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REVEALING THE ROLE OF RECEPTOR WSX1: A DOUBLE-EDGED SWORD IN TUMOR PROGRESSION

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Denada Dibra
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ABSTRACT

Tumor initiation and progression are dependent on both aberrant gene expression in tumor cells and the communication between tumor cells and its micro- and systemic environments. Many tumor suppressor genes and oncogenes have been characterized to suppress or promote tumor growth, but fewer genes in tumors are well-characterized as interacting with immune cells in the host to promote or inhibit tumor growth. The interleukin (IL) 27 receptor WSX1 is expressed in immune cells and induces an IL27-dependent immune response. Opposing this conventional dogma, our initial results reveal a much higher level of WSX1 expression in multiple types of epithelial tumor cells when compared to normal epithelial cells. These revelations suggest a role for WSX1 in tumor development, and thus a possible target in cancer immune-therapy. Using genetically modified tumor cells, our studies show that the expression of WSX1 in tumor cells regulates the communication between tumor and host cells resulting in two different consequences. In both the cervical cell line TC1 and the squamous carcinoma cell line AT84, overexpression of WSX1inhibited tumorigenicity both *in vivo* and *in vitro*. Sensitizing NK cell-mediated surveillance through upregulation of NKG2D ligands in tumor cells is the underlying mechanism by which WSX1 inhibits tumor growth. Further investigations into other cell lines, such as colon cancer (CT26) and Lewis Lungs Carcinoma (LLC), confirmed the role of WSX1 as a tumor suppressor *in vitro*. In contrast to the role that WSX1 plays in the aforementioned cells, aggressive LLC and melanoma AGS tumor cells expressing WSX1 grow faster than the control cohorts. These studies reveal that the principal mechanism by which WSX1 promotes tumor growth is the inhibition of T cell proliferation and production of the effector cytokine IFNγ both in the tumor microenvironment and distal lymphatic tissues. Our evidence reveals that this effect is initiated via direct tumor cell and immune cell contact. This important observation

reveals a new pathway of tumor-host interaction, which will ultimately lead to better strategies in immune therapy to reverse tumor tolerance.

CHAPTER 1

ROLE OF IL12 FAMILY IN REGULATION OF ANTITUMOR IMMUNE RESPONSE*

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INTRODUCTION

Tumor eradication or progression is dependent on interaction and communication with immune cells. Such a crosstalk between tumors and immune cells is partially conducted through cytokines. In reality, tumor microenvironment is frequently immunosuppressive and contributes to a state of immune tolerance(1). As such, delivery of potent immune enhancer cytokines such as IL12 may reverse immune tolerance, because IL12 is a potent proinflammatory immunoregulatory cytokine that plays a central role in tumor eradication via induction of IFNγ and cytotoxic T lymphocytes (2). IL12 therapy was successful in a variety of murine models (3-5). Although 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 efficiency. Only immunogenic cancers are susceptible to this cytokine, therefore, local expressions of this cytokine in the tumor microenvironment or alternative cytokines are needed in cancer therapy.

IL12 family is composed of IL12, IL23, IL27 and IL35. Although within the same family, these cytokines have different functions. IL12 is the key cytokine that promotes TH1 differentiation, while IL23 promotes TH17 (2, 7). IL35 enhances T regulatory functions, while IL27 exert pro and anti-inflammatory functions (8). Interestingly, these family members exert such diverse functions while at the same time share many receptors and subunits among each other. As such, IL12 and IL23 share subunit p40 and IL12Rβ1, IL27 and IL35 share subunit EBI3 and gp130 receptor, while IL12 and IL35 share p35 and IL12Rβ2 (9). Many reviews have described heavily how these cytokines affect autoimmune diseases (10-12). In addition, others have described how these cytokine affect the immune cells (13, 14). But the literature lacks reviews that focus on how IL12 family member signal in tumors and how they affect the crosstalk between tumors and immune cell. In summary, this review focused on the expression and function of IL12 family members and the cognate receptors in tumors, and how these cytokines affect T regulatory cells (Tregs).

ABERRANT EXPRESSION OF IL12 FAMILY MEMBER RECEPTORS AND SUBUNITS

The IL12 cytokine family is composed of IL12, IL-27 and IL-23 and IL35. IL-27 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). While gp130 is ubiquitously expressed and receptor of other cytokines such as IL-6, WSX1 is specific for IL-27(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 modulating 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 has not been 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 in epithelial cells overexpression of WSX1 delayed IL27-mediated tumor cell proliferation (22), WSX1 expression in leukemia cells transformed two leukemia cell line, 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, 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 point-mutation of Jak2 at V167F (27-29). WSX1-dependent transformation of the leukemia cells is dependent upon activation of JAK2-V617F. The co-expression of WSX1 and mutated JaK2, but not wildtype JAK2, results in phosporylation of STAT3 and JAK2. Therefore overexpression of WSX1 does not per se transform cells, but acts as a scaffold receptor to activate tumor cells with already mutated JAK's. 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 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). They also show the IL27 downstream signaling is inhibited, in accordance with other publications, showing 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 Epstesin-Barr Virus (EBV) and Human T cell Leukemia virus (HTLV) type associated lymphomas (26). EBV-Virus associated lymphomas are associated with several human malignancies such as Burkitt lymphoma, Hodgkin lymphoma and nasopharyngeal carcinoma (32). Although these tumors express antigen presenting molecules such as HLA-1, immune costimulatory molecules such as CD80 and CD86, and are susceptible to CTL in-vitro, cytotoxic T-cell specific against EBV are rarely found on 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- LPD's), 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 non-germinal 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-LPD's 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 (IL12p40 subunit was not expressed). In light of new discoveries of IL35 as an immunosuppressive cytokine associated with inhibiting effector T cell function, composed of EBI3/p35, it seems logical that immune evasion of these lymphoma cells would be attributed to IL35 function. In accordance, 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 not observed only in a pathogenic scenario, but is also found in normal settings such as in intestinal tract (36). The intestinal tract is the initial contact site between host and pathogens. In a balanced system, proinflammatory signals are balanced with anti-inflammatory 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 it would indicate several potentially important therapeutic targets. One potential use of these targets would be cancer, since immune cells develop tolerance towards tumor cells. Human mucosal epithelial cells produce EBI3, IL12p35 and IL23p19, but not their counterpart subunits, such as p28 and IL12p40 that 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). On the other hand, p35 is induced after IFNγ response and its expression was delayed when compared to EBI3 and p19. This model suggests that in a balanced system such as the intestinal tract, induction of p35 to make a functionally 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 above, 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 on (I) what are the pathways that are activated by WSX1 receptor, and (II) what are the critical pathways activated by this receptor are malfunctioning in tumor-bearing patients. As 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 TUMORS

IL23 is another member of IL12 family. This cytokine is a composed of two subunits: p19 and p40 (9). While IL23 shares p40 and receptor IL12Rβ1 with IL12, they drive quite different immune pathways. As IL12 drives the classical IFNγ pathway, IL23 is an essential factors required for the expansion already committed TH17 cells into pathogenic cells (37). Although many reviews and research articles have focused on the role of IL23 is autoimmune diseases, fewer articles have focused on role of IL23 in cancers (38-40).

The role of IL23 in tumor biology is dichotomous. While lack of IL23 showed protection against tumor initiation, IL23 used as a therapeutic or vaccine adjuvant reduced tumor growth (20, 41, 42). In order 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 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 and IFNγ production. In another study by Overwijk, overexpression of IL23 in non-immunogenic 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 non-immunogenic melanoma tumors (44). They used a gp100 peptide vaccination after adoptively transferring antigen-specific pmel CD8 T cells towards 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 of tumor specific T cells, they concludes that IFNγ production by CD8 T cells does not have a major role in enhancing IL23 role as an adjuvant; adoptive transfer of $IFN\gamma^{-/-}$ pmel T cells still remain responsive to IL23 therapy. In contrary to this statement that IFNγ is dispensable, others have shown that IFNγ is absolutely necessary for IL23 anti-tumor activity (45). In IFNγ knockout mice, IL23 antitumor effects were non-existent and partially abrogated in IL12 KO mice. 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 TH1 response is fully established, only then IL23 does exert its potent antitumor activity. This claim is not in disagreement with Overwijk study since their argument solely depends on $IFN\gamma^{-/-}$ 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 a 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 using IL23 systemically is weight loss. This toxicity is dependent on expression of TNFα (44). Depletion of TNFα reduces side effects; nonetheless it's not feasible as it mediates not only weight loss, but also anti-tumor 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, 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, macrophage 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 non-effective to eradicate tumors in $INF\gamma^{-/-}$ mice (45). IFNγ also has been shown to downregulate TH17 while promoting TH1 induction (47, 48). 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 TUMORS

One of the earlier functions attributed to this cytokine is its ability to synergetic induction of IFNγ with IL12, and proliferation of naive CD4+T cells (15). IL-27 also induces T-bet expression and IL12Rβ expression, key components to TH1 commitment, through STAT-1(49). Given its role in initiation of TH1 response and induction of IL12 receptor, several researchers evaluated the role of IL-27 in cancer immunotherapy. Function of IL-27 was examined in different tumor models such as: colon cancer 26 (CT26), neuroblastoma (TBJ), and aggressive melanoma (B16F10)(50-54)

IL-27 possesses T-cell and NK cell mediated antitumor activities. In immunogenic colon cancer system IL-27 mediated 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 for IFNγ and CD8. Interestengly, in Tbet$^{-/-}$ 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 IL-27 antitumor properties. In nude mice, CT26-IL27 tumor growth was retarded when compared to parent CT26; this phenomena was partially reversed upon administration of anti-asialo GM1 depletion antibody(50). Therefore possibly γδ T cells or NKT mediated antitumor effect. In highly aggressive melanoma B1F10, NK cells were mainly involved(53).

Hisada and Chiyo (2004, 2005) show that IL27 uses different subsets of immune cells to eradicate the same tumor model CT26 such as T and γδ/NKT respectively. The difference between these studies relies on different cell number and different amount of IL27 expression/cell. This suggests that higher amounts of IL27 in the microenvironment employ wider subsets of immune cells. Also this study shows that overexpression of either subunit alone does not enhance mice survival, but expression of both subunits does so, suggesting that IL27 but not its particular subunits are necessary for antitumor activity.

IL27 antitumor activity does not depend on either STAT4 or IL12(56). Since IL27 exerted antitumor effects in either IL12p40 or STAT4 knockout mice, IL12 is not necessary of 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 synergestic induction of IFNγ with IL12(56). While T-bet was important for induction of IL12Rβ2, perforin, granzyme B and synergestic induction of IFNγ with IL12, this transcription factor was not important for enhanced CTL activity, as in Tbet$^{-/-}$ mice IL27 enhanced allogeneic CTL activity in a dose dependent matter comparable to Wt mice (56). Although IL27 increased CTL activity in a Tbet independent matter, this transcription factor is important for IL27 mediated antitumor activities in vivo, as tumors grew much faster in Tbet$^{-/-}$ mice but not in Wt mice. This phenomena could be explained by a later study which emphasizes that Tbet and WSX1 expression in CD8 T cells are indispensable for IFNγ production in CD8 T cells in vivo but not in-vitro (57).

In neuroblastoma TBJ cell line, overexpression of IL27 eradicated more than 90% or tumors and rendered these mice resistant to tumor challenge(58). TBJ27 but not control TBJ 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. TBJ27 reduced number of metastatic tumor in the liver and furthermore, 40 % of the mice were tumor free of their metastatic tumors. The mechanism responsible for IL27 mediated tumor regression was dependent on CD8 but not NK or CD4 was. IL27 also enhanced IFNγ and MHC class I in the tumor microenvironment(58). Such great antitumor effect associated with IL27 production in the tumor could be attributed not only to IL27 signaling in the host, but also in the tumor itself. Although this study did not determine WSX1 levels on 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 on B16F10, a mouse melanoma that is a model of poor immunogenicity characterized by a low MHC class I expression. B16F10 that overexpress single chain IL27 show reduced tumor growth not only toward primary tumors, but also against pulmonary metastasis (59). Interestingly, IL27-mediated antimetastatic activities were not dependent only on the host as T, B, and NK deficient mice still retained tumor growth inhibition. The authors also showed that IL27 enhanced expression of antiangiogenic markers such IP10, MIG while it reduced in-vivo angiogenesis. What seems to be quite impressing is that IL27 acts independently of IFNγ to induce anti-angiogenesis markers. Even in IFN$\gamma^{-/-}$ mice, B16F10-IL27 showed reduced tumor growth and metastasis. Similarly, another group using the same tumor model B16F10 showed that IL27 exerts antitumor activities in 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 in synergy 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 tumor microenvironment (59, 60). Endothelial cells have a dual role during tumor progression either promoting or inhibiting tumor growth. 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 in T cells in CTL activity (61). In addition, they can upregulate certain receptors to recruit innate immune cells(62). The balance between anti and pro-tumor environment depends on cytokine balance secreted by the tumor and/or present there. IL-27 receptor WSX1 was present in endothelial cells and IL27 signaling directly on endothelial cells has increased anti-angiogenic 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 towards 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. As associated to previous tumor models, NK cells from mice overexpressing IL27 showed an increased IFNγ production and increase cytolytic activities when compared to 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 antiangiogenic markers MIG and IP10 in other reports (59), this study showed no change in IP10, MIG or vessel number. As one possibility could be that IFNγ induction in nude mice is limited, although other reports show that IL27 can has 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 aforementioned role of IL27 in reduce tumor growth, this cytokine has been shown to have anti-inflammatory role. Other groups have shown that IL27 receptor knockout mice have a prolonged cytokine expression, while DC have a prolonged expression of activation markers CD80/86 after LPS stimulation(66). In addition, IL27 directly downregulated these activation markers in LPS-stimulated DC. Also IL27 receptor WSX1 was upregulated in DC cells after LPS stimulation. 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 an in-vivo tumor model 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 cells reside in the lymphoid tissue to interact with antigen-specific T cells. One of the problems associated with tumor microenvironment is lack of maturation DC 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, 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, 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 T_H3 also contribute to suppression in the periphery. Tr1 and T_H3 are characterized by secretion of immunosuppressive IL10 and TGFβ, respectively (74, 75). In a clinical setting, high number Treg's is an indication of poor prognosis for cancer patients (76-78). Many tumors enhance number of iTregs as a mechanism to evade tumor recognition. A high accumulation of Treg's in the tumor microenvironment, lymph nodes or blood is not a result of high trafficking into these areas, but rather is 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 cells. Tumors release TGFβ or induce immature DCs to release TGFβ that can enhance the number of Tregs (80). Tregs not only suppress CD8 T cells 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 Tregs. Induction of $Foxp3^+$ Treg cell via TGFβ is completely inhibited by IL6 (81, 82). The combination of IL6 and

TGFβ diverts induction of Foxp3 Tregs into TH17 cells. Not only IL6, but also IL27 has a prominent role in induction of Tregs. Recent reports have shown that IL27 not only suppressed TH17 induction via TGFβ and IL6, but also suppress number of inducible regulatory T cells in vitro (83, 84). IL27 suppresses TGFβ-induced number of 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 iTreg while IL27 retained iTreg suppression in $STAT1^{-/-}$ splenocytes (83).

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

In addition to Foxp3 inducible Tregs, other regulatory T cells such as Tr1 contribute to active suppression in the periphery (Fig. 1.1). Tr1 cells express IL10 and these cells exert their immune suppression mainly through this cytokine (87). IL27 not only suppresses the induction TGFβ-mediated iTregs, but also enhances generation of Tr1 like cells to produce IL10 (88). The presence of TGFβ produced from Tregs converts immature Dc cells into tolerogenic ones. These modified DC produce IL27 and TGFβ. Production of IL27 and TGFβ by these tolerogenic DC cells in turn converts 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.

Not only IL27 neutralizes 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.

