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Innate Immunity in Interleukin-33-Mediated Tumor Suppression: Competing Roles of Natural Killer Cells and Type 2 Innate Lymphoid Cells

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Abstract

Interleukin 33 (IL-33) is a complex inflammatory cytokine with diverse functions. In the context of cancer development, depending on the model, the implementation and dosage, and the immune cells involved, IL-33 can elicit dramatically different outcomes. Recent studies, including our own, have explored definitive contributions of the adaptive immunity in IL-33-mediated antitumor effects. Innate immune involvement, however, has been poorly characterized. Utilizing $Rag1^{-/-}$ mice (lacking T and B lymphocytes), we show that either systemic administration of recombinant IL-33 or ectopic expression of IL-33 effectively inhibits tumor growth independent of adaptive antitumor immunity. We further demonstrate that IL-33-mediated antitumor effects depend on expansion and activation of NK cells. Additionally, IL-33 promotes the intratumoral expansion of active type 2 innate lymphoid cells (ILC2s) via its receptor, ST2. The role of ILC2s in cancer is almost entirely unexplored. Interestingly, IL-33-treated ILC2s exacerbated tumor growth. These ILC2s directly inhibited NK activation and cytotoxicity. IL-33-induced ILC2 activity coincided with greater expression of the immunosuppressive ecto-enzyme CD73. Absence of CD73 expression in ILC2s resulted in increased activation levels in NK cells, offering a potential mechanism by which ILC2s might suppress NK cell-mediated tumor killing. Thus, our data reveal a novel contribution of IL-33-induced ILC2 to tumor growth by weakening NK cell activation and tumor killing, regardless of adaptive immunity.

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Finally, I'd like to dedicate this work to the many people in my life—and their loved ones—who have had to contend with cancer: to a cousin, a grandmother, a grandfather, an uncle, a mentor,

and others who have been lost; to an aunt who continues to fight; and to my father who has made it safely through his struggles. Cancer touches too many lives, and stories of struggle and loss are too frequently heard. Progress is always being made to change that, but never feels fast enough. Still, this tiny contribution of mine is dedicated to you.

Table of Contents

Figures

Chapter 1: Introduction and Background

1.0 Introduction

The immune system stands as an immensely refined and complex protective mechanism capable of distinguishing self from altered or non-self. This capability, coupled with the means to effectively eliminate 'non-self', poises the immune system to serve as an effective tool in mitigating cancer development. This potential has been increasingly appreciated in recent years as the field of cancer immunology continues to rapidly grow.

The focus of cancer immunology is two-fold—(1) to explore the dynamic interactions between the immune system and cancer that can ultimately lead to immune escape and (2) to develop interventions that can manipulate the immune system to foster effective antitumor responses. The results of the substantial efforts made towards these goals have led to major advancements in our understanding of cancer-immune interactions which has inspired the development of many new therapeutic strategies to treat clinically-apparent tumors. This paper serves to review the major topics in cancer immunology while providing new insights on a particular therapeutic strategy. Additionally, this work highlights a novel role for a newly identified member of the innate immune system in the context of cancer development.

1.1 Cancer and the immune system

Cancer initiation

Currently, cancer is the second leading cause of death in the United States, falling just behind heart disease. Malignant neoplasms were responsible for an estimated 595,690 deaths in 2016, accounting for approximately 20% of all death in the United States that year.[1, 2] With another 1.7 million new cases are estimated to be reported this year[3]—representing the latest in an ever-increasing rate of diagnoses—cancer has understandably maintained its status as a major focus of biomedical research for decades.

The difficulty in developing effective treatments for this persistently common yet lethal disease lies in its incredible diversity—there are more than 100 different types—and the many characteristics it can acquire to circumvent current treatment strategies. Cancer occurs when normal cells become transformed and break from their normal physiological programing. This transformation is driven by the acquisition of various mutations, which can occur via environmental means such as ultraviolet light, diet, and tobacco smoke. Viruses such as Epstein-Barr, human papilloma, hepatitis B and C, and others can similarly disrupt cell functions by integrating their genetic material into a host cell's DNA. Genetic irregularities may also be inherited or occur randomly during DNA replication.

These mutations, which can occur indiscriminately throughout the genome, run the risk of disrupting the homeostatic equilibrium between cell growth and death, ultimately eliciting malignant behavior. Loss of genes such as p53 and BRCA1 and 2, which are involved in monitoring and repairing DNA, can permit the accumulation of mutations and further promote genetic instability. Disruption of genes involved in controlling apoptosis like Bcl-2 may prevent a

cell from properly self-terminating. Concurrently, changes in cell growth signaling, such as the oncogenic activation of K-ras, may force a cell to ceaselessly enter cell division, leading to uncontrollable proliferation. Other acquired 'hallmarks' of malignancy include upregulation of angiogenesis, alterations in metabolism to accommodate rapid growth, and promoting extravasation and metastasis.

