of the stratified epithelium of the skin and airway epithelium are also important sites of IL-18 production [13, 14].

IL-18 Receptor

Interleukin-18 receptor (IL-18R) belongs to the IL-1 receptor/TLR family, which has three conserved extracellular immunoglobulin-like domains and a TIR (Toll-IL-1 receptor) motif in cytoplasmic domain. IL-18R is composed of a constitutive ligand-binding α chain, which binds IL-18 with low affinity (Kd 20–40 nM) and inducible β chain which does not bind IL-18. IL-18Rα and IL-18Rβ chains form high affinity IL-18R. The tricomplex of IL-18, IL-18Rα, and IL-18Rβ forms a high affinity complex (Kd 600 nM) (Fig. 1) [15–17].

The principle cellular targets of IL-18 are NK cells and T cells. IL-18R is expressed on various cell types such as T lymphocytes, B lymphocytes, natural killer cells, and nonlymphoid cells such as macrophages, endothelial cells, fibroblasts, melanocytes, cardiomyocytes, and numerous epithelial cells. IL-18R expression is increased after activation by IL-2, IL-12, and mitogen whereas IL-4 inhibits its expression [9].

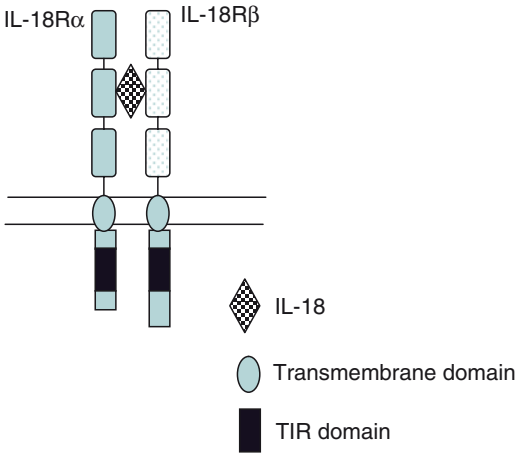

Fig. 1 IL-18 receptor: IL-18R is composed of three extracellular Ig-like domains. The cytoplasmic TIR domain is indicated by the black box. Upon binding of IL-18 to the IL-18Rα chain, the IL-18β chain is recruited to the complex and a high affinity receptor is formed

IL-18 Receptor Signal Transduction

IL18Rα, previously known as IL-1R-related protein (IL-1Rrp), is a member of the IL-1R family and represents the ligand-binding chain of IL-18R. Upon IL-18Rα binding to IL-18, IL-18Rβ, the second chain, is recruited to the signaling complex. IL-18Rβ is structurally related to IL-18Rα; however, it will not bind to IL-18 unless IL-18α is already bound to IL-18. Since the IL-18Rβ structure is also related to the IL-1 signal-transducing chain, the IL-1R accessory protein, IL-18Rβ was originally termed the IL-18R accessory protein-like chain (IL-18RAcP).

IL-18 signals IL-18R through a pathway shared with the IL-1R pathway. IL-18R is a member of the IL-1R family. Receptors in this family contain a Toll/IL-1 receptor (TIR) homology domain in the cytoplasmic tail, which is crucial for signal transduction. The IL-18R extracellular domain is composed of three Ig-like domains. Both IL-18Rα and IL-18Rβ are required for signal transduction.

The IFN-γ promoter region has binding sites for various transcription factors such as AP-1, NF-kB, NFAT, and STAT4 [18–20]. After binding of IL-18 to IL-18Rα, the IL-18Rβ is recruited to the signaling complex. Signal transduction includes recruitment of an adaptor molecule, MyD88 (myeloid differentiation factor 88) and the kinase IRAK (IL-1 receptor-associated kinase). Phosphorylated IRAK then dissociates from the IL-18R complex and interacts with TRAF-6 (TNF receptor-associated factor-6). TRAF-6 phosphorylates NIK (NF-kB-induced kinase), which in turn phosphorylates IKK resulting in NF-kB and AP-1 activation. Binding of IL-18 to IL-18R also stimulates the MAPK pathway involved in IFN-γ production (Fig. 2) [21].

Mice deficient in MyD88 do not produce acute phase protein, and do not respond to IL-1 or IL-18 [22]. Mice deficient in IRAK lack responsiveness to IL-18, and activation of NF-kB and IFN-γ production induced by IL-18 are significantly reduced [23].