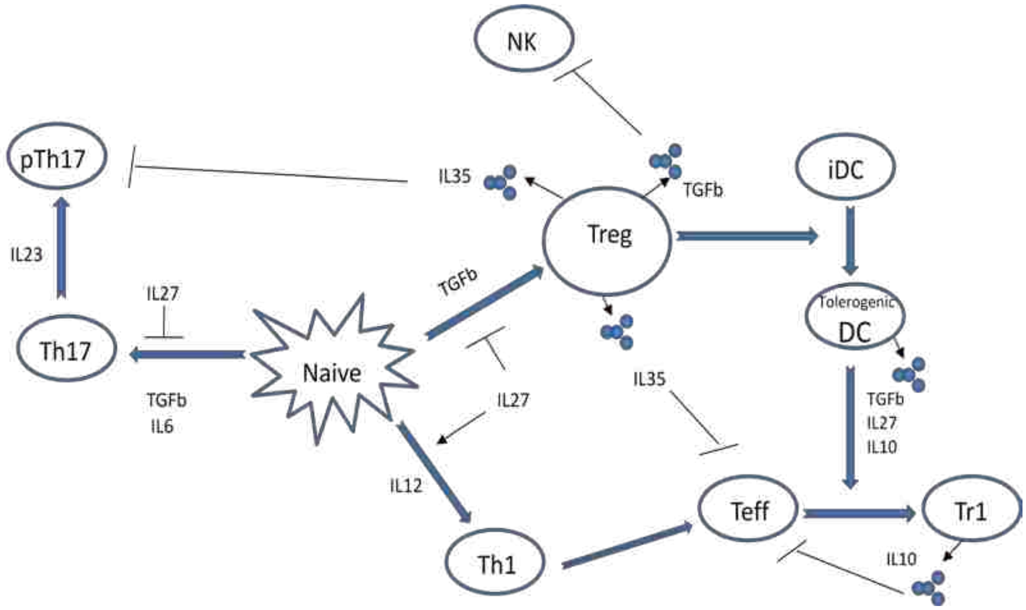

Figure 1.1. IL12 family affects different pathways of immune system

The mechanistic cues on how IL27 in some instances promotes a pro-inflammatory environment by reducing Treg numbers(83, 84), while on other's promotes an anti-inflammatory 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 pro-inflammatory environment; addition of this cytokine later on when the Treg cells are already acquired Fox3p enhances Treg function <u>indirectly</u> by converting IL-6 mediated refractory T cells into T cells susceptible to Treg cytolysis. In addition, production of IL27 in presence of TGFβ gears effector cells towards 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 Tregs. First, Villarino at al shows that WSX1 expression is modulated during an immune response. IL27 receptor, WSX1 is increased not only in activated T cells and memory T cells, but also on Treg CD62L^{high}CD25^{high} when compared to naïve CD62L^{high}CD25^{low} (85) . Although others have shown that IL27 does not affect Treg suppression, why is there a higher expression in Tregs rather than in naïve T cell. Next, there is no evidence whether IL27 suppresses inducible Tregs in vivo or whether IL27 exerts antitumor effects partially by effecting Tregs. 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 of IL27 will determine whether IL27 suppresses or enhances regulatory T cells. 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 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 presence of Tregs. In this prospective, IL27 might promote an immunosuppressive environment. Therefore, use of IL27 as a therapeutic agent alone might not be effective. Administration of IL27 in addition with Treg suppressor such as cyclophosphamide might prove a synergistic effect. The timing and sequence of administration between these two agents should be considered as well. The first phase should consist of cyclohexamine, followed by IL27 after a lag period. In the presence of lower Tregs, IL27 might stimulate T cells and NK to produce cytotoxic molecules against tumors. The lag period should provide enough stimulus 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 cells would translate in fewer tolerogenic DC's; therefore there is less chance that IL27 might enhance induction of Tr1 cells. In light of new anti-inflammatory properties, further in-depth studies are needed to explore the anticancer role of IL27.

ROLE OF IL35 IN TUMOR AND IMMUNE CELLS

The ever-growing complex IL12 family just acquired a new cytokine IL35 to increase ways in which modulates and shapes the immune system (8). IL35 is composed of IL27 subunit EBI3 and IL12p35, 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, but not p19 or p28 are expressed by Treg cells. Treg cells upregulate this cytokine only during active suppression of T cells while in contact with T effector cells, suggesting that close proximity to Teff is required for induction in trans of this cytokine(92). IL35 suppresses effector T cell proliferation and IFNγ production in response to activation in a antigen-specific and non-specific 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 IL12 family. Although both IL27 and IL35 compete for EBI3, the only known preferential expression difference these two cytokines is location, as IL27 is mainly constraint to APC's while IL35 is to Treg cells. 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 interaction.

STATEMENT OF THE PROBLEM AND SPECIFIC AIMS

While the function of IL-27 receptor WSX1 in immune cells is well-studied, the function of this receptor in tumor cells is not well characterized. Also, the function of the receptor WSX1 is exclusively associated with IL27 and IL27's role in immune cells, thus an IL27-independent function and its association with cancer biology is largely unknown. Our initial results reveal a much higher level of WSX1 expression in multiple types of human epithelial tumor cells when compared to normal epithelial cells. Moreover, the high level of WSX1 expression in epithelial tumor cells does not correlate to the IL-27-mediated STAT1 phosphorylation in these cells. These preliminary data led to our hypothesis that WSX1 has a function in tumor development independently of IL-27, and thus a possible target in cancer immune-therapy.

In order to explore the function of WSX1 in tumor biology, we performed a series of *in vivo* and *in vitro* experiments.

Aim 1: Determine if IL27 receptor WSX1 affects tumor growth.

Aim 2: Investigate whether WSX1 has a function in tumor biology independently of IL27.

Aim 3: Understand the mechanism via which WSX1 affects tumor growth.

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CHAPTER 2

EXPRESSION OF WSX1 IN TUMORS SENSITIZES IL27-SIGNALING INDEPENDENT NK CELLS SURVEILLANCE*

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INTRODUCTION

IL27 receptor WSX1 is most homologous to the IL12 receptor β2 chain (1). WSX1 together with gp130 constitute a functional signal-transducing receptor for IL27, whereas lack of either subunit attenuates IL27-mediated signaling (2). WSX1 is reported to be expressed in immune cells such as monocytes, dendritic cells, T and B lymphocytes, NK cells, mast cells, and endothelial cells (1).

In a patent application (PCT/2007/0280905), we reveal that WSX1 is detectable in breast epithelial tumor cells. This discovery is further supported by a recent report which revealed that WSX1 is expressed in another type of epithelial tumor, melanoma cells (3). The same as found in immune cells, WSX1 is functional in these epithelial tumors cells as indicated by the IL27-mediated activation of STAT1 and STAT3 (3).

Clearly, the reports found in the literature suggest that WSX1 plays a role through the IL27 signaling pathway, but the IL27-independent role of WSX1 in promotion or inhibition of tumorigenesis has not been reported yet. Using genetically modified tumor cells, we present evidence that the expression of WSX1 in epithelial tumor cells suppresses tumor growth both *in vitro* and *in vivo*. Such inhibition of tumor growth is dependent on NK cells but independent of IL27 signaling. Our results reveal a novel function of WSX1 in epithelial tumor cells, which is to sensitize NK-mediated antitumor immunosurveillance in an IL27-independent manner.

MATERIALS

Cell culture and reagents. Human cancer cell lines from different tissue origins including Hela, HT29, HCT116 and 4T1 were purchased from ATCC. Human breast cancer cell lines MDA468, MDA231, and MCF7 were provided by Dr. Bolin Liu (University of Colorado Denver School of Medicine at Aurora, CO). The normal colon cell line NCM460 was purchased from INCELL Cooperation, LLC. UM-SCC11A, UM-SCC11B, UM-SCC17A, and UM-SCC17B are head and neck squamous cell carcinomas provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI) (4). Mouse HPV-associated tumor cell line TC1 was provided by Dr. T. C. Wu (John Hopkins University, Baltimore, MD) (5).The mouse squamous cell carcinoma cell line AT84 was provided by Dr. Edward Shillitoe (State University of New York Upstate Medical School, Syracuse, NY). Recombinant mouse IL27, NKG2D/Fc, monoclonal anti-human WSX1, and anti-human MICA-Pe antibody were purchased from R&D Systems. Anti-mouse pSTAT1-701, anti-mouse IgG-PE, actin, anti-human IgG-PE, and anti-hamster IgG-PE were purchased from Santa Cruz Biotechnology. Anti-NKG2D C7 was provided by Dr. Wayne Yokoyama (Washington University School of Medicine, St. Louis, MO).

Quantitative real-time PCR. Total RNA was isolated from cells using TRIzol Reagent (Invitrogen). Residual genomic DNA was removed from total RNA using the TURBO DNA-*free*™ kit (Applied Biosystems/Ambion). Two micrograms of RNA were used for cDNA synthesis using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). The relative gene expression levels were determined using quantitative Real-time PCR and SYBR green labeling method in an ABI 7300 Sequence Detector (Applied Biosystems). The reaction contained 2 μL of cDNA, 12.5 μL the SYBR Green PCR Master Mix (Applied Biosystems), and 200-250 nM of primer in a total volume of 25 μL. The PCR cycling conditions were as follows: 40 cycles of 15 s at 95°C, 60 s at 60°C. All samples were run in duplicates. PCR amplification of ß-actin was performed using 0.1 μL of cDNA. The C_T value of each sample was acquired, and the relative level of gene expression was calculated by the Delta C_T method, which was normalized to the endogenous control of ß-actin. Data were expressed as an n-fold relative to control. The forward and reverse primer sequences for the human ß-actin and WSX1 detection are:

5’AGAGGGAAATCGTGCGTGAC3’ and 5’CAATAGTGATGACCTGGCCGT3’, respectively. WSX1: 5’GAGCCCCCTCCGAGTTACAC3’ (forward) and 5’AGCTGTTCCCGAGGAATGG3’ (reverse).

Establishing stable WSX1 expressing cell lines. The murine WSX1 gene was purchased from Open Biosystems and subcloned into pBMN-GFP plasmid (Phoenix™ Retrovirus Expression System, Orbigen, Inc.). The retrovirus was produced by transfecting mWSX1/GFP constructs into Phoenix eco packaging cells. AT84 and TC1 cells were infected with retroviral containing supernatant derived from the transduced HEK293 cells. The transduction was confirmed by detecting green fluorescent protein (GFP) expressing cells under the fluorescence microscope. Cell colonies with GFP expression from a single cell were picked, expanded, and further confirmed for WSX1 expression using Flow Cytometry. Using this approach, both WSX1/GFP and GFP positive TC1 and AT84 cells were obtained.

Animal procedures. All the animal procedures were approved by the IACUC at Louisiana State University. Six- to eight-week-old mice were used for this study. The subcutaneous tumor models were generated by subcutaneously inoculating TC1 and AT84 tumor cells ($2x10^5$ in a 30-µL volume per mouse) into mice. Tumor measurement and calculation were the same as described previously (6). C57Bl/6 WSX1 knockout mice were provided by Dr. Fred de Sauvage **(**Genentech), and C57Bl/6 EBI3 knockout mice were provided by Dr. Mark P. Birkenbach (Temple University School of Medicine, Philadelphia, PA). C57Bl/6, Balb/c SCID, C3H, C57Bl/6 perforin, and Rag deficient mice were purchased from commercial sources.

NK cytotoxicity assay. NK cell activity was evaluated using the CyToxiLux kit (OncoImmunin,Inc.), a single-cell-based fluorogenic cytotoxicity assay (7, 8). Effector cells were prepared from spleens as previously described (8) and incubated with red fluorescence-labeled target cells at a ratio 100:1, 50:1, and 25:1 in 200 µL cell culture media. Target cells alone were used as control for spontaneous cell death. Sixteen hours after incubation, adhesive target cells were washed with PBS. Alive red target cells (input target cells) were counted using Olympus BX41 fluorescence microscope. NK activity was calculated using the following equation: % NK cell activity = 100 x (input target cells – output target cells) / (input target cells).

Flow Cytometry. Cells were stained with the indicated primary and secondary antibodies for 30 min at 4° as indicated in each figure. The expression of the indicated genes was analyzed on FACS Calibur and Cellquest graphics software (BD Biosciences).

Cell proliferation. Cell proliferation assays were performed using the luminescence ATP Lite assay detection system (PerkinElmer). Briefly, 500 cells were seeded in a 96 well plate; cells were lysed on days 0, 2, and 4 for measuring the ATP levels. The cell proliferation index was calculated using the following equation: Cell proliferation index= [ln (d)] / [ln (d0)], where ln (d) = natural log at the day when cells were lysed; ln (d0) = natural log at the day when cells were seeded.

Soft agar growth assay. The clonogenic assay was performed as previously described (9). Briefly, genetically engineered cells ($5x10^3$ for TC1-GFP and TC1-WSX1 cells and $1x10^3$ for AT84-GFP and AT84-WSX1 cells) were suspended in 0.34% agar in cell culture media (Sigma, St Louis, MO). The mixture solution was layered on solid agar support prepared from 0.9% agar in cell culture media. The cells were seeded in triplicates on a 6-well plate and grown for 2 weeks. Colonies were counted under a 10x dissecting microscope after staining with 0.05% Crystal Violet for 1 h. Images were captured using Molecular Image Gel Dox XR (Biorad).

Western blot. Cells ($5x10^5$), growing in 10% heat-inactivated FBS-containing culture media, were treated with or without IL27 (20 ng/mL) for the indicated times. Protein extract was obtained by directly lysing cells using 60 μL of Laemmli sample buffer. Twenty microliters of total protein extract from each sample were separated by SDS-PAGE, transferred to a nitrocellulose membrane and incubated with primary antibody overnight at 4° C (750x dilution for anti-pSTAT1/701 and 1000x fold dilution for anti-Actin).

Statistical analysis. For *in vivo* experiments, Univariate Repeated Measures ANOVA was used to analyze the difference among treatments using SAS version 9.1.3. When appropriate, Tukey's HSD test was performed for interaction affects. For *in vitro* results, student's T test analysis was conducted.

RESULTS

A much higher level of functional WSX1 is expressed in most of the tested epithelial tumor cells than in normal epithelial cells. It is well known that the IL27 receptor WSX1 is expressed mainly in immune cells and the only other type of cells expressing this gene is endothelial cells. However, recently we and others have found WSX1 expression in breast and melanoma epithelial tumor cells lines (3). To determine whether the WSX1 expression in epithelial tumor cells plays an important function, we have compared the magnitude of WSX1 expression between normal and tumor epithelial cells. The quantitative analysis result demonstrated that WSX1 was present not only in breast cells but also in colon, cervical, and squamous cell carcinoma tumor cells, suggesting that WSX1 was expressed in most human epithelial tumor cells (Fig. 2.1*A*). However, the expression level of this gene varied greatly among the different cell lines when compared to the normal epithelial cell line NCM460 (Fig. 2.1*A*). A few cell lines such as HT29 and UM-SCC17A showed 6.9-8.4-fold lower expression of WSX1 when compared to a normal epithelial cell line, NCM460, while most of the cell lines such as Hela, HCT116, and UM-SCC11A showed much higher levels of expression (ranging from 13-78 times higher than NCM460). The high level of WSX1 expression in most of the epithelial tumor cells but not in the normal epithelial cells suggests that WSX1 may play a role in regulating tumor progression. The level of WSX1 protein expression was positively associated with the level of mRNA (Fig. 2.1*B*).

Since WSX1 is the receptor of IL27, one obvious question is whether the high level of gene expression is associated with a high level of function. We used phosphorylation of STAT1 by IL27 as a functional WSX1 end point. After 10 min of incubation with IL27 (the second lane in each panel, Fig. 1*C*), IL27 induced phosphorylation of STAT1; such an increase correlates well with the presence of WSX1 expression but does not correlate with the absolute level of WSX1 expression (Fig. 2.1*B*). The human cell lines HT29 and UM-SCC17A lacking WSX1 expression showed very low to no detectable STAT1 phosphorylation, while cell lines such as Hela, HCT116, and UM-SCC11A with WSX1 expression showed an increase in phosphorylation of STAT1. However, a similar level of phosphorylation was detected in both the low (NCM460 and UM-SCC17B) and high (HCT116 and Hela) level WSX1-expressing cells (Fig. 2.1*C* vs. 2.1*A*).

WSX1 reduces tumorigenecity and proliferation of epithelial tumor cells. The results from others exclusively illustrate that WSX1 plays a role in inducing an immune response through IL27-signaling in immune cells. The result from Fig. 1*B* confirms that the IL27/WSX1 signaling occurs in epithelial tumor cells. However, the high level of WSX1 expression does not correlate to the STAT1 phosphorylation in most epithelial tumor cells (Fig. 2.1*C*). Moreover, the endogenous IL27 is undetectable in either serum or splenocytes and, therefore, may not initiate any signaling in either tumor or host cells during tumorigenesis and development.