As mentioned before, such cancerous traits depend on the random acquisition of mutations in genes governing these functions. It requires a perfect storm of genetic mutations that can ironically be thought of as an aberrant form of natural selection; the transformed cells gain new traits, improving their fitness, and enhancing their survival. However, instead of reaching an equilibrium with their environment, the malignant cells continue to grow uncontrollably, ultimately reaching an unfortunate outcome for them and their host.

Immune involvement

A more recently accepted hallmark of cancer is the ability to evade the immune system.[4] The inclusion of this hallmark acknowledges the immune system as a major hurdle cancer must overcome to achieve malignancy. The immune system possesses an immense complexity with which it can defend the body against countless threats. With a large plethora of different types of immune cells, many displaying great plasticity and a number of subsets, and a nearly limitless potential repertoire of different antibodies and T cell receptors, the body's immune system stands poised to repel seemingly innumerable threats, including cancer.

The concept of the immune system's involvement in cancer development can trace its origins back to Rudolf Virchow and John Hughes Bennet. In the mid 1840s, they correctly characterized leukemia by an increase in white blood cells, giving rise to the name of the disease. For many

years after, the involvement of the immune system in cancer was merely theorized, with most researchers remaining skeptical. Only in the last few decades, with advances in understanding of immunology and cogent clinical evidence, has the concept gained mainstream acceptance. Transplant patients receiving immunosuppressants to avoid graft rejections are four times as likely to develop cancer.[5] Patients with human immunodeficiency virus are at far greater risk of developing certain cancers: 500 times more likely to develop Kaposi sarcoma, 12 times as likely to be diagnosed with non-Hodgkin's lymphoma, and 3 times more likely for cervical cancer.[6] Patients with primary immunodeficiencies impacting various immune components also demonstrate elevated susceptibility to malignancies.[7]

1.2 Antitumor immunity

Immunosurveillance and tumor recognition

The theory of immunosurveillance was introduced by Frank Burnet and Lewis Thomas (independently) in 1970 stating that the immune system is capable of recognizing and eliminating malignant cells in the body.[8] The hypothesis was released in an attempt to explain the involvement of the immune system in mouse models[9] and spontaneous regression of some tumors in humans. Researchers have since discovered a variety of ways in which the immune system can protect against malignancy.

Tumor cells and associated stromal cells can express a wide variety of proteins that function as antigens including mutated proteins, fusion proteins, developmentally and tissue-restricted proteins, as well as tumor-selectively over-expressed proteins, all termed as tumor-associated

antigens (TAAs).[10] When presented in the context of MHC, TAA-specific lymphocytes can read these TAAs as a form of 'non-self' and mount a response against the tumor cells that express them. Some TAAs may be conserved with general tumor types. Prostate-specific membrane antigen PSMA (prostate-specific membrane antigen)[11] and MART-2 (melanoma Ag recognized by T cells-2)[12] are upregulated on prostate tumor cells and in melanoma respectively and offer a targetable antigen by cytotoxic T lymphocytes.

Even in the absence of targetable TAAs, innate immune cells such as NK cells can identify tumors via other means. Recognition of tumor cells by cytotoxic T cells requires that TAAs be presented on the surface of the tumor cell via MHC. However, tumors can frequently downregulate MHC expression to avoid detection. MHC expression is typically impaired in many tumors.[13] NK cells possess inhibitory receptors that bind MHC. When NK cells engage with cells that lack functional MHC, their cytotoxic machinery is engaged, resulting in the release of cytotoxic granules, eliminating cells with impaired MHC expression.[14]

Tumor cells can be thought of chronic sources of inflammation and injury, and commonly express stress-induced molecules and danger signals. Recognition of these molecules by innate immune cells like NK cells, macrophages and dendritic cells elicits cytolytic behavior that can help eliminate tumor cells.[15] Stress-induced expression of MICA by epithelial tumor cells can directly bind to receptor NKG2D on NK cells, prompting their activation and promoting tumor cell-killing.[16]

Mediators of antitumor immunity

Dendritic cells (DCs) are antigen-presenting cells (APCs) that serve as a bridge between the innate and adaptive immune system. DCs are a diverse population of APCs responsible for presenting

antigens to other immune cells and stimulating immune responses. For several years, DCs have been known to be involved in immune responses to cancer and their infiltration of tumors typically correlates with reduced tumor growth.[17] DCs are early initiators of antitumor responses, and their removal in early stages of cancer accelerates tumor growth.[18] After phagocytosis and processing of the elements in the tumor environment, DCs present tumor-associated antigens (TAA) to antigen-specific naïve $CD4^+$ T cells via MHC II. Both the presentation of TAAs and secretion of cytokines including IL-2 and IL-12 skews T cells toward a Th1 phenotype and results in a robust antigen-specific response against TAA-expressing tumor cells. Activated type 1 $CD4^+$ T helper cells (Th1) produce cytokines such as IFN-γ and TNF-α which in turn help arrest tumor growth and promote the activity of $CD8^+$ cytotoxic T lymphocytes (CTLs) and NK cells.[19, 20, 21] CTLs can directly eliminate tumor cells via interactions with their target TAAs presented by tumor cells via MHC I. This interaction prompts the CTLs to release of granules containing cytolytic molecules such as perforin and granzymes.