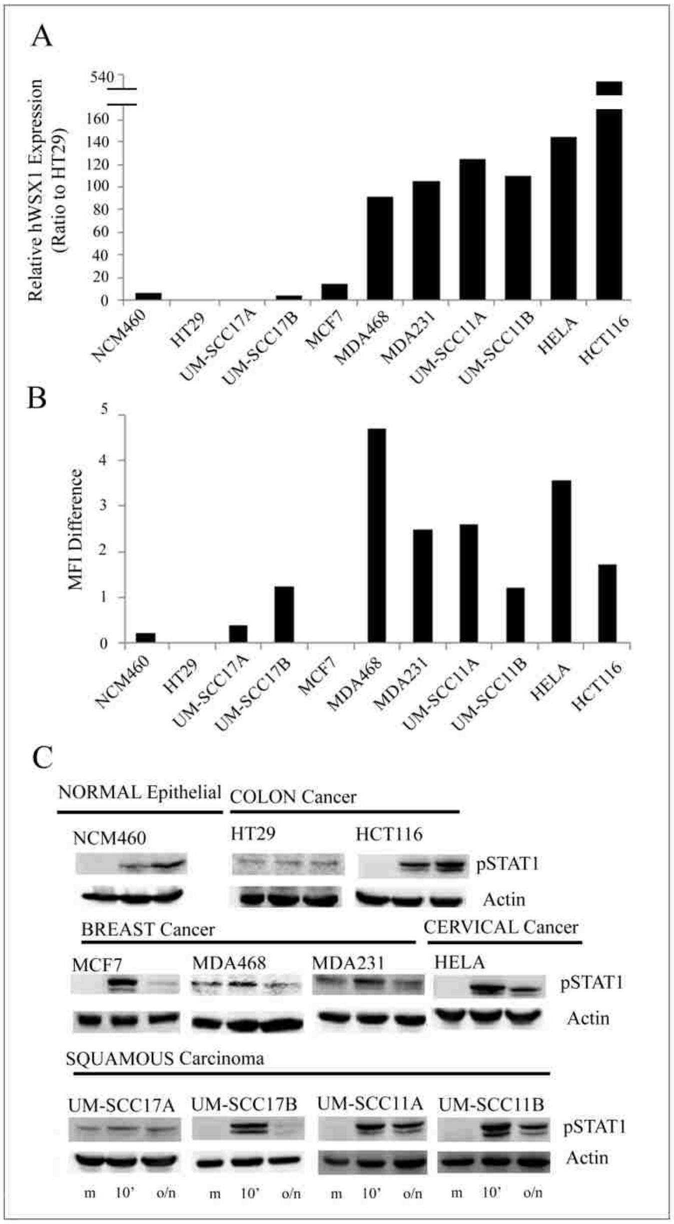

Figure 2.1. Quantitative and functional analysis of WSX1 in a variety of human epithelial cell lines. *A*, determination of the level of WSX1 expression using Quantitative Real-time PCR. The levels of WSX1 mRNA are normalized to actin mRNA, and the data shown is the relative expression of each cell line to HT29. *B*, detection of WSX1 expression at the protein level via flow cytometry. Cells were stained with the isotype control or with anti-human WSX1 antibody followed by anti-mouse-PE and median fluorescence intensity (MFI) difference was calculated as difference in MFI between isotype control and WSX1-stained cells. *C*, detection of functional WSX1 in tumor cells. Cells were treated with IL27 for 10 min (10'), overnight (o/n), or left untreated (m). Cell extracts were analyzed using western blot technique and probed with anti-pSTAT1 and actin antibodies.

These facts suggest that a high level of WSX1 expression alone may affect tumorigenesis, which is the central hypothesis to be tested below.

To determine whether increased WSX1 expression alone may regulate IL27 signaling-independent tumor development, a clonogenic assay was performed to determine the tumorigenecity and proliferative ability of tumor cells engineered with WSX1 or GFP control genes. This method has been known to be effective in determining these end points (10). Flow cytometry analysis confirmed the expression of WSX1 in the stable transfected cell clones (Fig. 2.2*A*). The clonogenic assay results illustrated that expression of WSX1, but not GFP, dramatically reduced the ability of cells to grow in soft agar in both TC1 and AT84 cells (Fig. 2.2*B and 2C*).

To further confirm the inhibitory effect of WSX1 expression on tumor cells, cell proliferation was determined. Similar to the clonogenic assay, WSX1 significantly reduced the proliferation of TC1 and AT84 cells, but the inhibition of AT84 proliferation by WSX1 expression was at a much lower magnitude when compared to TC1 (Fig. 2.2*D*).

WSX1 suppresses tumor growth *in vivo* in both TC1 and AT84 tumor models. Although the clonogenic assay is a good predictor of tumorigenecity *in vitro*, it does not read any host cell-induced cytotoxic end-points that occur in a true tissue environment (11). To avoid this problem, the effect of WSX1 expression on tumor growth was tested in syngeneic mice by subcutaneously inoculating with GFP or WSX1-expressing tumor cells. In agreement with the *in vitro* assay result, WSX1 expression almost completely abolished TC1 tumor growth (Fig. 2.3 A). Likewise, WSX1 expression also inhibited AT84 tumor growth in a different mouse strain (Fig. 2.3 B). The remarkable difference in the tumor growth rate shown in TC1 and confirmed in AT84 strongly indicates that WSX1 has a tumor suppressive role in epithelial tumor cells.

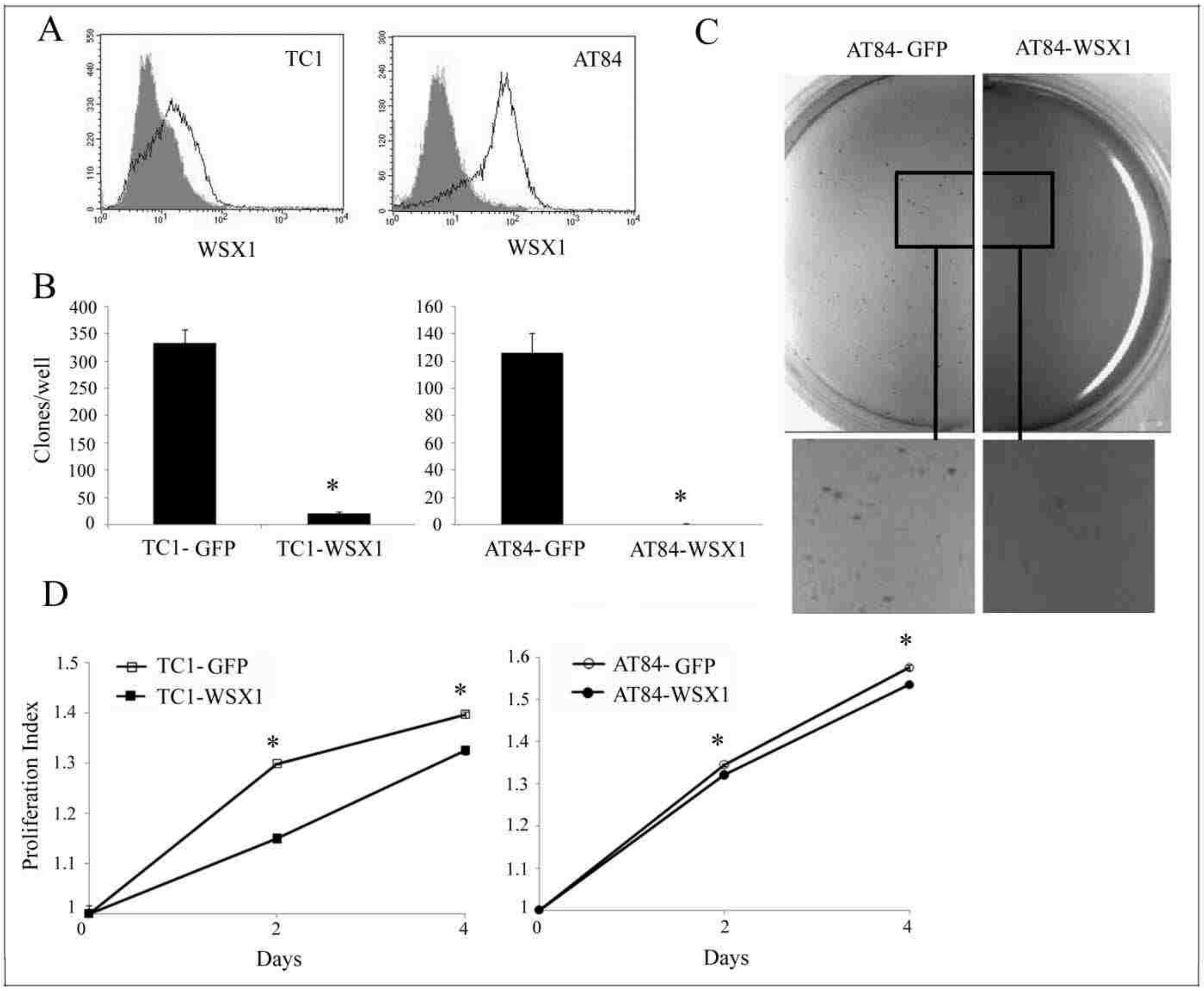

Figure 2.2. WSX1 reduces tumorigenecity and proliferation of epithelial tumor cells. *A*, detection of WSX1 expression in TC1 and AT84 tumor cells with flow cytometry. TC1 and AT84 tumor cells were transduced with retroviruses containing either control GFP (gray) or WSX1 gene (not shaded). The established stable cell lines were stained with a monoclonal WSX1 antibody followed by an anti-hamster-PE antibody. The data is representative of two independent clones. *B*, comparative analysis of soft agar growth assay between GFP and WSX1 in two different cell lines, TC1 and AT84. The data is representative of two independent clones, each performed in triplicate. *C*, a low-magnification photograph of clones formed of AT84-GFP vs. AT84-WSX1. *D*, GFP and WSX1 positive TC1 and AT84 cells were harvested on indicated days and analyzed for ATP release N=4. Error bars are smaller than symbols. *Columns*, mean; *bars*, SE *, $P < 0.05$

WSX1-mediated suppression of tumor growth is dependent on NK cells. The direct antitumor mechanism by WSX1 was not found in the literature, but one possible explanation

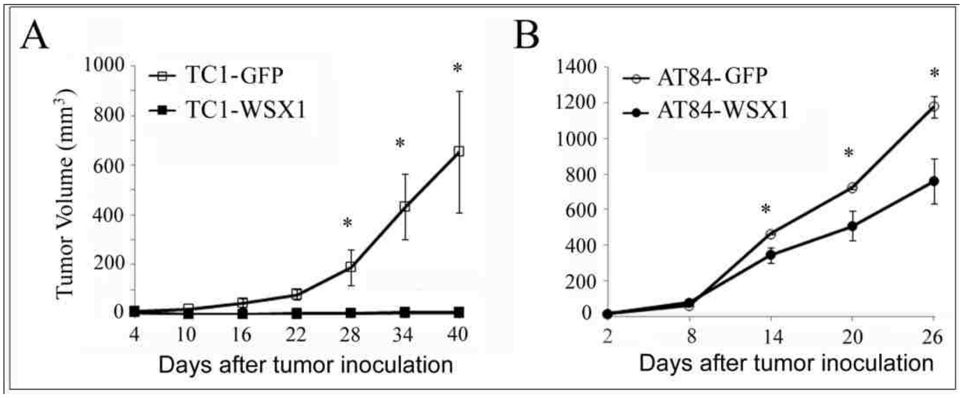

Figure 2.3. WSX1 suppresses tumor growth *in vivo* in both TC1 and AT84 tumor models. *A*, comparison of tumor growth between TC1-GFP and TC1-WSX1 in C57Bl/6 mice. *B*, comparison of tumor growth between AT84-GFP and AT84-WSX1 in C3H mice, N=4-5. *Points*, mean; *bars*, SE. *, $P < 0.05$.

could be due to the reduction of tumor cell proliferation as observed *in vitro* (Fig. 2.2*D*). However, the dramatic difference in tumor growth *in vivo* (Fig. 2.3*A*) when compared to the small difference of proliferation *in vitro* (Fig. 2.2*D*) between TC1-WSX1 and TC1-GFP indicates that cell proliferation differences alone may not be the major cause for the diminished tumor growth in the presence of WSX1. An alternative assumption is that WSX1 expression in tumor cells may enhance the immune surveillance by the host immune cells. To distinguish whether the effect of cell proliferation or the immune system might be the major mechanism that accounts for the WSX1-dependent tumor growth inhibition *in vivo,* we tested tumor growth in wild-type, perforin (NK), and Rag (T, B) knockout mice.

Similar to wild-type mice, tumor growth reduction between TC1-GFP and TC1-WSX1 was found also in T and B knockout mice (Fig. 2.4*A*, *left* vs. *middle*). However, the absence of perforin almost completely impaired the ability of WSX1 to inhibit tumor growth as there is no statistically significant difference in tumor growth between GFP and WSX1 tumors (Fig. 2.4*A*, *right*). This result suggests that WSX1 may sensitize NK cell surveillance for inhibiting WSX1 positive tumor growth. To further support this statement, tumors engineered with control GFP gene grew at a similar rate regardless of the presence or absence of NK or T cells (Fig. 2.4*B*, *left*). In contrast, WSX1-positive TC1 tumors disappeared in three out of four wild-type mice, grew very slowly in T and B deficient mice (reaching 50 mm^3 by day 40 after tumor inoculation), and developed aggressively in NK deficient mice (averaging 450 mm^3 by day 40, almost nine times higher than in T and B knockout mice) (Fig. 2.4*B*, *right*). To confirm that this observation was not dependent on a single clone, another independent clone (TC1-WSX1-CL2) was tested *in vivo*. Similar to the other engineered WSX1 positive TC1 clone, TC1-WSX1-CL2 was eradicated in 4 out of 4 wild-type mice, while it reached 200 mm^3 by day 38 in NK deficient mice (Fig. 2.4*C*). These findings suggest that our observation is not clone-dependent.

Similar to the TC1 model, the WSX1-positive AT84 tumor cells grew slower than the control AT84-GFP in both the wild-type and T and B cell deficient mice (Fig. 2.4*D, left vs. middle*). To test the WSX1-mediated NK cell dependence for the AT84 tumor model, STAT1 knockout mice were used because STAT1 is an essential transcription factor for NK cell function. In the absence of STAT1, these mice demonstrate impaired NK activity *in vitro* and fail to reject NK-sensitive tumors *in vivo* (12). As expected, no difference in the growth of control and WSX1-positive AT84 tumors was detected in these NK-defective STAT1 deficient mice (Fig. 2.4*D*, *right*).

WSX1 suppression of tumor growth is independent of IL27. The presented results strongly suggest that WSX1 may play an antitumor role independent of IL27 since the endogenous level of IL27 is undetectable in mice. To exclusively confirm the role of IL27, since the cooperation among WSX1 expression in tumor cell and NK cell is needed for antitumor activity, and IL27 signals in both tumor and immune cells, WSX1-mediated tumor growth inhibition was compared in EBI3 knockout mice. Because EBI3 is a subunit of IL27, the lack of

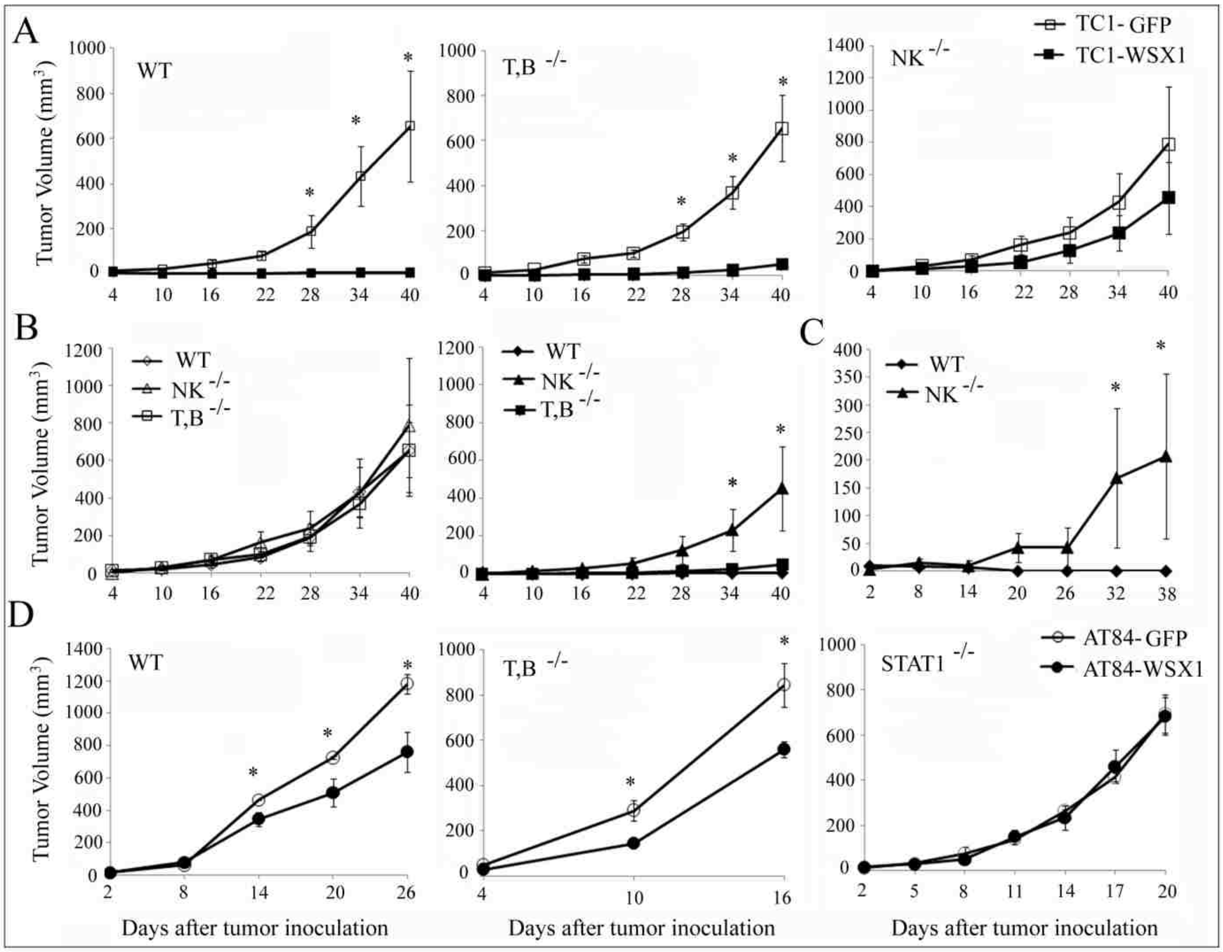

Figure 2.4. WSX1-mediated suppression of tumor growth is dependent on NK cells. *A*, comparison of tumor growth between TC1-GFP and TC1-WSX1 in C57Bl/6 wild-type (WT) mice (*left*), Rag (T,B$^{-/-}$) (*middle*), and perforin (NK$^{-/-}$) knockout mice (*right*). *B*, differential display of tumor growth rate of TC1-GFP (*left*) or TC1-WSX1 (*right*) in different immune knockout models such as C57Bl/6 wild-type (WT), Rag (T,B$^{-/-}$), and perforin (NK$^{-/-}$) knockout mice. *C*, comparison of tumor growth in C57Bl/6 wild-type (WT) mice and perforin knockout (NK$^{-/-}$) mice for an independent clone of TC1-WSX1 (TC1-WSX1 CL2). *D*, comparison of tumor growth between AT84-GFP and AT84-WSX1 in C3H wild-type (WT) (*left*), SCID (T,B$^{-/-}$) (*middle*), and STAT1 (STAT1$^{-/-}$) knockout mice (*right*), N=3-5. *Points*, mean; *bars*, SE. *, $P < 0.05$.