While Th1-mediated antitumor immunity elicits NK cell responses, NK cells belong to a group called innate lymphoid cells that contribute to the first line of defense in antitumor immunity. The significance of NK cells in attenuating tumor growth has been known for decades and is now becoming increasingly appreciated in clinics. [22, 23] As a part of the innate immune system, NK cells possess a host of natural cytotoxicity receptors (NCRs) that prime them for rapid cytolytic responses. These include NCRs like NKp46, NKp44, and NKp30, for which the ligands are weakly expressed by normal cells but are upregulated in stressed and diseased cells.[24] Another activating receptor, NKG2D, recognizes stress-induced expression MICA and MICB, which are class I-like molecules frequently found on cancerous and virally infected cells.[16, 25] NK cells also lyse cells

opsonized by antibodies via detection by CD16, a low-affinity receptor for the Fc portion of immunoglobulin (FcγRIII).[26, 27]

1.3 Immune evasion

Immunoediting

The relationship between the immune system and cancer is dynamic. While many transformed cells may be eliminated by the immune system before ever truly becoming cancerous, some ultimately progress beyond the immune system's ability to control. The theory of immunoediting was introduced by Robert Schreiber to expand on the idea of immunosurveillance to explain this phenomenon.[28] In earlier stages of tumor development—described as the 'elimination' phase—tumor cells may be more immunogenic. This is similar to the idea of immunosurveillance which claims NK cells, $CD4^+$ and $CD8^+$ T cells are able to effectively target and remove tumor cells. Next, however, during the 'equilibrium' phase, tumors begin to acquire resistance to immune targeting. Variant tumor cells may arise that exhibit impaired expression of MHC molecules or reduced production of tumor-associated antigens or may acquire features that directly suppress immune effector cells. Over time, tumors may be "edited" by the immune system by permitting the outgrowth of these resistant cells. Again, this phenomenon seems to resemble natural selection, where selective pressure by the immune system favors the growth of the "fittest" cells. Over the course of this equilibrium phase, the resistant variant cells eventually 'escape' immune control and become malignant tumors resistant to immune influence.

To summarize, the theory of immunoediting suggests highly immunogenic cancer cells are effectively eliminated, while weakly immunogenic cells grow under less scrutiny by the immune system and eventually give rise to immune-resistant tumors that escape immune control. Evidence for this theory is provided by Kim et al. (2007) and Teng et al. (2008).[29, 30] Cancer cells from immunodeficient mice failed to develop secondary tumors when transplanted in immunocompetent syngeneic mice. Conversely, cancer cells from immunocompetent mice were just as likely to develop secondary tumors when transferred into either immunodeficient or immunocompetent mice.

Tumor-mediated immunosuppression

The tumor microenvironment includes the tumor cells, stromal cells such as fibroblasts and macrophages, vasculature, and extracellular components (i.e. enzymes, cytokines and growth factors). All of these elements can be manipulated by cancer cells to foster an environment that favors tumor growth while simultaneously suppressing antitumor immune responses.

Tumors may express immunosuppressive molecules such as PD-L1 or CTLA-4 to impair lymphocyte activity. These molecules are part of a tolerance mechanism to prevent autoimmunity. Engagement of PD-L1 with its receptor, PD-1, found on lymphocytes, impairs their proliferation and activity.[31] PD-L1 and CTLA-4 are expressed by many tumors and strongly correlate with weaker antitumor immune responses and poor prognoses.[32] Similarly, tumor cells may also express proapoptotic ligands such as FasL and TRAIL.[33] Engagement of these ligands with their receptors on T cells can drive the lymphocytes to self-terminate and arrest tumor rejection. Some tumors produce enzymes which can deplete the surrounding environment of metabolic substrates and further attenuate immune effector functions. Indoleamine 2,3-dioxygenase (IDO) is a tryptophan-

consuming enzyme produced by many tumors and is associated with fewer immune infiltrates with high proportions immunosuppressive T regulatory lymphocytes.[34]

Fibroblasts, together with a number of innate immune cells including macrophages, secrete various extracellular components to promote wound healing. Cancers resemble non-healing wounds in that they can elicit wound-healing responses. Through the secretion of growth factors and cytokines like VEGF, TGF-β, HGF, IL-6, and SDF-1, cancer-associated fibroblasts promote inflammation, angiogenesis, invasion, metastasis, and even oncogenesis.[35] The contributions of stromal cells are instrumental for tumor development. Inhibition of fibroblast activation protein (FAP) from stromal cells has been shown to severely impede tumor stromagenesis and growth.[36]