EBI3 would result in inhibition of IL27 signaling in both the host and tumor. As expected, lack of endogenous IL27 did not affect WSX1-mediated tumor growth suppression and a similar difference in tumor growth between TC1-GFP and TC1-WSX1 was found in wild-type mice as was found in IL27 EBI3 knockout mice (Fig. 2.4*A left* vs. 2.5*A*).

To further extend this exclusive confirmation, tumor growth in WSX1 knockout mice was also tested. Because WSX1 is a specific receptor for IL27, the lack of WSX1 in these mice should impede IL27 signaling in the immune cells. Similar to EBI3 knockout mice, WSX1-mediated tumor growth suppression was retained in WSX1 knockout mice (Fig. 2.5*B*). Moreover, WSX1 positive tumors grow at a similar rate in wild-type, EBI3, and WSX1 knockout mice (Fig. 2.5*C*). These results support our hypothesis that WSX1 retains its ability to impede tumor growth in the absence of IL27 signaling in either tumor or host cells.

WSX1 sensitizes NK cell-surveillance by inducing NKG2D ligand expression in tumor cells. Our data above clearly demonstrate that the ability of WSX1 to suppress tumor growth is dependent on NK cells and independent of IL27 signaling in either the tumor or the host. The question is whether the presence of WSX1 in tumor cells directly sensitizes NK cell-mediated cytotoxicity. To accomplish this goal, NK cell cytotoxicity was compared between TC1-GFP and TC1-WSX1 cells. These assays revealed that TC1-WSX1 cells are more efficiently lysed when compared to the TC1-GFP cells (Fig. 2.6*A*, *left*). To rule out the role of CD8 T cells, we used perforin and Rag knockout splenocytes. Similar to wild-type splenocytes (Fig. 2.6*A*, *left*), splenocytes from Rag knockout mice lysed TC1-WSX1-positive cells more efficiently than TC1-GFP (Fig. 2.6*A*, *middle*). Contrarily, the lack of perforin eliminated the enhanced cytolytic activity against TC1-WSX1 (Fig. 2.6*A*, *right*). Similarly, cytotoxicity against AT84-WSX1 was significantly higher than AT84-GFP (Fig. 2.6*B*). These results indicate that WSX1 expression in tumor cells provokes a direct NK cell surveillance.

One possible hypothesis is that WSX1 increases NK cell cytotoxicity by promoting the interaction between tumor cells and NK cells. Given that NKG2D is one of the primary receptors that promotes tumor cell surveillance (13), we determined whether the expression of WSX1 in

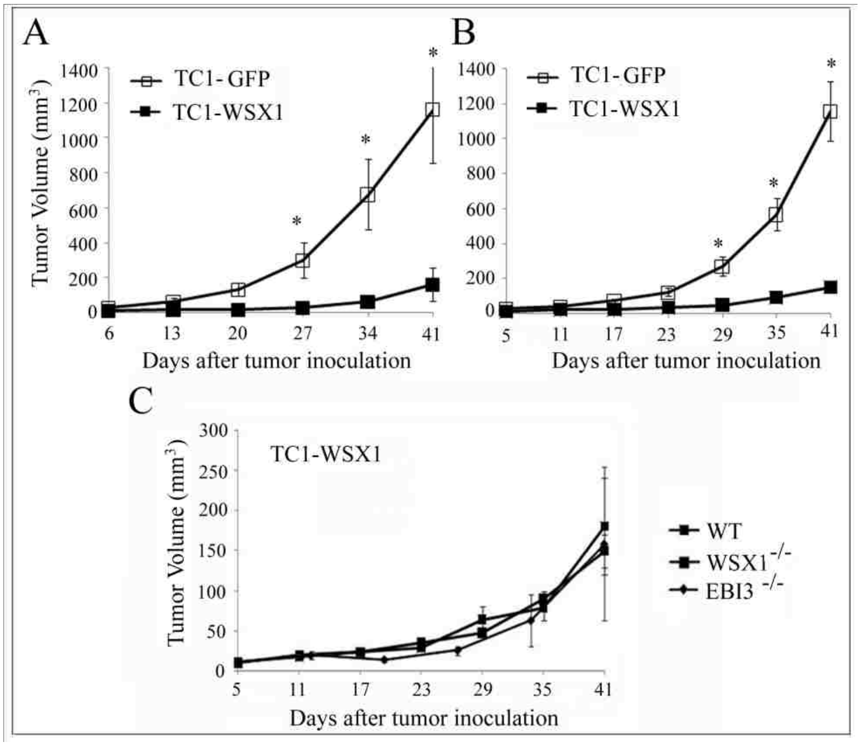

Figure 2.5. WSX1 suppression of tumor growth is independent of IL27. *A* and *B*, Comparison of tumor growth between TC1-GFP and TC1-WSX1 in (*A*) EBI3 knockout (EBI3$^{-/-}$) and (*B*) WSX1 knockout (WSX1 $^{-/-}$) mice. *C*, comparison of TC1-WSX1 tumor growth in C57Bl/6 (WT), WSX1$^{-/-}$, and EBI3$^{-/-}$ mice, N= 4-5. *Points*, mean; *bars*, SE. *, $P < 0.05$.

tumors upregulates expression of NKG2D ligands. Flow cytometry analysis using an NKG2D/Fc binding assay (a reagent that detects cell-surface expression of all known NKG2D ligands) confirmed the hypothesis that WSX1, but not GFP, greatly enhanced the expression of NKG2D ligands (Fig. 2.6*C, left and middle*). Such ability of WSX1 to induce the expression of NKG2D ligands is more pronounced in the TC1 model than in the AT84 model (Fig. 2.6*C*, *right*).

To definitely confirm the hypothesis that upregulation of NKG2D ligands by WSX1 expression is the central mechanism to enhance NK-mediated cytolytic activity against WSX1 positive tumor cells, NKG2D receptors on NK cells were blocked using anti-mNKG2D C7 in the cytolytic assay (14). As expected, addition of an NKG2D neutralization antibody, anti-mNKG2D

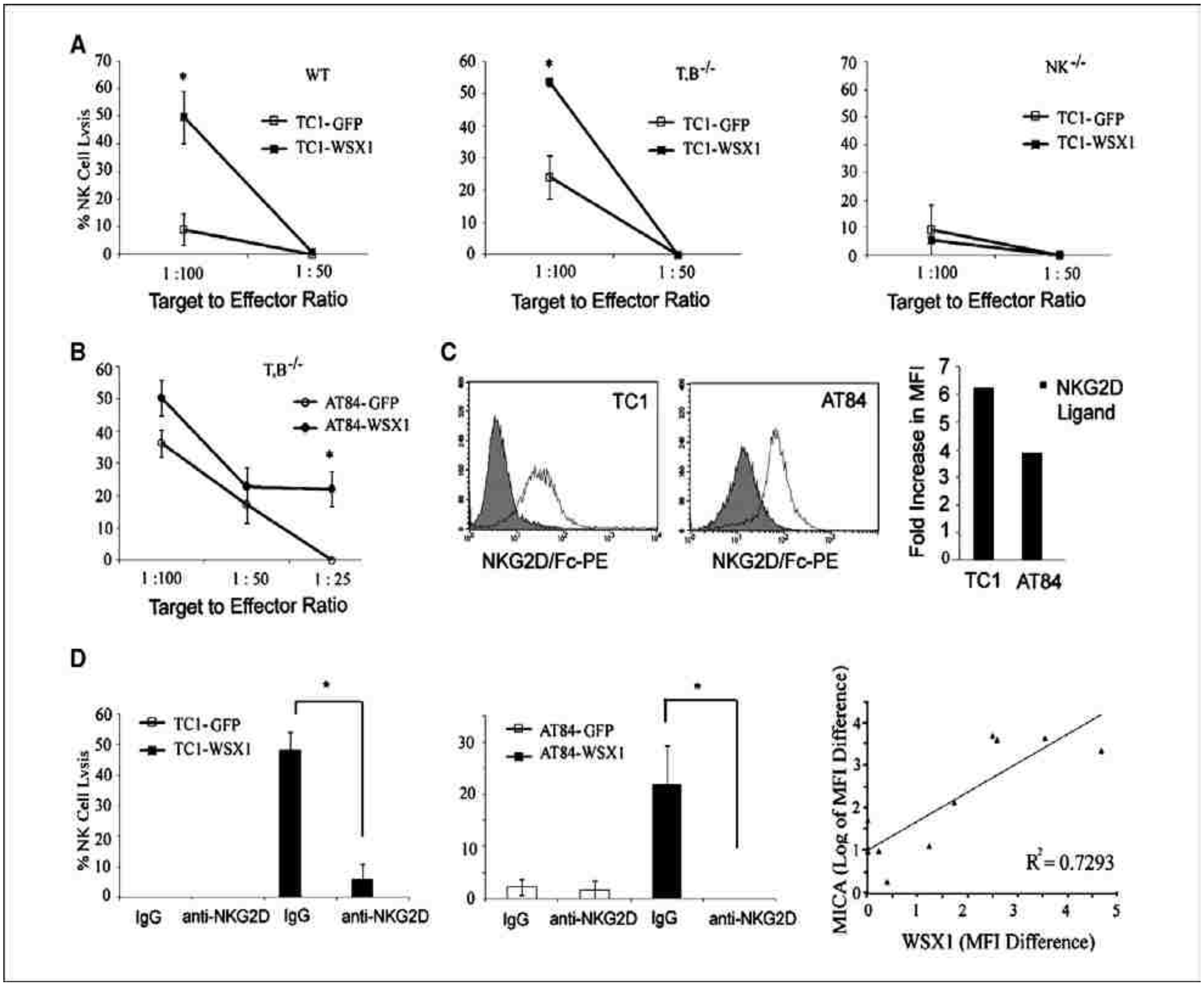

Figure 2.6. WSX1 sensitizes NK cell lysis *in vitro* via an NKG2D pathway. *A*, NK cell activity against TC1-GFP and TC1-WSX1 was analyzed from lymphocytes derived from (*A)* C57Bl/6 wild-type (WT) mice (*left*), Rag (T,B$^{-/-}$) (*middle*), and perforin (NK$^{-/-}$) knockout mice (*right*). Viable target cells from triplicate wells were counted using a fluorescence microscope. *B*, NK cell activity against AT84-GFP vs. AT84-WSX1 was analyzed from lymphocytes derived from SCID mice (T,B$^{-/-}$). The graphs represent data from three independent experiments. *C*, comparison of NKG2D ligands expression in TC1 (*left*) and AT84 (*middle*) tumor cells transduced with GFP (gray) or WSX1 gene (not shaded). The indicated cells were stained with NKG2D/Fc followed by anti-human IgG-PE and fold induction in MFI was calculated between each pair (*right*). *D*, NK cell activity in the presence of anti-NKG2D antibody or isotype control against TC1-GFP and TC1-WSX1 (*left*) at 1:100 target to effector ratio, or AT84-GFP and AT84-WSX1 *(middle*) at 1:25 target to effector ratio. Correlation of WSX1 and MICA expression in the human tumor cells indicated in Fig 1. Cells were stained with the isotype control, WSX1, or MICA and MFI difference between isotype and WSX1 or MICA antibody was plotted (*right*). The plotted values of MFI for MICA expression were log-transformed. *Points*, mean; *bars*, SE. *, $P < 0.05$.

C7, substantially reduced NK cell lysis against both TC1-WSX1 and AT84-WSX1 target cells to levels comparable of GFP counterparts (Fig. 2.6*D, left and middle*). Furthermore, WSX1 expression is strongly correlated (R^2= 0.7293) to MICA expression in human cancer cell lines (Fig. 2.6*D*, *right*).

DISCUSSION

Although it is well known that WSX1 is expressed in T and NK cells and is a critical receptor for triggering immune responses via IL27 signaling in these cells (1, 2, 15), our recent results revealed that WSX1 is also expressed in epithelial tumor cells such as breast tumor cell lines, while others have found its expression in human melanoma (3) and leukemia cells (16). Neither us nor others have quantified the level of WSX1 expression and compared the level of expression between epithelial tumor cells and normal epithelial cells. In this study, using a quantitative real-time PCR assay, we surprisingly found that WSX1 is not only expressed in most of the tested epithelial cells (8 out of 10), but is also expressed 13- to 78-fold higher than normal epithelial cells (Fig. 2.1 A).

While the function of WSX1 in immune cells has been studied extensively, its function in epithelial tumor cells has hardly been studied. In immune cells, it is generally accepted that IL27 possesses both pro- and anti-inflammatory properties (17-25). For example, IL27 induces key components to Th1 commitment such as synergistic induction of IFNγ with IL12, proliferation of naive $CD4^+$T cells, induction of T-bet and IL12Rβ expression, and possesses T-cell and NK cell mediated antitumor activities (15, 20-25); however, parasitic studies show that the absence of WSX1 triggers aberrant cytokine production (26, 27). In contrast to previous studies in immune cells, our findings assign a new role to WSX1 in epithelial cancer cell biology, that expression of WSX1 inhibits epithelial tumor growth *in vitro* and *in vivo* (Figs. 2.1, 2.3, and 2.4). Such an observation was confirmed in two independent tumor models, TC1 and AT84. However, this observation is different in tumors derived from immune cells in which WSX1 elicited antiapoptotic and mitogenic signals (16) and transformed two leukemia cell lines, 32D and BaF3.

Different from the recent report in which WSX1 expression in epithelial melanoma cells requires IL27 to inhibit its tumor cell proliferation and tumor growth (3), our results provide a strong case of IL27-independent antitumor activity by WSX1 (Fig. 2.2 and 2.5). First, IL27 independence was demonstrated by our trial showing that WSX1 expression inhibited tumor growth in mice that lacked the IL27 subunit EBI3, the same as found in wild-type mice (Fig. 2.5 A). Second, WSX1 expression in epithelial tumor cells inhibits tumor growth in WSX1 knockout mice (Fig. 2.5 B).

Currently, no linkage between WSX1 expression in tumor cells and NK cell-mediated surveillance is reported in the literature. Using two different tumor models, TC1 and AT84, we show that WSX1 suppresses tumor growth *via* NK cells. In perforin and STAT1 knockout mice, WSX1-dependent suppression of tumor growth is impaired (Fig. 2.4), while *in vitro* WSX1 tumor cells are more sensitive to NK cell cytotoxicity (Fig. 2. 6). Such phenomena are dependent on the NKG2D pathway, as the presence of WSX1 directly enhances the expression of NKG2D ligands (Fig. 2.6) thereby enhancing NK cell-mediated recognition and release of cytotoxic molecules towards tumor cells.

Considering that WSX1 expression in our tumor models inhibits tumor growth while expression of WSX1 in certain leukemia cell lines confers transformation (16), further work is needed to investigate the molecular mechanism downstream of WSX1 that leads to opposing consequences between epithelial and blood-derived tumors. This link between WSX1 and NKG2D ligands, which are the intrinsic sensors of oncogenic transformation that induce innate immunosurveillance (28), suggests that WSX1 expression in epithelial tumor cells might play a role to prevent tumorigenesis but was eventually overridden by the defaulted pathway or immune escape mechanism. Such a scenario is also seen with the MICA-NKG2D surveillance system in which a high level of MICA expression in tumor cells were subjected to NKG2D surveillance (29, 30), but this surveillance system was overridden by the aggressive tumors due to the development of multiple immune escape mechanisms (31, 32). The connection of WSX1 to innate immunosurveillance could explain the observed discrepancy among the two tumor models used in this study: WSX1 is more effective in inhibiting tumor growth and increasing NKG2D ligand expression in TC1 model, a HPV-transformed normal cell line, than in aggressive AT84 models, a spontaneously arising oral squamous cell tumor of C3H mice, albeit WSX1 expression is higher in AT84 than in TC1.

In summary, this study exposed a novel function of WSX1 in epithelial tumor cells, linked WSX1 to NK-mediated antitumor immunosurveillance, and, most importantly, revealed that WSX1 induces an antitumor function by upregulating the expression of NKG2D ligands and this process is independent of the IL27 signaling pathway.

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CHAPTER 3

WSX1 INDUCES TUMOR TOLERANCE VIA AN IL27-INDEPENDENT MECHANISM

INTRODUCTION

Extensive research done on IL27 indicates that this cytokine plays both an inflammatory and anti-inflammatory role in regulating the immune response. Primarily, this cytokine was attributed pro-inflammatory properties, as TCCR (IL27 receptor, also known as WSX1) deficient mice showed reduced Th1 responses in both *in vivo* and *in vitro* assays (1-3). Also, IL27 induced T cell proliferation, the expression of IL12 receptor β2, and the Th1 transcription factor T-bet and attenuated induction of Treg cells (4-6). Opposite to this pro-inflammatory effect, IL27 is also an anti-inflammatory cytokine as it induces IL10 production in T cells (7-10). In multiple parasitic and autoimmune disease models, mice lacking WSX1 showed an exacerbated immune response and enhanced pro-inflammatory cytokine production (11-14).

The function of the IL27 receptor WSX1 is exclusively associated to IL27 and IL27's role in immune cells, thus IL27-independent function and its association with cancer biology is largely unknown. One reason is that WSX1 is primarily expressed in immune cells such as monocytes, dendritic cells, T and B lymphocytes, NK cells, mast cells, (1). The other reason, perhaps, is that WSX1 needs to pair with gp130 to constitute a functional signal-transducing receptor for IL27 signaling, whereas lack of either subunit attenuates IL27-mediated signaling (15).

Recently, we have revealed that WSX1 has an important role not only in immune cells but also in multiple cancers of epithelial origin via an IL27-signaling-independent pathway (16). In TC1 and AT84 tumor cells, overexpression of WSX1 inhibits tumor growth via NK-cell-dependent immune surveillance in vivo, and this effect is independent of IL27 signaling. This discovery is further supported by a recent report which revealed that WSX1 is expressed in another type of epithelial tumor, melanoma cells (17).

The findings that WSX1 suppresses tumor growth suggest that the levels of WSX1 expression in tumors cells should be reduced. Opposite to this assumption, many of the human cell lines have a high level of WSX1 expression when compared to a normal control cell line, NCM460 (16). Thus, many facets of this receptor in tumor biology remain to be discovered. Using genetically modified tumor cells, we present clear evidence that the expression of WSX1 in two independent tumor models, such as aggressive Lewis Lung Carcinoma (LLC) and melanoma cell line AGS, promotes tumor growth independent of IL27 signaling. The underlying mechanism by which WSX1 promotes tumor growth is through inhibition of T cell proliferation and inhibition of production of the effector cytokine IFNγ both in the tumor microenvironment and distal lymphatic tissues. Our conclusive evidence reveals that this effect is initiated via direct tumor cell and immune cell contact. This important discovery reveals a totally new mechanism on how tumor cells and host immune cells communicate to promote tumor growth.

METHODS

Cell culture and reagents. Mouse cancer cell lines AGS and LLC were kindly provided by Dr. William E. Carson (The Ohio State University, Columbus, Ohio) and Dr. Augusto C. Ochoa (Louisiana State University, School of Medicine, New Orleans, LA), respectively. Recombinant mouse IL27, NKG2D/Fc, and anti-CD3 were purchased from R&D Systems (Minneapolis, MN); anti- pSTAT1-701, anti-STAT1, anti-actin, anti-hamster IgG-FITC, and anti-hamster IgG-APC from Santa Cruz Biotechnology (Santa Cruz, CA), anti-NKG2D-Pe, anti-NK1.1-FITC, anti-CD4-APC, anti-CD4-FITC, and anti-CD28 from Biolegend (San Diego, CA); anti-CD8-FITC from BD Biosciences (San Jose, CA), and anti-IFNγ-APC, from eBiosciences (San Diego, CA). Mouse anti-WSX1 was kindly provided by Dr. Fred de Sauvage (Genentech, San Francisco, Ca). RPMI or DMEM media were purchased from Invitrogen (Carlsbad, CA).

Establishing stable WSX1 and dominant negative WSX1 (DN-WSX1) expressing cell lines. The murine *WSX1* full length gene (gene bank # BC032878) was purchased from Open Biosystems and subcloned into *pBMN-GFP* (Phoenix™ Retrovirus Expression System, Orbigen, Inc.) for generating the *WSX1-IRES-GFP* retroviral construct. The murine *WSX1* full length gene was first subcloned into *pDsRed-Express* vector (Clontech Laboratories, Inc. Mountain View, CA) to make the *WSX1-DsRed* fusion gene (referred to as *fWSX1* throughout the manuscript). The fusion gene was subsequently cloned into *pBMN-GFP*, resulting in the *WSX1-DSRed-IRES-GFP* retroviral construct. The *Lac Z* gene in *pBMN-Z* plasmid (Phoenix™ Retrovirus Expression System, Orbigen, Inc.), was replaced with the *WSX1-DsRed* fusion gene (referred to as *rWSX1* throughout the manuscript) from above for generating the *WSX1-DSRed* retroviral construct. *WSX1* without cytoplasm domain was PCR amplified from *WSX1* gene and subcloned to *pDsRed-Express* to make the *WSX1-DN-DsRed* fusion gene (referred as *DN-WSX1* throughout the manuscript). The fusion gene was subsequently cloned to *pBMN-GFP* for generating the *WSX1DN-DSRed-IRES-GFP* retroviral construct. The retrovirus was generated by transfecting *mWSX1-IRES-GFP*, *WSX1DN-DSRed-IRES-GFP*, or control *GFP* and *DsRed* constructs into Phoenix Eco packaging cells. LLC and AGS cells were transduced with retrovirus containing the gene of interest. The transduction was confirmed by detecting fluorescent cells under the fluorescence microscope. To differentiate between transduced and non-transduced cells, positive fluorescent cells were sorted using a BD FACS Aria III cell sorter.

Animal procedures. All the animal procedures were approved by the IACUC at Louisiana State University. Six- to eight-week-old mice were used for this study. The subcutaneous tumor models were generated by subcutaneously inoculating LLC and AGS tumor cells (2×10^5 in a 30-µL volume per mouse) into mice. Tumor measurement and calculation were the same as described previously (18). C57Bl/6 WSX1 (TCCR) knockout mice were provided by Dr. Fred de Sauvage (Genentech), and C57Bl/6, and Balb/c SCID, deficient mice were purchased from commercial sources.

Flow Cytometry. Cells were stained with the indicated antibodies for 30 min at 4˚C as indicated in each figure. The expression of the indicated genes was analyzed on FACS Calibur (BD Biosciences, San Jose, CA) and FCS Express 3 (De Novo Software, Los Angeles, CA). For intracellular staining, BD Biosciences intracellular kit was used according to manufacturer's instructions. Caspase 8, Caspase 9, and Annexin V kits were purchased from Biovision (Mountain View, Ca), and staining was performed according to manufacturer's instructions.

Cell proliferation. Cell proliferation assays were performed using the luminescence ATP Lite assay detection system (PerkinElmer, Waltham, Ma). Briefly, 500 cells per well were seeded in a 96 well plate; cells were lysed on days 0, 2, 4, and 6 for measuring the ATP levels. The cell proliferation index was calculated using the following equation: Cell proliferation index= [ln (d)] / [ln (d0)], where ln (d) = natural log at the day when cells were lysed; ln (d0) = natural log at the day when cells were seeded.

Soft agar growth assay. The clonogenic assay was performed as previously described (19). Briefly, genetically engineered cells ($2x10^3$ for LLC-GFP and LLC-WSX1 cells) were suspended in 0.34% agar in cell culture media (Invitrogen, Carlsbad, CA). The mixture solution was layered on solid agar support prepared from 0.9% agar in cell culture media. The cells were seeded in triplicates on a 6-well plate and grown for 2 weeks. Colonies were counted under a 10x dissecting microscope after staining with 0.05% Crystal Violet for 1 h.

Western blot. Cells ($5x10^5$), growing in 10% heat-inactivated FBS-containing culture media, were treated with or without IL27 (20 ng/mL) for the indicated times. Protein extract was obtained by directly lysing cells using 60 µL of Laemmli sample buffer. Twenty microliters of total protein extract from each sample were separated by SDS-PAGE, transferred to a nitrocellulose membrane and incubated with primary antibody overnight at 4° C (750x dilution for anti-pSTAT1/701, 1000x dilution for anti-STAT1, 500x for anti-WSX1, and 1000x fold dilution for anti-Actin).

Statistical analysis. For *in vivo* experiments, Univariate Repeated Measures ANOVA was used to analyze the difference among treatments using SAS version 9.1.3. When appropriate, Tukey's HSD test was performed for interaction affects. For *in vitro* results, student's T test analysis was conducted.

Cell harvesting/purifications. Spleens were mashed through a 70 µm cell strainer (Fisher) to obtain single cell suspensions. $CD3^+$ T cells were purified using negative selection according to manufactures instructions (Stem Cell Separation, Vancouver, BC, Canada). Splenocytes or $CD3^+$ T cells were labeled with 20µM CFSE for 15 minutes at 37ºC (Invitrogen, Carlsbad, CA). Tumor-infiltrating lymphocytes were obtained by removing tumors, chopping them into pieces, and resuspending the mixture in sterile PBS (without Ca^{2+} and Mg^{2+}) in the presence of a digestion enzyme mixture of collagenase IV, hyaluronidase V (Sigma-Aldrich, St. Louis, MO), and DNase II (Fisher, Pittsburgh, PA). The tissue/enzyme mixture was placed in a shaker at 37ºC for 1-2 hours, and then poured through a 70 µm cell strainer, followed by twice washing in PBS with Ca^{2+} and Mg^{2+}. After the last wash, the cells were stained for flow cytometry.

Confocal microscopy. To analyze the distribution of WSX1 within the cell, TC1 and LLC cells expressing fWSX1 were seeded onto glass slides and fixed. The slides were visualized and photographed using **Leica TCS SP2** confocal microscope at the Microscope Center with the help of Dr. Xiaochu Wu. (Louisiana State University School of Veterinary Medicine).

***In vitro* co-incubation assays.** $2*10^6$ CFSE-labeled or unlabeled splenocytes were co-incubated with $2*10^5$ tumor cells in a 12 well plate for 72 hours in total of 2 mL RPMI media. $1*10^6$ CFSE-labeled $CD3^+$ T cell were co-incubated with $1*10^5$ or $2.5*10^4$ tumor cells in a 12 well plate for 48 hours in total of 2 mL RPMI media, and cells were analyzed for CFSE dilution. Lymphocytes or $CD3^+$ T cells were activated with anti-CD3 and anti-CD28 antibodies at concentrations of 2.0 µg/mL and 0.5 µg/ml, respectively.

Adoptive T cell transfer. $5*10^6$ CFSE-labeled $CD3^+$ T were suspended in 100 µL of PBS and injected i.v. into mice bearing either LLC-GFP or LLC-WSX1 tumors at 300 mm^3. The mice were sacrificed 16 hours and 6 days post adoptive transfer, tumors were harvested and processed via enzyme digestion, and CFSE-positive cells in the tumor microenvironment were analyzed via flow cytometry.

RESULTS

The paradoxical functions of WSX1 between *in vivo* and *in vitro* assays. We have previously shown that WSX1 is expressed not only in immune cells as reported by others (1) but also in epithelial tumor cells (16). WSX1 expression in tumor cells reduced tumorigenecity and cell proliferation *in vitro* in two different cell lines, AT84 and TC1. We wanted to further extend this discovery and determine whether WSX1 has the same function in Lewis Lung Carcinoma (LLC). Therefore, LLC cells were engineered to express either WSX1 or control GFP (Fig. 3.1*A*). The clonogenic and ATP light assays confirmed our previous discovery and showed a 5-fold reduction in clonogenic ability when compared to control cohorts (Fig. 3.1*B and* 3.1*C*). Likewise, the same finding was also observed in CT26 and 4T1 (data not shown). In summary, a total of 5 different cell lines yielded the same result: WSX1 expression in tumor cells inhibits tumorigenecity.

To confirm that WSX1 is properly expressed, the localization of this receptor was determined via confocal microscope in LLC or TC1 expressing fWSX1 (full length WSX1 fused to DSRed). As expected, the data showed that WSX1 is expressed in the membrane, similarly in both TC1 and LLC (Fig. 3.1*D*). Furthermore, flow cytometry confirmed that there is abundant expression of this receptor on the membrane (Fig. 3.1*E*).

To test whether the same conclusion can be made in vivo, we compared the tumor growth difference between GFP- or WSX1-positive LLC tumor cells. Contrary to the *in vitro* results and the other two tumor models (16), WSX1 promotes tumor growth in LLC (Fig. 3.1*F*). Similarly, WSX1 enhanced tumor growth in another independent tumor model, melanoma cell line AGS (Fig. 3.1*G*). Therefore, the fact that WSX1 promotes tumor growth in two independent tumor models suggest that this receptor plays a dual function in tumor development.

WSX1 promotes tumor growth independently of IL27. Our previous data suggested that WSX1 inhibited tumor growth independently of IL27 (16). To determine whether WSX1 is a promoter of tumor growth independently of IL27, we assessed tumor growth differences between full length WSX1 and dominant negative WSX1 (DN-WSX1, lacking intracellular domain). We expected that DN-WSX1 should shut down the ability of IL27 to signal in these cells. If DN-WSX1-expressing tumors grow slower than full-length WSX1 in wild-type mice, then IL27 promotes tumor growth; otherwise, IL27 does not explain pro-tumorigenic abilities of WSX1. To confirm that IL27 can not signal in DN-WSX1, we determined the ability of IL27 to induce Stat1 phosphorylation and Stat1 expression. As expected, IL27 is able to signal in full-length WSX1 expressing cells, while in DN-WSX1 cells IL27 has lost such ability (Fig. 3.2*A*).

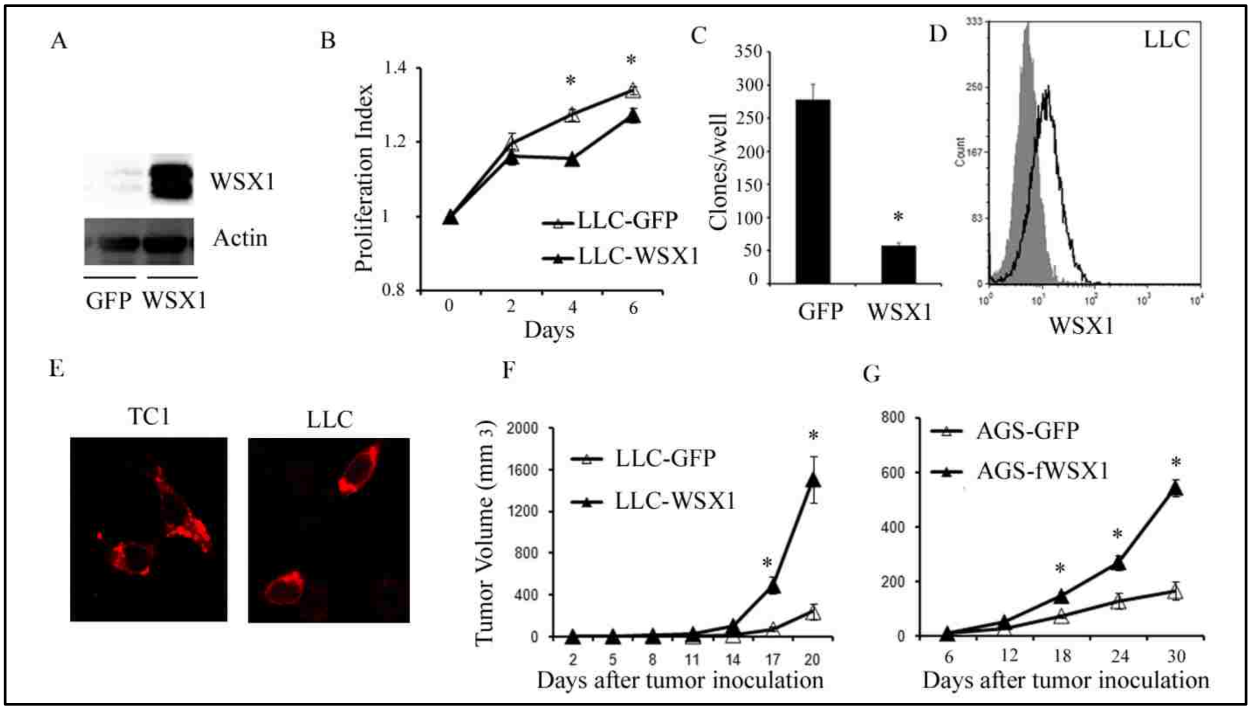


Figure 3.1. The paradoxical functions of WSX1 between *in vivo* and *in vitro* assays. *A*, detection of WSX1 expression in LLC tumor cells via western blot analysis**.** LLC tumor cells were transduced with retrovirus containing either control *GFP* or *WSX1* gene. Cell extracts of the established cell lines were analyzed using western blot techniques and probed with mouse WSX1 and actin antibodies. *B*, detection of the role of WSX1 in tumor cell proliferation. GFP or WSX1 positive LLC cells were harvested on the indicated days and were analyzed for ATP release. N=4. Error bars are smaller than symbols. *C*, comparative analysis of soft agar growth assay between LLC-GFP and LLC-WSX1 cells. Data is representative of two independent experiments, each experiment performed in triplicate. *D*, detection of WSX1 expression in LLC cells via flow cytometry. Cells transduced with either *GFP* (*shaded in gray*) or *WSX1* genes (*not shaded*) were stained with monoclonal WSX1 antibody followed by an anti-hamster-PE antibody. *E*, detection of WSX1 localization in the cell. Images of TC1 (*left*) or LLC (*right*) cells expressing *fWSX1* gene were captured using a confocal microscope. *F*, comparison of tumor growth between LLC-GFP and LLC-WSX1 in C57Bl/6 mice, N=4. *G*, comparison of tumor growth between AGS-GFP and AGS-rWSX1 in C57Bl/6 mice, N=5. *Points*, mean; *bars*, SE. *, P <0.05.