Inflammatory conditions promote the recruitment of activated immune cells. One could predict that increased immune activity might correlate to a more potent antitumor response. In actuality, through the recruitment of immunoregulatory cells and the upregulation of immune checkpoint receptors, tumors can foster highly immunosuppressive environments. T regulatory cells (Tregs) promote self-tolerance and help mitigate auto-immune diseases.[37] Stimulated by TGF-β and IL-2, populations of $CD4^+$ $Foxp3^+$ Tregs have been found to be heavily increased in many malignancies and frequently correlate with unfavorable patient outcomes.[38, 39, 40] Through direct contact and secretion of cytokines like IL-10, Tregs suppress inflammatory and cytotoxic capabilities of other immune cells such as NK cells and impair the maturation and function of dendritic cells.[37] Tregs also express CTLA-4 which can block CD80 and CD86 engagement in DCs, impairing typical costimulatory interactions needed for priming cytotoxic T cells.[41]

Phenotypic shifts in dendritic cells can also be seen when tumors break equilibrium with their environment and begin rapidly growing. Elevated presence of soluble factors such as TGF-β and IL-10 can elicit a tolerogenic phenotype in DCs.[42] In one study, DCs initially appeared to be

instrumental in mitigating the growth of ovarian cancer, but over time eventually adopted an immunosuppressive nature that facilitated tumor expansion.[18] Macrophages also frequently undergo a phenotypic change during tumor development. Classically activated macrophages, or M1 macrophages, exhibit antitumor properties. They possess cytotoxic capabilities, capable of secreting immunostimulatory cytokines such as IFN-γ and IL-12 and express co-stimulatory molecules that improve lymphocyte responses.[43] Tumor-associated macrophages (TAMs or M2 macrophages), however, secrete IL-4, IL-10, and other factors promoting angiogenesis, tumor growth and metastasis [44, 45, 46]. The mechanisms driving TAM polarization are the source of much investigation.

Myeloid-derived suppressor cells (MDSCs) are a population of immature immune cells of myeloid origin that also frequently expand in response to tumors and have been associated with tumor progression and poor prognoses.[47, 48] MDSCs produce a number of factors including arginase 1 and nitric oxide synthase to suppress the expansion and activity of cytotoxic T cells, NK cells, and DCs.[49]

CD73, or ecto-5′-nucleotidase, is another immunosuppressive molecule strongly linked to tumor growth and poor patient outcomes.[50] CD73 is an ecto-enzyme responsible for the catabolization of AMP into free adenosine. CD73, adenosine and adenosine receptor signaling has well-described immunosuppressive functions.[51, 52, 53] CD73 has been found to be overexpressed in many different cancers including colon, ovarian, leukemia, gastric cancers and is broadly expressed on melanoma cells.[50]

Understanding the different members of the tumor microenvironment, and the myriad potential immunosuppressive mechanisms they can employ, is critical to designing effective therapeutics.

1.4 Immunotherapies

Though the immune system is well-equipped to protect against cancer, some tumors still navigate the complexity of the immune system to evade it and foster a suppressive environment. However, the potential to eliminate malignancy is still inherent in the immune system, and many creative immunotherapeutic strategies seek to similarly navigate that complexity to reestablish an effective antagonistic relationship with cancer.

Dr. William Coley (1862-1936) at the Memorial Hospital in New York is acknowledged for pioneering one of the first successful immunotherapies. Observing one sarcoma patient, he noticed a surprising regression in tumor growth coinciding with erysipelas, a bacterial skin infection. Hypothesizing that certain elements of the infection were responsible for the regression, he injected other sarcomas with pus collected from the bacterial lesions of erysipelas-infected patients. Amazingly, some cases resulted in tumor regression and greater survival. Though the field of immunology was poorly understood in the early 1900s, the basic concept of cancer immunotherapy had still been established: to utilize the intrinsic capabilities of the immune system to target and eliminate cancer. Coinciding with advancements in our understanding of immunology, cancer immunotherapy has seen impressive advances in the last several years. Promising and creative strategies have emerged, taking advantage of the complexity and diversity of the immune system.

Cancer vaccines

Vaccine-based approaches to cancer treatment have recently seen an enormous surge in attention. Cancer vaccine strategies are designed to build targeted immune responses against tumor-specific targets. In most methodologies, dendritic cells (DCs) are generated from a patient's peripheral blood mononuclear cells (PBMCs). Cytokines like granulocyte macrophage colony-stimulating factor (GM-CSF) are used to further expand and mature DCs. DCs are then pulsed *ex vivo* with a relevant tumor-related protein or peptide prior to reinfusion into the patient. The hope is that the primed DCs will then engender an adaptive immune response against antigen-expressing cancer cells. Sipuleucel T, or Provenge by Dendron, is the first therapeutic cancer vaccine to receive FDA approval for use in patients with metastatic, castration-resistant prostate cancer. Autologous PBMCs from prostate cancer patients are primed with a fusion protein between the prostate antigen prostatic acid phosphatase (PAP) and GM-CSF before being reintroduced to the patient.