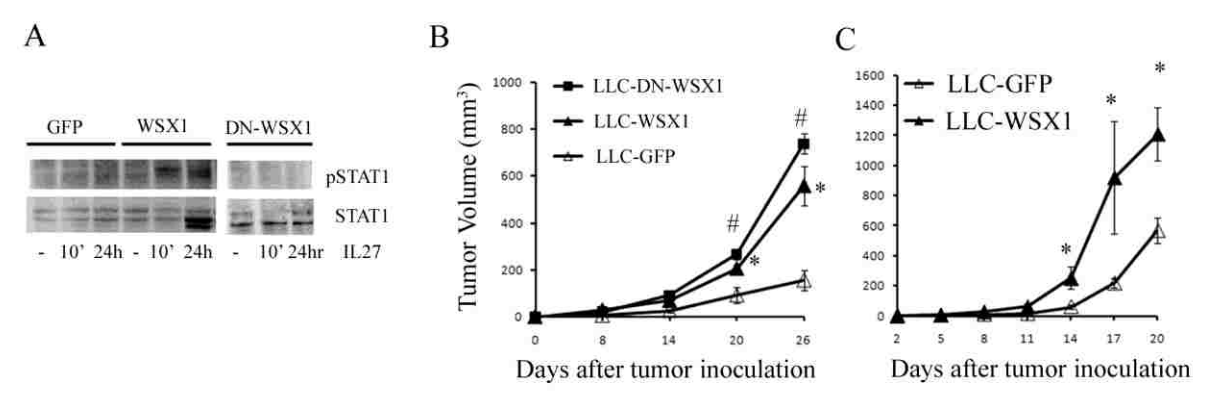


Figure 3.2. WSX1 promotes tumor growth independently of IL27. *A*, detection of functional and truncated WSX1 in tumor cells. LLC cells expressing GFP, WSX1, or DN-WSX1 were treated with IL27 for 10 minutes (10’), overnight (24hr), or left untreated (-). Equal amounts of cell extracts were analyzed using WB techniques and probed with pSTAT1 and STAT1 antibodies. *B*, comparison of tumor growth between LLC-GFP, LLC-WSX1, and LLC-DN-WSX1 in wildtype C57Bl/6 mice. N=5. *C*, comparison of tumor growth between LLC-GFP, LLC-WSX1 in TCCR $^{-/-}$ mice. N=4. *Points*, mean; *bars*, SE. *, P <0.05 between LLC-WSX1 and LLC-GFP. #, P<0.05 between LLC-DN-WSX1 and LLC-GFP.

In vivo, DN-WSX1-expressing tumors grow faster than either full-length and control cohorts, excluding the role of IL27 as a tumor promoter (Fig. 3.2*B*). Previous studies have shown that IL27 possesses anti-tumor activities via signaling in immune cells (20-23). Thus, to exclude the possibility that WSX1 would act as a decoy receptor, thereby competing with WSX1-expressing immune cells for IL27, we compared tumor growth rates between GFP and WSX1-positive tumors in TCCR$^{-/-}$ mice. An increase in tumor growth rates in WSX1-positive tumors compared to control counterparts in TCCR$^{-/-}$ mice (IL27 cannot signal into host) suggests that WSX1 expression in tumors does not act as a decoy receptor for IL27 but rather promotes tumor growth by itself. Indeed, similar to wildtype mice, WSX1 promotes tumor growth in TCCR$^{-/-}$ mice (Fig. 3.2*C*).

WSX1-mediated tumor growth is NKG2D independent. The NKG2D pathway not only plays a role in the anti-tumor immune response but also induces immune escape via multiple mechanisms: NKG2D-ligands are shed by Erp5, which results in NKG2D receptor internalization in NK^+ and $CD8^+$ cells (24, 25). Similar to soluble NKG2D ligands, the release of exosomes from tumor cells downregulates the NKG2D receptor in immune cells (26). Moreover, immunosuppressive $NKG2D^+CD4^+$ cells expand upon ligation with NKG2D ligand and inhibit $CD4^+$ cell proliferation (27). Since we previously found that WSX1 induces NKG2D ligand expression in tumor cells and NKG2D ligand internalization can induce immune tolerance, we hypothesized that WSX1 might promote immune-evasion via an NKG2D-dependent pathway. Contrary to our previous results, WSX1 does not induce NKG2D ligand upregulation (Fig. 3.3*A*). Next, we investigated whether WSX1-positive tumors affected NKG2D expression by NK^+ or $CD8^+$ cell *in vivo*. Our results showed that there is no significant reduction of NKG2D receptor in either NK^+ or $CD8^+$ cells by WSX1; in contrast, WSX1 enhanced expression of the NKG2D receptor in these cells (Fig. 3.3*B*). Moreover, the percentage of immunosuppressive $NKG2D^+CD4^+$ T cells is similar between GFP- and WSX1-positive tumors. Studies comparing the total cellular (cells were permeabilized) or membrane expression of NKG2D in the splenocytes of mice bearing LLC-GFP or LLC-WSX1 revealed that extracellular and total cellular NKG2D expression was increased rather than decreased by WSX1 (Fig. 3.3*C*) suggesting that internalization of NKG2D is not the mechanism that explains the pro-tumor properties of WSX1.

WSX1 triggers immunosuppression in the tumor microenvironment and in the spleen. WSX1 inhibits tumorigenicity and cell proliferation *in vitro*, while it promotes tumor growth when inoculated subcutaneously in syngeneic mice (Fig. 3.1). This prompted us to think that WSX1 in these tumor cells regulates tumor-host communication, therefore inducing a state

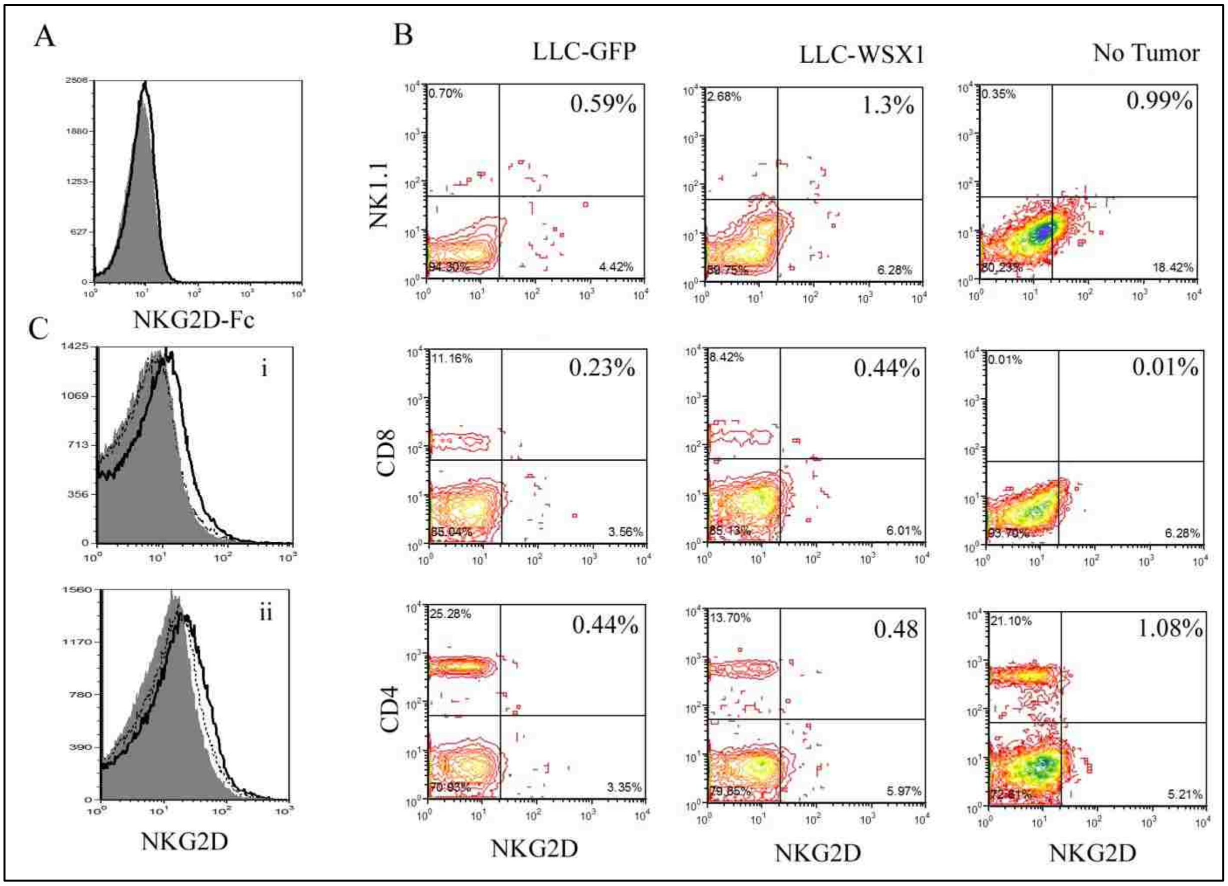


Figure 3.3. WSX1-mediated tumor growth is NKG2D independent. *A*, comparison of NKG2D ligand expression in LLC cell transduced with either GFP (*shaded gray*) or WSX1 (*not shaded*). The indicated cells were stained with NKG2D-Fc followed by anti-human IgG-PE. *B*, comparison of NKG2D expression in NK$^+$, CD8$^+$, and CD4$^+$ cells in the splenocytes of mice bearing LLC-GFP or LLC-WSX1 tumors, or mice without tumors. Splenocytes from tumor bearing mice were pooled (5 mice/group), processed, and stained with the following antibodies: NK1.1-FITC, CD8-FITC, CD4-APC, and NKG2D-PE. *C*, comparison of membrane-only or total cellular expression of NKG2D in the splenocytes of mice bearing LLC-GFP (*dotted line*) or LLC-WSX1 tumors (*solid line*) or mice without tumors (*shaded in gray*). Processed splenocytes were either permeabilized (*bottom panel*, *ii*) or left untreated (*top panel*, *i*) and stained with NKG2D-PE antibody.

of immunosuppression and suppression of tumor growth. In other words, such tumor growth differences may be diminished in immune-compromised mice. To confirm such a hypothesis, we compared tumor growth rates between control, WSX1, and DN-WSX1 expressing tumors in immune compromised SCID mice. As seen in Fig. 3.4*A*, absence of T and B cells completely

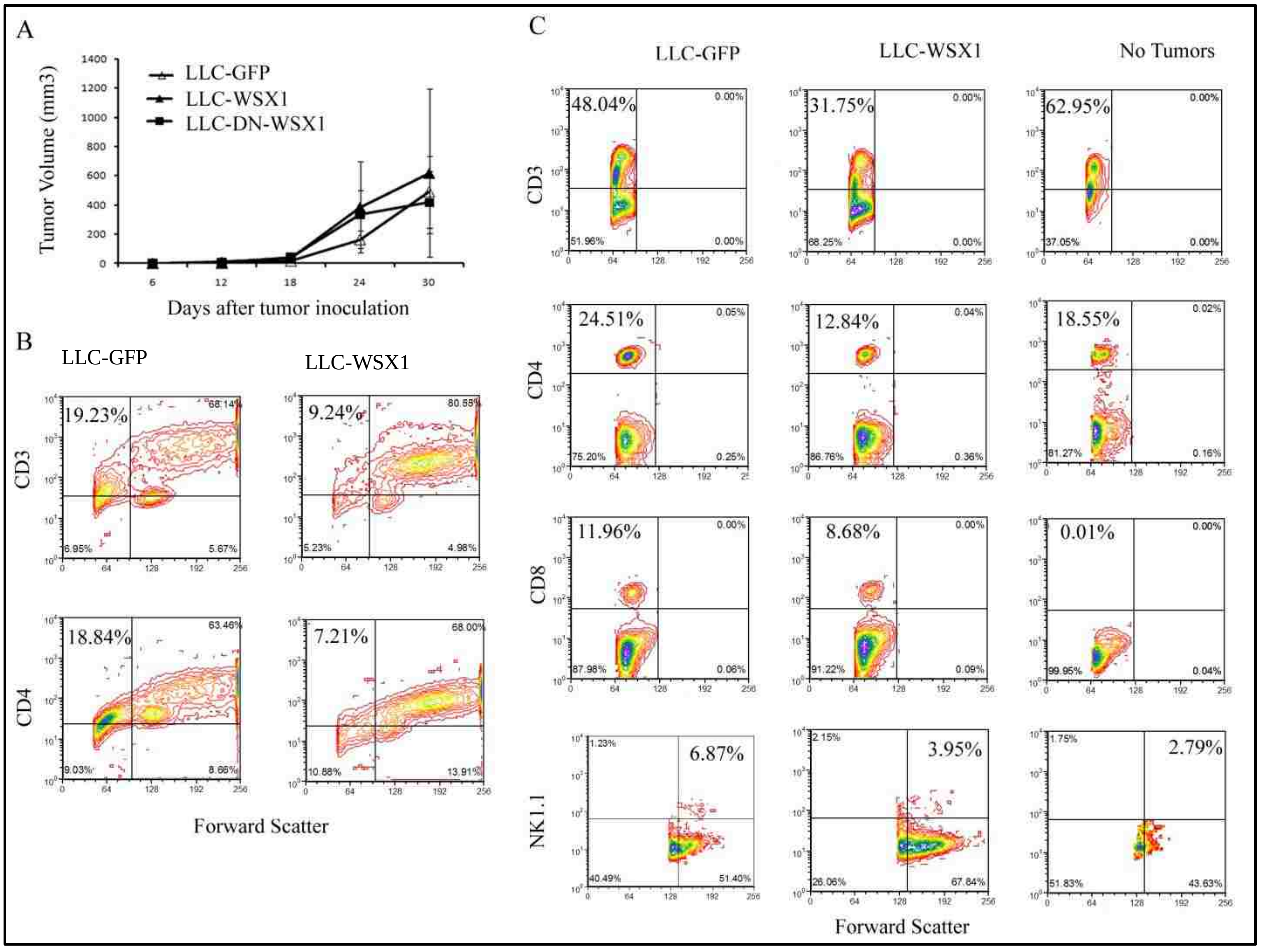


Figure 3.4. WSX1 triggers immunosuppression in the tumor microenvironment and the spleen. *A*, comparison of tumor growth between LLC-GFP, LLC-WSX1, and LLC-DN-WSX1 in SCID mice. N=3-4. *B*, comparison of the number of T cells in the tumor microenvironment between LLC-GFP and LLC-WSX1 tumor-bearing mice. Tumors from 5 mice were pooled, processed, and then stained with CD3-APC or CD4-PE. *C*, comparison of the number of immune cells in the spleen between mice bearing LLC-GFP or LLC-WSX1 tumors or mice without tumors. Splenocytes of tumor-bearing mice were pooled (N=5), processed, and then stained with CD3-FITC, CD8-FITC, CD4-APC, and NK1.1-FITC. N=4. *Points*, mean; *bars*, SE. *, P <0.05.

abolished the ability of WSX1 to promote tumor growth. To further support this statement, we investigated the phenotype of immune cell infiltration in the tumor microenvironment. The total number of $CD3^+$ and $CD4^+$ T cells present in the tumor microenvironment was drastically reduced in WSX1- positive tumors (Fig. 3.4*B*). Such an observation was not due to lack of T cells infiltration in tumor microenvironment, as systemic transfer of CFSE-labeled $CD3^+$ T cells had similar tumor-infiltrating ability 16 hours post-transfer (data not shown). WSX1 does not only affect immune cells via paracrine signaling in the local tumor microenvironment, but also in an endocrine manner in distal organs such as the spleen. Multiple immune cell populations, $CD3^+$, $CD4^+$ $CD8^+$, and NK^+ cells, in the spleen were reduced in LLC-WSX1 tumors, suggesting the release of secretable factors.

WSX1 induces immunosuppression in a cell contact-dependent manner. To further characterize how WSX1 regulates immune cells, tumor cells expressing WSX1 or GFP were directly co-incubated with splenocytes. Similar to the *in vivo* results, the frequency of $CD3^+$, $CD4^+$, $CD8^+$, and NK^+ cells were reduced by 40-60% in LLC-WSX1 tumors compared to LLC-GFP in culture systems (Fig. 3.5*A*). Likewise, WSX1 inhibited the frequency of T and NK cells in an independent tumor model, AGS (Fig. 3.5*B*).