Identification of relevant tumor antigens represents a major hurdle to the efficacy of cancer vaccines. Antigens are very rarely conserved across tumor types and, even within a given type, antigens, such as PAP in prostate cancer, may not frequently be shared.

Adoptive cell therapy

Adoptive cellular therapy offers another rapidly-growing approach to treatment. In general, leukophoresis of PBMCs or tumors and lymph nodes is used to collect tumor-reactive lymphocytes. Following *ex vivo* enrichment, manipulation and stimulation, these cells are reinfused back into the body where they exhibit strong antitumor behavior. Isolating tumor-specific $CD8^+$ cytotoxic lymphocytes from circulating PBMCs requires multiple rounds of pheresis to isolate and great effort to sufficiently expand and activate.[54] Tumor infiltrating

lymphocytes (TILs), collected directly from tumors or draining lymph nodes, are among the most potently reactive against tumor cells, but require continued administration of potentially toxic levels of IL-2 to overcome tumor-induced suppression and maintain their cytotoxicity.[55] Another strategy utilizes T cells engineered to express chimeric antigen receptors (CARs) that replace the extracellular domain of a T cell receptor with the variable region of an antibody. CAR T cells are uniform in their ability to respond to a specific whole antigen and are not restricted to responding solely to peptide presented by MHC. They too are currently limited in their effectiveness as a treatment option by potential toxicity and transduction efficiency, though advances continue to be made.[56, 57]

Taking a departure from the more common T cell-mediated therapy, adoptive natural killer (NK) cell therapy has recently yielded promising results in clinics.[58, 59, 60] As a member of the innate immune system, NK cells do not possess antigen-specific receptors and therefore do not require TAA to elicit their cytotoxic functions. Instead, NK cells detect danger signals produced by cancer cells and tumor stroma cells.[15] They also possess receptors that recognize MHC I such as Ly49, which inhibit their cytolytic functions when bound to MHC.[14] Tumor cells frequently have defects in MHC expression, and so are unable to inhibit NK-mediated killing. Interestingly, NK cell line NK-92, which was derived from a NK lymphoma patient, has yielding an effective way of producing significant quantities of NK cells for adoptive transfer. Infusions of NK-92 have shown promising efficacy in clinical trials.[61] Another cutting-edge strategy involves the development of CAR NK cells, whereby a chimeric antigen receptor similar to those used in CAR T cells is inserted into autologous NK cells. Early results using mouse xenograft experiments indicate CAR NK cells engendered similar antitumor efficacy as CAR T cells but did not elicit the same levels of toxicity.[62]

Monoclonal antibodies and checkpoint inhibitors

The use of monoclonal antibodies (mAb) is the most common form immunotherapy seen in clinics. Monoclonal antibodies can effectively eliminate targeted cells or impair signaling pathways by blocking ligands from activating surface receptors. The first use of antibodies in patients occurred in the late 90's when antibodies against CD20 in lymphoma patients were used to promote destruction of $CD20^+$ tumor cells via antibody-dependent clearance.[63]

The most common use of mAbs in clinics is as checkpoint inhibitors. In a testament to the significance of checkpoint inhibitors in immunotherapy, the Nobel Prize in Physiology and Physics was jointly awarded in 2018 to James P. Allison and Tasuku Honjo for their independent studies in the 1990s leading to the discoveries of two immune checkpoints, PD-1 and CTLA-4. Many tumor cells, tumor-associated macrophages, and tolerogenic dendritic cells express PD-L1.[32, 64, 65] Binding of PD-L1 with its receptor, programmed death-1 (PD-1), expressed on lymphocytes, leads to impaired immune activity and drives exhaustion and dysfunction. The use of monoclonal antibodies to block PD-1/PD-L1 has achieved notable success in improving antitumor immune responses in clinics. Neutralizing antibodies against PD-1 like pembrolizumab from Merck and nivolumab from Bristol-Myers Squibb have both demonstrated appreciable success in treating melanoma.[66] A mAb against cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), ipilimumab, now owned by Bristol-Myers Squibb, gained approval for use in late stage melanoma even earlier. Tumor-expression of CTLA-4 is responsible for suppressing T cell function—while increasing Treg function—and levels of CTLA-4 are typically elevated in tumor environments. Ipilimumab has seen great success in blocking CTLA-4 signaling and improving T-cell activation in tumors.[41,

[67] Immune related adverse effects, such as dermatitis and inflammatory bowel disease, are potential complications with this treatment.[68]