A hallmark of T and NK cell activation is expression of IFNγ. WSX1-positive tumors cells reduced the percentage of $CD4^+$ T cells that were producing IFNγ when compared to cohorts (3% versus 23.5%) in splenocytes *in vitro*. Moreover, the percentage of $IFN\gamma^+$ $CD8^+$ and $IFN\gamma^+$ NK^+ cells was also largely decreased in the presence of LLC-WSX1 (Fig. 3.5*C*) compared to the presence of LLC-GFP. This observation was also extended to the AGS model (Fig. 3.5*D*).

Although WSX1 affects T and NK cell number and cytokine production, we wanted to assess whether WSX1 does so in a contact-dependent manner or via release of secretable factors. To answer this question, LLC-tumor cells where co-incubated with splenocytes directly or separated via a transwell, and T cell numbers and cytokine production was determined. The use of a transwell reversed the ability of WSX1 to induce immunosuppression in T cells (Fig. 3.6*A* and 3.6*B*), suggesting that WSX1 induces immunosuppression via a direct receptor-receptor interaction.

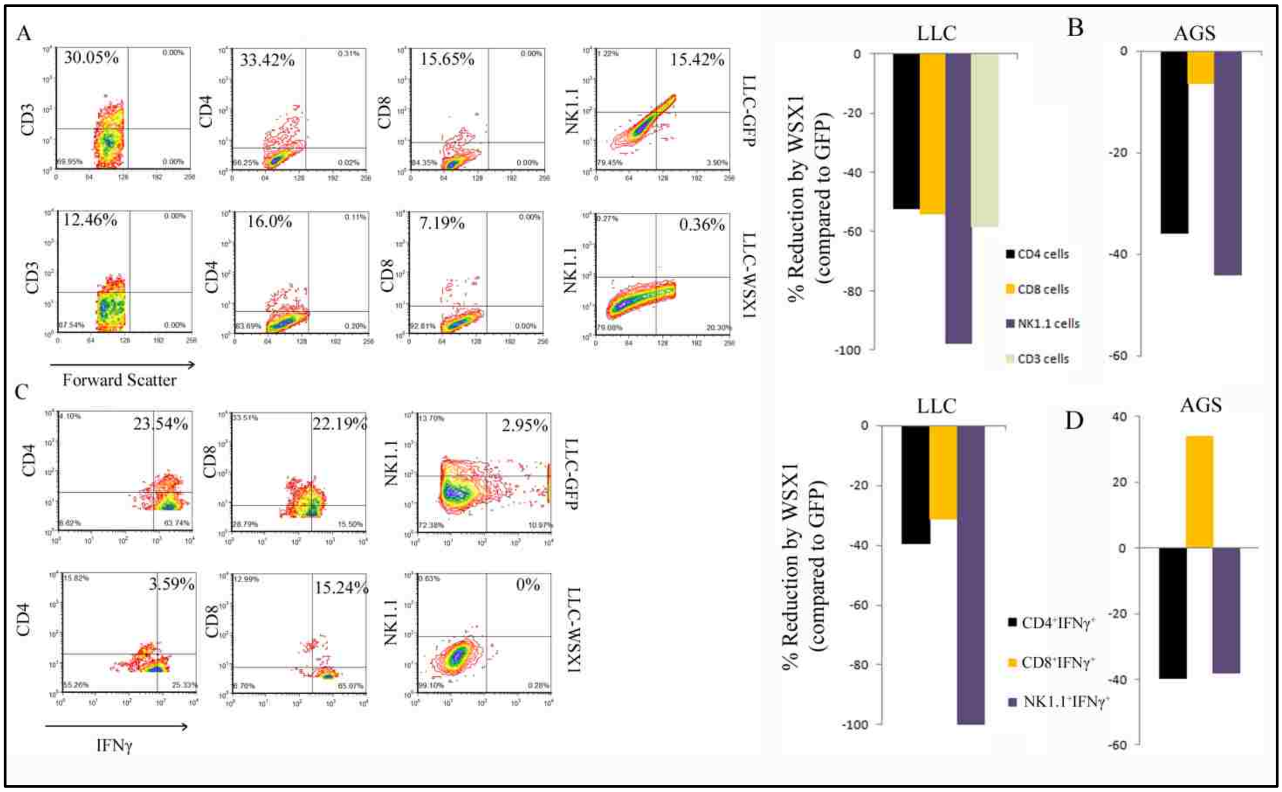


Figure 3.5. WSX1 induces immunosuppression in immune cells in a cell contract-dependent manner. *A*, comparison of the effect of LLC-GFP or LLC-WSX1 tumor cells on the number of immune cells in vitro. Splenocytes were co-incubated with the tumor cells mentioned above in the presence of anti-CD3 and anti-CD28 antibodies for 72 hours and stained with the following antibodies: CD3-FITC, CD8-FITC, CD4-FITC, and NK1.1-FITC. *B*, AGS-GFP or AGS-fWSX1 effect on immune cells were analyzed similarly to 3.5A. *C*, IFNγ expression in CD4, CD8, and NK cells was compared in splenocytes co-cultured with either LLC-GFP or LLC-WSX1 tumor cells. *D*, IFNγ expression in CD4, CD8, and NK cells was compared in splenocytes co-cultured with either AGS-GFP or AGS-fWSX1 tumor cells.

WSX1 directly affects T cell proliferation. Our findings so far support the idea that WSX1 expression in tumors reduces T cell numbers and IFNγ production. Next, we wanted to assess whether WSX1 induces T cells death or whether WSX1 inhibits T cells proliferation. To address this question, splenocytes were stimulated with CD3 and CD28 in the presence WSX1-positive tumor cells. After 72 hours, $CD4^+$ and $CD8^+$ cells were analyzed for expression of apoptotic markers: Caspase 8, Caspase 9, and Annexin V. The percentage of $CD4^+$ and $CD8^+$ cells expressing apoptotic markers were similar when incubated with WSX1-expressing tumor

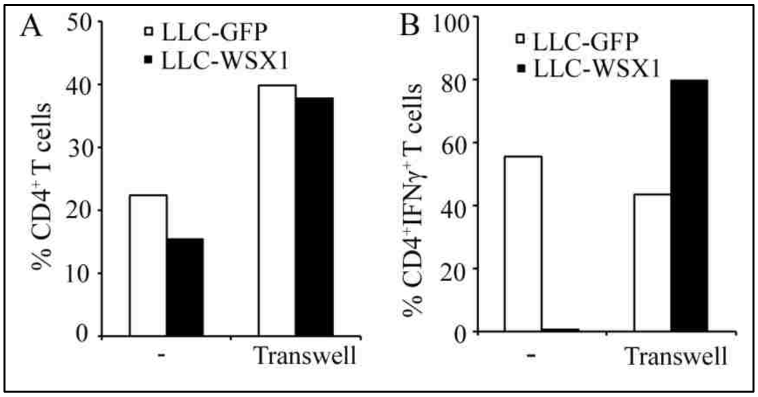


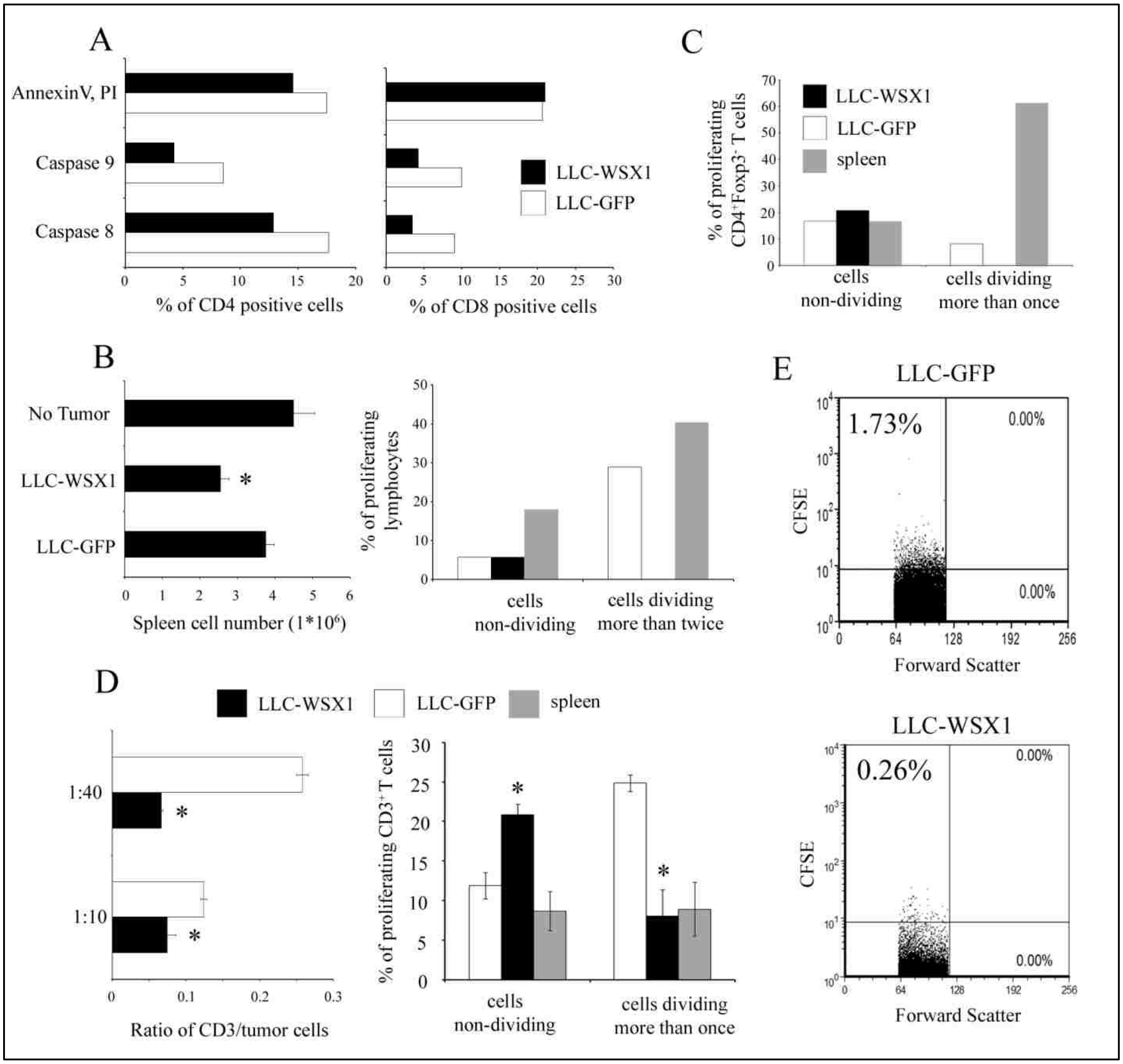
Figure 3.6. WSX1 induces immunosuppression in a cell contract-dependent manner. *A*, detection of the effect of LLC-GFP or LLC-WSX1 tumor cells on CD4$^+$ T cells in the presence or absence of a transwell. Cells were seeded and analyzed similarly as in point 3.5*A*. *B*, detection of the effect of LLC-GFP or LLC-WSX1 tumor cells on IFNγ expression in CD4$^+$ T cells in the presence or absence of a transwell. Cells were seeded and analyzed similarly as in point *B*.

cells than control cohorts (Fig. 3.7*A*), suggesting that WSX1 affects T cells proliferation rather than apoptosis of T cells.

As a measure of T cell proliferation, CFSE –labeled splenocytes were co-incubated with tumor cells. Cell proliferation was determined on both lymphocytes and CD4$^+$Foxp3$^-$ populations. Since we cannot rule out the possibility that tumor cells affect T cell proliferation via APC cells, total splenocytes rather than purified T cells were used. Analysis of CFSE staining showed that lymphocyte cell proliferation was affected, where 29% of lymphocytes in the presence of LLC-GFP had divided more than twice, while in the presence of LLC-WSX1, none of the cells had divided more than twice. Similar results were obtained for CD4$^+$Foxp3$^-$ cells, as a larger percentage of non-dividing cells were seen in presence of LLC-WSX1 than LLC-GFP tumor cells (21% vs. 16%, Fig. 7*C*). More importantly, only 10% had divided more than once in the presence of LLC-GFP, and none had divided in the presence of WSX1.

This data further supports the idea that WSX1 affects T cell proliferation but does not address the question of whether WSX1-tumors affect T cell proliferation directly or indirectly

Figure 3.7. WSX1 directly affects T cell proliferation. *A*, comparison of apoptosis markers in CD4 and CD8 T cells when co-incubated with LLC-GFP or LLC-WSX1 tumor cells. Splenocytes and tumor cells are co-incubated as in Fig. 5*A*, and then cells gated for $CD4^+$ (*left*) or $CD8^+$ (*right*) markers were analyzed for Caspase 8, Caspase 9, or double positive cells in both Annexin V and propidium iodine (PI) expression. *B*, comparison the differences in splenocytes cell numbers and the differences in lymphocyte proliferation in the presence of either LLC-GFP or LLC-WSX1 tumor cells. Splenocytes and tumor cells were co-incubated as in Fig. 5*A*, and viable splenocytes were counted via tryptophan blue exclusion (*left*). In the right panel, CFSE-labeled splenocytes were seeded as in Fig. 5*A*, and then cells gated for lymphocytes (based on size) were analyzed for CFSE dilution. *C*, CFSE-labeled splenocytes were seeded as in Fig. 5*A*, and cells gated on expression of CD4 but excluded from FOXP3 expression were analyzed for CFSE dilution. *D*, comparison of $CD3^+$ T cell numbers and proliferation in the presence of either LLC-GFP or LLC-WSX1 tumor cells. $CD3^+$ T cells were purified, labeled with CFSE, co-incubated with either LLC-GFP or LLC-WSX1 at a 1:10 or 1:40 tumor to T cell ratio, and then analyzed for CFSE dilution. *E*, Purified $CD3^+$T cells were CFSE- labeled and injected systemically into LLC-GFP or LLC-WSX1 tumor-bearing mice. 6 days post-transfer, CFSE-positive lymphocytes within the tumor microenvironment were analyzed. *Points*, mean; *bars*, SE. *, $P < 0.05$.


A
AnnexinV, PI
Caspase 9
Caspase 8
0 5 10 15 20
% of CD4 positive cells
0 5 10 15 20 25 30
% of CD8 positive cells
LLC-WSX1
LLC-GFP
C
% of proliferating CD4+Foxp3- T cells
70 60 50 40 30 20 10 0
LLC-WSX1
LLC-GFP
spleen
cells non-dividing
cells dividing more than once
B
No Tumor
LLC-WSX1
LLC-GFP
*
0 1 2 3 4 5 6
Spleen cell number (1*10^6)
% of proliferating lymphocytes
50 40 30 20 10 0
cells non-dividing
cells dividing more than twice
E
LLC-GFP
1.73%
0.00%
0.00%
CFSE
Forward Scatter
LLC-WSX1
0.26%
0.00%
0.00%
CFSE
Forward Scatter
0 64 128 192 256
D
LLC-WSX1
LLC-GFP
spleen
1:40
1:10
*
0 0.1 0.2 0.3
Ratio of CD3/tumor cells
% of proliferating CD3+ T cells
30 25 20 15 10 5 0
cells non-dividing
cells dividing more than once

via another cell type such as APC. Thus, purified CFSE-labeled CD3$^+$ cells were directly co-incubated with tumor cells expressing either GFP or WSX1 at different ratios. After 48 hours, the ratio of CD3$^+$ cells/tumor cells was significantly reduced by WSX1 (Fig. 3.7*D*, *left*). Moreover, in LLC-WSX1, 20% of CD3$^+$ cells were non-dividing, whereas in LLC-GFP only 12% of these cells were non-dividing (Fig. 3.7*D*, *right*). Likewise, the number of cells dividing more than once was significantly reduced by WSX1 (25% vs. 8%, $p<0.05$), further demonstrating that WSX1 expression in tumor cells directly affects T cell proliferation. Such inhibition was also shown *in vivo*, as systemic transfer of CFSE-labeled CD3$^+$ T cells was approximately 10 fold lower in the tumor microenvironment of LLC-WSX1 than in control GPF tumor-bearing mice 6 days post-transfer (Fig. 3.7*E*).

DISCUSSION

According to the CDC, lung cancer remains the major cause of cancer-related deaths in United States. In 2006, the number of deaths of cancer patients related to lung cancer was larger than the sum of breast, colon, and prostate cancer combined. Such phenomenon is partly due to the fact that these tumors are highly unresponsive to immunological-based therapies (28, 29). This trend is partly explained by the high immunosuppressive tumor microenvironment in these tumors (30, 31), which in return induces a tolerant state and poor ability to respond to any immune-based therapies. Revealing these suppressive mechanisms is critical for developing effective immune therapies in treating both primary and metastatic tumors by reversing the tumor tolerance.