CD73 offers another interesting target in mAb therapy. CD73 is an ectoezyme expressed on the surface of many tumor cells, immune cells, and endothelial cells where it catalyzes the rate-limiting step in the production of extracellular adenosine from AMP. It acts in conjunction with CD39, which catabolizes of ATP into ADP and then AMP. CD73 and extracellular adenosine have well-characterized tumor-promoting effects.[51, 52, 53] In this way, CD73 can be considered a metabolic immune checkpoint. Numerous clinical trials using antagonistic mAbs against CD73 have yielded promising results.[69]

In contrast to "removing the breaks" on immune activity via checkpoint blockade, antibody therapy may also "step on the gas" by directly promoting immune activation through agonistic binding of activating receptors. 4-1BB, or CD137, is an inducible costimulatory receptor expressed on lymphocytes and DCs.[70] Ligation of 4-1BB on CD8 T cells and NK cells enhances their proliferation, cytokine production, and cytotoxicity. Anti-4-1BB agonistic mAbs have demonstrated promising efficacy in a variety of tumor models and clinical trials.[71, 72] Similar results have observed in other agonist mAb treatments targeting costimulatory molecules like CD40 and OX40, albeit with mild to medium toxicities.[73, 74]

Cytokine therapy

Cytokines are cell-signaling proteins used to coordinate immune activity and act as non-specific activators of immunity. Many tumors develop an immunosuppressive or tolerant environment, impairing the body's intrinsic antitumor immune capabilities. Cytokine treatment seeks to stimulate immune cells present in the body and reestablish antitumor responses. Cytokine therapy,

delivered as a monotherapy, also forgoes much of the expense and labor involved in vaccine and adoptive cell therapy, while offering the potential to fine-tune the dosage and treatment schedule. Over the last few decades, a number of studies have explored the effects of particular cytokines in eliciting widespread antitumor responses.

IL-2, originally acknowledged for its function as a T and NK cell growth factor *ex vivo*, was the first cytokine to be used in patients to enhance antitumor responses in melanoma.[75, 76, 77] Recombinant IL-2 was used in combination with adoptive T cell therapy. Though treatment was effective in some patients, the effect was not consistent, and did not outperform standard treatment methods. One explanation considers the infidelity of IL-2 signaling. The IL-2 receptor is expressed on many lymphocytes like CD8 T cells and NK cells, in addition to immunomodulatory immune cells like T regulatory cells (Tregs). The increased presence and activity of Tregs may impair the effector functions of CD8 T cells and NK cells during IL-2 treatment. Still, IL-2 has demonstrated durable responses in patients, albeit at a low rate, and is currently FDA-approved for use in metastatic melanoma and metastatic kidney cancer.

Interferon-α (IFN-α), first used to treat hairy-cell leukemia (HCL) in 1986, is a more specific activator of type 1 immunity. As a type I interferon, IFN-α involved in Th1 differentiation, the generation and preservation of activated cytotoxic T lymphocytes, and the differentiation and activation of dendritic cells.[78, 79] IFN-α can also directly induce apoptosis of cancer cells and impair the outgrowth of certain tumors.[80] In early applications, 90% of HCL patients treated with IFN-α demonstrated at least minor responses, in addition to substantially improved granulocyte, hemoglobin, and platelet levels in 77% of patients.[81] Use of IFN-α is also approved in treatment of hairy cell leukemia, AIDS-related Kaposi sarcoma, and non-Hodgkin's lymphoma and as adjuvant therapy in patients with stage II and II melanoma.

IL-12 promotes the differentiation of Th1 cells, directly enhances effector functions of CD8 T cells and NK cells, and can inhibit angiogenesis. Use of IL-12 as immunotherapy in mouse models has yielded promising results, and combinations with IL-18 have reversed NK cell anergy in tumors, improving tumor-killing capacity.[82] Unfortunately, outcomes in clinics have not reflected this success, where severe issues of toxicity have limited the use of IL-12.

Numerous other cytokines, such as IL-15 and IL-21 have been explored as potential immunotherapies. However, a number of cytokines are prone to elicit toxic effects, which can limit the amount that can be administered and the overall effectiveness of the treatment.[83] The choice of cytokine, the dosing, and the method of implementation are critical factors that must be considered when evaluating the therapeutic efficacy of cytokine therapy.

1.5 Interleukin 33

Interleukin 33 is a fairly new member of the IL-1 superfamily,[84] joining IL-1α, IL-1β, IL-1Ra and IL-18.[85] Originally discovered as an intracellular transcriptional regulator, in 2005 it was recognized as the ligand for the then-orphaned receptor, T1/ST2.[85] Secreted by fibroblasts, epithelial cells, and endothelial cells in response to cell injury,[86] IL-33 is thought to act as an early inducer of inflammation. IL-33 coordinates a wide variety of immune responses mediated by Th2 cells, mast cells, eosinophils and type 2 innate lymphoid cells, which all stably express ST2.[84, 87] More recently, IL-33 has even demonstrated the ability to activate dendritic cells, amplify Th1 reponses, and promote cytoxicity in $CD8^+$ T cells and natural killer (NK) cells.[88, 89] Our own

previous data supports these findings.[90] Considering the wide range of reponses IL-33 can mediate, IL-33 is deserving of its recent attention.

IL-33 gene and structure

In humans, the IL-33 gene is located on chromosome 9 at 9p24.1 and at 19qC1 in mice.[85, 91] IL-33 cDNA encodes 270 amino acids (aa) in humans and 266aa in mice.[85] The full length form of IL-33 can be separated into three distinct regions. The N-terminus (IL-33_{1-65} in humans) possesses a predicted helix-turn-helix domain, containing a chromatin-binding motif (CBM) at IL-33_{40-58}. An intermediate region follows (IL-33_{66-111}), containing cleavage points for proteases for generation of active short forms of IL-33. The C-terminal, IL-$33_{112-270}$, is described as an IL-1-like domain. The region takes on a β-trefoil similar to that of IL-1α, IL-1β, and IL-18.[92] Though it shares the most sequence homology with IL-18, it is functionally more similar to IL-1α. This region also contains cleavage sites for caspases 1, 3, and 7 which renders it functionally inactive.

Processing and release of IL-33

IL-33 was originally described as being expressed in the nucleus of high endothelial venules.[91] It was also reported to be produced by epithelial cells, fibroblasts, and smooth muscle cells.[86] Under normal physiological conditions, the full-length form of IL-33 is confined to the nucleus by the chromatin-binding motif. Interestingly, though IL-33 has been characterized as an inflammatory cytokine, overexpression of nuclear IL-33 in steady-state conditions can actually repress NF-κB signaling.[93] In this way, IL-33 may actually serve to regulate inflammation in situations of hyperactivation of inflammatory signals.

During cell injury or necrosis, nuclear IL-33 is released into the cell's surroundings. IL-33 released in this way retains its full-length form, complete with the CBM of its N-terminus. In contrast to other cytokines, IL-33 is biologically active in its full-length form. IL-33 does not share activating cleavage sites present in pro-IL-1β and pro-IL-18.[94] Recent evidence even demonstrates released IL-33 can be bound to chromatin.[95] This chromatin-bound IL-33 elicits variable signaling properties.

Once released, numerous proteases are able to cleave IL-33 in its intermediate region ($IL\text{-}33_{66\text{-}111}$). Extracellular elastases from human neutrophils are capable of cleaving within this region. Recently, allergens such as pollen, cockroach allergen and *A. alternata* have demonstrated the ability to cleave IL-33 at various sites within this domain.[96] These cleaved, short forms of IL-33 preserve their functional IL-1-like domains which can elicit different signaling capabilities.

The functional IL-1-like domain possesses numerous caspase cleavage sites. Unlike the proteolytic sites in the intermediate region, these caspase cleavage sites render the IL-33 functionally inactive. In this way, the release of IL-33 during cell injury and necrosis contrasts apoptosis, where release of caspases cleaves the functional IL-1-like domain, rendering the cleavage products of IL-33 inactive.

IL-33 receptor, ST2

The IL-33 receptor, ST2 (suppressor of tumorigenicity 2), was originally studied for many years as an orphan receptor involved in inflammation and autoimmune responses.[97] The receptor remained orphaned until 2005 when IL-33 was discovered as a functional ligand.[85] The IL-33 receptor exists as a dimer of ST2 and the IL-1 receptor accessory protein (IL-1RAcP).[98, 99] Though it does not directly bind to IL-33, IL-1RAcP has been shown to be required for IL-33-mediated

signaling.[99] Mice deficient in IL-1RAcP failed to respond to IL-33 and demonstrated impaired IL-33-mediated induction of NF-κB.[98] Binding of IL-33 to ST2 recruits myeloid differentiation factor 88 (MyD88), resulting in activation of both MAPK and NF-κB signaling.[84]

ST2 possesses numerous isoforms. A long transmembrane form, ST2L, is the most studied form and is commonly found among hematopoietic cells. Two variant forms, ST2V and ST2LV, have been reported. ST2V, which results from alternative splicing in the C-terminal portion, was found predominantly in gastrointestinal organs such as the intestines and stomach, in addition to the spleen.[100] A second variant, ST2LV, was found to be expressed in the brain, eyes, liver, lung, and heart during embryogenesis.[101] The significance of these variant forms has yet to be closely explored. A soluble form, sST2, results from alternative splicing that removes the transmembrane and cytoplasmic domains.[102] This form appears to be highly inducible under inflammatory conditions such as those elicited by TNF, IL-1α and IL-1β, and in skin in response to UV radiation.[103] sST2 binds IL-33, but in the absence of any transmembrane and cytoplasmic components, is unable to elicit typical IL-33 mediated signaling. In this way, sST2 is thought to act as a decoy receptor to curb IL-33 responses.[104]