Several immune suppressive molecules secreted or expressed from tumor associated macrophages, myeloid-derived suppressor cells, tolerogenic DC cells, Treg cells, and tumor cells in the tumor microenvironment may be responsible for the suppressive effect (32-34). For the first time, this study shows that WSX1 expression in tumors stimulates immune suppression (Fig. 3.1) and this receptor might be a new target in immune therapy to reverse tumor tolerance.

The underlying mechanism by which the known immune suppressive molecules regulate immune suppression have been previously explored. It was found that upregulation of PD-L1 in multiple tumors, which, upon binding to its receptor expressed in activated T cells, induces apoptosis, anergy, and exhaustion of effector or memory T cells (35-37). Expression of indoleamine 2,3-dioxygenase (IDO) by tumor cells is another such mechanism (38, 39). Upregulation of this tryptophan-degrading enzyme induces tryptophan depletion in the local surroundings, which severely downregulates different facets of immune responses, such as effector T cell population, DC cells, and NK cells. Moreover, tumor cells induce expression of co-stimulatory receptors such as CD80 and CD86, inducing proliferation of not only effector T cells but also and more importantly induction and maintenance of Treg cells via CTLA-4 (40).

In this study, WSX1 plays a crucial role in regulating the communication between tumor cells and immune cells. This communication is via a direct interaction between WSX1 expressing tumor cells and T cells, resulting in inhibition of IFNγ production and T cell proliferation (Fig. 3.4), and this mechanism occurs both in vitro and in vivo (Fig. 3.4 and 3.5). The communication between WSX1 expressing tumor cells and T cells is seen not only in an aggressive lung cancer model, LLC, but also in melanoma cell line AGS, indicating that WSX1 is as important as other immune suppressive molecules in regulating anti-tumor immune responses. Since most human tumor cells express high levels of WSX1 (16), such an immune-suppressive mechanism may be very common in human patients and needs to be tested in future studies.

Similar to these receptors, WSX1 attenuates the immune response in the tumor microenvironment and in other distal organs such as the spleen, characterized by lower numbers of T cells and NK cells. This study further confirms that WSX1 inhibits T cell proliferation *in vitro* and *in vivo*. WSX1 inhibits the number of CFSE-positive T cells in the tumor microenvironment. The decreased presence of CFSE-positive T cells in the tumor microenvironment is not due to T cell infiltration in tumors, as the number of CFSE-labeled T cells after 16 hours post-injection were similar between GFP and WSX1- positive tumors (data not shown). In these tumor models, the function of this receptor is directly associated with immune suppression but does not affect the intrinsic pathways within tumor cells. In contrast, tumor cells which overexpress WSX1 grow slower in multiple tumor models and have lower clonogenic ability when compared to control cohorts (Fig. 3.1) (16). Additionally, WSX1 cannot promote tumor growth in immune-compromised mice which once again confirms the ability of WSX1 to influence immune tolerance (Fig. 3.4*A*).

A hallmark of an effective immune response is the ability of T and NK cells to effectively secrete IFNγ. Secretion of IFNγ plays a key role in macrophage activation, inflammation, and host defense against intracellular pathogens, T helper 1 (Th1) cell responses, tumor surveillance, and immunoediting (41). WSX1-positive tumors cells have an inhibitory affect on the number of T cells and the percentage of T and NK cells secreting IFNγ, similar to the effect of CTLA and PD-1 ligands (35, 42, 43). Indeed, such inhibition is also observed in both tested tumor models.

WSX1 expressing tumor cells induce tumor tolerance via direct cell-contact inhibition. The ability of WSX1 to reduce the number of T and NK cells and cytokine production is reversed in the presence of transwell barrier. Such an observation suggests that WSX1 does not inhibit T cells proliferation via expression of IL10 or TGFβ directly but needs another receptor on immune cells. That the physical separation between tumors and immune cells reverses tolerogenic properties of WSX1 and tumor cells directly affect the proliferation of T cells suggests the existence of another inhibitory receptor on activated T cells. Although we cannot exclude that WSX1 could affect the expression of either CTLA or PD-1 ligands in tumor cells, which in turn act on T cells, this hypothesis is not as plausible since even WSX1 lacking the intracellular domain promotes tumor growth. Besides directly affecting T cells, NK cell number and the ability of these cells to produce IFNγ are affected by WSX1. In SCID mice the NK cell population is intact and WSX1's ability to promote tumors is abolished (Fig. 3.4), which suggests that T cells are the primary cells affected by WSX1. Conversely, the effect on NK cells might be indirect via T cells or another cell type such as tolerogenic APC cells or Treg cells, both of which play crucial roles in tolerance and cancer (44-46). So, finding out how WSX1 affects T cells and their subsequent roles on other cells would be of crucial interest in developing effective strategies to break tolerance.

Similar to our previous work, we again see that WSX1 promotes tumor growth independently of IL27 (16). Tumor cells expressing a truncated version of WSX1 that lacks the intracellular domain, which renders IL27 unable to signal into the tumor cells, grow much faster than tumor cells containing full-length WSX1. This discovery reaffirms other findings that IL27 inhibits tumor growth (22). To further exclude the concept that WSX1 promotes tumor growth by acting as a sequestering receptor, therefore limiting IL27 from signaling into the host, we used TCCR$^{-/-}$ mice so IL27 is unable to signal into immune cells. Even in the absence of IL27 signaling in immune cells, WSX1 expression in tumors retains the ability to promote tumor growth. Others have also found that WSX1 does promote tumor growth in leukemia tumor models; however, this was the case only if the JAK2 was mutated in these tumors (47). Under this special condition, WSX1 was acting as a scaffold receptor to maintain activation of JAK2. This mechanism does not explain our observation, as the truncated WSX1 receptor enhances tumor growth, regardless of whether it can serve as a scaffold receptor or not for JAK2. Also, WSX1 attenuates cell proliferation without IL27. In figure 6, direct co-incubation between WSX1-positive tumor cells and purified CD3$^+$ T cells inhibits T cells proliferation, whereas neither T cells nor tumor cells express IL27. In summary, WSX1 has a function in tumor biology independent of IL27; therefore, caution should be exerted in interpreting any data from TCCR$^{-/-}$, as multiple functions that were previously attributed to IL27 might actually be applied to WSX1 itself.

Downregulation of the NKG2D receptor in immune cells is another immune-evasive mechanism: immunosuppressive CD4$^+$NKG2D $^+$ cells or shedding of NKG2D ligands leads to immune evasion via downregulation of NKG2D receptors in NK$^+$ or CD8$^+$ cells (24, 25, 27). Previously, our group identified a connection between WSX1 and NKG2D ligand expression in two independent tumor models, AT84 and TC1 (16). There is no downregulation of the NKG2D receptor in NK$^+$ and CD8$^+$, and there is a higher percentage of NKG2D positive NK$^+$ and CD8$^+$ cells. WSX1 did not change immunosuppressive CD4$^+$NKG2D$^+$ cells, and, more importantly, the levels of NKG2D ligands are similar between GFP-expressing and WSX1expressing LLC tumors. Moreover, there is higher expression of both extracellular and total NKG2D in lymphocytes in WSX1-positive tumor bearing mice.

Identifying the mechanism which determines whether WSX1 inhibits tumor growth via induction of the NKG2D ligand, as in TC1 and AT84 tumors, or inhibits T cell proliferation and cytokine production resulting in immune tolerance, as in LLC and AGS, is of great interest. Of further importance would be to find out how WSX1 interacts with T cells, and, specifically, which mechanisms this receptor uses to limit T cell population expansion. Understanding such regulatory mechanisms may provide new targets for potential therapies in multiple fields such as cancer, anti-aging, autoimmune diseases, and transplant acceptance. In summary, this report identifies for the first time that WSX1, independently of IL27, promotes tumor growth and immune tolerance in multiple tumor models via direct inhibition of T cell proliferation and cytokine production both in the tumor microenvironment and at distal sites.

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CHAPTER 4

CONCLUDING REMARKS

OVERALL SUMMARY OF FINDINGS

It is well known that a strong immune response inhibits tumor growth and metastasis. Expression of pro-inflammatory cytokines in the tumor microenvironment such as IL12 and IFNγ inhibit tumor growth in a variety of mouse and human tumors models, while expression of anti-inflammatory cytokines such as IL10 and TGFβ are associated with disease relapse and progression (1-4). Therefore, elucidating such immune suppressive mechanisms is critical in developing effective immune therapies in treating tumors. The interleukin (IL) 27 receptor WSX1 is expressed in immune cells and induces an IL27-dependent immune response (5). Opposing this conventional dogma, our initial results revealed a much higher level of WSX1 expression in multiple types of epithelial tumor cells when compared to normal epithelial cells, suggesting a role for WSX1 in tumor development, thus a possible target in cancer immune-therapy (6).

In chapter 2, we reveal that expression of exogenous WSX1 in epithelial tumor cells suppresses tumorigenicity *in vitro* and inhibits tumor growth *in vivo*. Different from the role of WSX1 in immune cells, the antitumor activity of WSX1 in epithelial tumor cells is independent of IL27 signaling and is mainly dependent on NK cell surveillance. Deficiency of either the IL27 subunit Epstein–Barr virus-induced gene 3 (EBI3) or WSX1 in the host animals had no effect on tumor growth inhibition induced by WSX1 expression in tumor cells. Expression of WSX1 in epithelial tumor cells enhances NK cell cytolytic activity against tumor cells, while the absence of functional NK cells impairs the WSX1-mediated inhibition of epithelial tumor growth. The underlying mechanism by which WSX1 expression in tumor cells enhances NK cytolytic activity is dependent on upregulation of NKG2D ligand expression. Our results reveal an IL27-

independent function of WSX1—sensitizing NK cell-mediated antitumor surveillance via an NKG2D-dependent mechanism.

In chapter 3, further analysis of WSX1 expression in tumors reveals a paradox: expression of WSX1 in Lewis Lung Carcinoma (LLC) cells enhances tumor growth *in vivo*, but it reduces tumor cell proliferation and clonogenic ability of these cells *in vitro,* which is also observed in the TC1 and AT84 cell lines. The phenomenon in LLC tumors is dependent on suppression of the immune response, as WSX1-positive tumors inhibit T cell proliferation and the ability of T and NK cells to produce IFNγ. Such observations were confirmed both *in vivo* and *in vitro*. This effect is initiated via direct contact between tumor and immune cells. Such immune suppression is mediated independently of the NKG2D and IL27 pathway: WSX1 expression does not induce NKG2D ligand expression, and the lack of IL27 signaling in either the tumor or the host does not reverse the ability of WSX1-positive tumors to promote tumor growth. Thus, our discovery reveals a new channel through which cells and host immune cells communicate to promote tumor development.

SIGNIFICANCE OF RESEARCH

In 1909, Paul Aldrich predicted that the immune system would inhibit the initiation of tumorigenesis; otherwise, the rate of tumorigenesis would be much higher than the current rate. One of the key immune populations in immunosurveillance consists of NK cells, as they possess the ability to “see” and destroy the transformed cells; therefore, harnessing the ability of these cells to destroy tumors is an attractive strategy in cancer patients. The NKG2D-NKG2D ligand pathway is a dominant pathway that makes tumor cells visible to the NK cell attack (7). Modulating the expression of NKG2D ligands in the tumor cells will enhance the ability of NK cells to eradicate tumors. Thus, understanding how these ligands are upregulated in tumors will lead to better strategies in developing anti-cancer therapies.

In the past decade, however, evidence has shown that the complex relationship between tumors and immune cells is not a one way street (8, 9). Further iresearch into this relationship has shown that immune cells play a crucial and necessary role in tumor progression. Indeed, several reports have shown that although immune cells can destroy transformed cells, certain immune cells in the tumor microenvironment can also promote escape of tumor cells from the immune system, and/or downregulate the transition from a pro-inflammatory immune response into a tolerant state (10). Multiple tumors have adapted such pathways to downregulate the immune response, thus facilitating tumor growth and immune evasion. Several regulatory mechanisms include: upregulation of PD-1L, IDO, galectin 3, co-stimulatory receptors CD80 and CD86, downregulation of MHC class I and so forth (11-16); .

So far, no current link between WSX1 expression in tumors and its affect on the tumor microenvironment has been reported in the literature. For the first time we introduce WSX1 expression in tumors as a modulator of immune cells. Overexpression of WSX1 in tumors has a complex role: WSX1 expression in TC1 and AT84 epithelial tumors inhibits tumor growth via an NKG2D-dependent mechanism, while in LLC and AGS tumors WSX1 promotes tumor growth and induces immune tolerance via a T cell dependent mechanism. Several genes have such a complex nature in carcinogenesis. TGFβ is a clear example as it was definitively shown in multiple models of skin carcinogenesis to inhibit papilloma growth, while expression at a later stage promotes rapid epithelial-mesenchymal transition and metastasis (17-19). Similarly, SIRT1 has been shown to downregulate p53 activity and act as a tumor suppressor in cells where p53 is mutated (20)

The role of NKG2D ligand expression in tumors as a sensor of transformation has been well documented in several chemically-induced tumors; however, other than ATM/ATR pathways, additional mechanisms exist that detect alterations in tumor cells (21), (22). Here we show another such mechanism and link WSX1 expression in tumor cells to NKG2D ligand expression. Similar results are shown in two independent tumor models, suggesting that WSX1 indeed has such a role. Thus, tumors expressing WSX1 could translate into a good tumor prognostic marker for NK cell therapy.

T cell anergy is a common occurrence in tumor microenvironment (23). As tumors progress to more malignant stages, the cells go through a journey that requires the acquisition of intrinsic and extrinsic characteristics that promote tumor growth. One of such mechanisms to induce T cell anergy is upregulation of PD-1L or IDO by tumors cells (12, 14, 15). Here, we describe another such event and we show that WSX1 induces immune suppression in both LLC and AGS tumor models via reduction of T and NK cells at the tumor microenvironment and at distal sites. Therefore, together with previously mentioned immune-evasion mechanisms such as upregulation in tumors of PD-L1 or IDO expression has on T cell function, WSX1 shows similar properties in immune evasion. Moreover, since multiple human samples have high expression of WSX1, it will certainly remain a focus for therapeutic purposes.

In addition to the fact that WSX1 expression affects immune cells and the tumor microenvironment, these studies have made significant contributions to understanding IL27 biology. In both chapters 2 and 3, we present clear evidence that although WSX1 is a receptor for IL27, this receptor has functions independent of IL27. As WSX1 expression in tumor cells shows that it inhibits T cells proliferation directly without the need of IL27; however, caution should be exerted in interpreting any data from TCCR$^{-/-}$, since multiple functions that were previously attributed to IL27 might actually belong to WSX1. Indeed, many different phenotypes are observed between EBI3$^{-/-}$ (IL27 subunit knockout) and TCCR$^{-/-}$ mice (24, 25). Although many of these differences could be attributed to either IL35 or p28/CLF complex, WSX1 could also attribute to these differences. Therefore, better knockout models are needed to sort out the differences between the subunits and the receptors of IL12 family.

FUTURE DIRECTION

Although we believe that our aforementioned studies have made significant contributions in identifying novel pathways through which tumors cells affect the tumor microenvironment, further studies are required to better understand the underlying mechanisms of the molecular switch for WSX1 to inhibit tumor growth in certain tumors, such as TC1 and AT84, while enhancing tumor growth in others, such as LLC, and AGS. Our data shows that tumors expressing WSX1 affect not only T cells but also NK cells. This observation prompts us to ask whether WSX1-positive tumor cells have a similar effect on other immunosuppressive cells such as myeloid-derived suppressor cells, tolerogenic dendritic cells, and tumor associated macrophages. Moreover, the intricacy of cross-talk among immune cells creates further interest in investigating how T cells in the presence of WSX1-positive tumors affect other cell populations, such as DC, NK cells, MSDC, and immune inhibitory factors such as IL10, TGFβ, VEGF and so forth.

WSX1 function as a tumor promoter needs cell-to-cell contact with immune cells and tumor cells, but the downstream effects on T cells are unclear, suggesting that WSX1 has a corresponding receptor in T cells, and signaling via this receptor induces T cells tolerance. Further studies could explore the identity of this receptor, and how this receptor affects TCR signaling, SHP-1 and SHP-2 phosphatases, and IL2 production from T cells. In summary, better understanding of how WSX1 expression affects immune cells and tumor growth will lead to better and more effective therapies for cancer patients.

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APPENDIX

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Denada Dibra was born in Shkoder, Albania. After attending the first semester of high school in Albania, she decided to go abroad as an exchange student. She received her high school diploma from Minden High School in May 2000, and decided to further pursue higher education in the United States. In December 2004, she received her bachelor's degree from Louisiana State University in biochemistry with a minor in chemistry. While attending her undergraduate courses, she decided to take a graduate course in cell and molecular biology offered by the Department of Comparative Biomedical Sciences at the Louisiana State University School of Veterinary Medicine. After hearing all the interesting research being done at this department, she decided to apply to graduate school. In January 2005, she was fortunate to be accepted into the doctoral program in the Department of Comperative Biomedical Sciences, under the mentorship of Dr. Shulin Li, to pursue her interest in cancer research. She will receive her Doctor of Philosophy degree in spring 2010.