IL-33 signaling

Downstream signaling of IL-33 is mediated through the ST2—IL-1RAcP dimer that forms the IL-33 receptor.[98] Mice deficient in either ST2 or IL-1RAcP do not demonstrate any inflammatory response to IL-33 treatment.[98] Binding of the IL-33 receptor triggers the recruitment and activation of the MyD88 adaptor protein, in addition to IRAK1,4 and TRAF6.[85] MyD88 is critical for IL-33-mediated cytokine production and proliferation in mast cells.[105, 106] The recruited factors form a scaffolding, allowing the activation of NF-κB and MAP kinase.[85] Several different kinases

downstream of MAPK are activated by IL-33 administration including ERK1/2, JNK, and p38 in a manner similar to IL-1β signaling.[85]

1.6 IL-33 in disease

Allergy and asthma

IL-33 has now been strongly linked to a wide variety of allergic[107, 108] and Th2-mediated responses including anaphylaxis[109] and asthma.[110] Early work on IL-33 was conducted in the context of allergy and asthma. IL-33 was upregulated heavily in endobronchial biopsies of asthmatic patients.[111] *In vitro*, IL-33 directly promoted allergic responses via DCs and Th2 cells.[111] IL-33-activated bone marrow-derived DCs promote Th2 differentiation by priming CD4 T cell development towards a Th2 phenotype characterized by IL-5 and IL-13 production.[112] Intranasal administration of IL-33 promoted accumulation of IL-5$^+$ T cells and promoted allergic-induced airway inflammation.[110] In a model of anaphylaxis, IL-33 upregulated in keratinocytes in response to peanut allergen.[109] ST2 blockade altered the Th2-promoting phenotype of DCs and impaired Th2 induction.

Rheumatological diseases

IL-33 has been linked to rheumatological diseases including rheumatoid arthritis (RA), osteoarthritis (OA), and psoriatic arthritis (PsA). Endothelial cells and fibroblasts in synovial tissue of RA, PsA and OA patients exhibit elevated levels of IL-33 protein.[113] Circulating IL-33 was also detected in several RA patients' serum by ELISA, though remained undetectable in healthy

serum.[114] In mouse models of RA, driven by collagen-induced arthritis (CIA), ST2$^{-/-}$ mice demonstrated reduced severity of CIA coupled with attenuated *ex vivo* induction of inflammatory cytokines and anti-collagen antibody production.[115] IL-33 treatment exacerbated CIA and increased production of inflammatory cytokines and antibodies in a mast cell-dependent manner.

Alzheimer's disease

Reduced IL-33 expression in the brain has been observed in patients with Alzheimer's disease (AD).[116] Administration IL-33 has also demonstrated the capacity to ameliorate symptoms in mouse models of AD.[117] In the context of the brain, IL-33 has been shown to be indispensable for tissue homeostasis and the repair of aging neurons.[118] IL-33 was critical for neuron viability in the cerebral cortex and hippocampus, where it promoted repair of DNA double-strand breaks and autophagic clearance of amyloid plaques. Results like these have encouraged the initiation of phase one clinical trials testing IL-33 in patients with AD.

Viral responses

Though IL-33 has classically been considered an inflammatory and Th2-promoting cytokine, IL-33 also mediates Th1-related antiviral responses. Release of IL-33 from virally damaged barrier tissues elicits antiviral immune responses required for clearance of viral infections. During lymphocytic choriomeningitis virus infection, Th1 effector cells express ST2—albeit transiently—in both *in vivo* and *in vitro* settings.[119] ST2 expression in Th1 cells depended on T-bet and STAT4 and denoted highly activated effector cells. Loss of ST2 led to reduced LCMV-specific Th1 effector responses and impaired expansion, differentiation, and cytokine production. IL-33 and ST2 signaling was also indispensable for effective CD8 T cell responses to prototypic RNA and

DNA viruses in mice. IL-33 directly promoted CD8 T cell expansion and enhanced their effector functions.[120]

1.7 Role of IL-33 in cancer

Myriad studies demonstrate the IL-33/ST2 axis plays a role in tumor immunity. However, depending on the context several protumor[121, 122, 123] and antitumor [88, 124, 125] functions have been reported. In numerous studies of breast cancer, IL-33 has typically supported tumor growth. As previously described, IL-33 can elicit a wide range of immunomodulatory functions and depending on the context—the tumor tissues, the implementation of IL-33, and the immune cells involved—the impact of IL-33 on tumor growth can vary greatly.

Dendritic cells

Although DCs express low levels of ST2 at baseline, IL-33 increases surface expression of MHC-II, CD40, CD80, CD86 CCR7 and OX40L and drives the production of numerous cytokines (IL-1β, IL-4, IL-5, IL-13, and TNF-α).[126, 127, 128, 129] In most of these studies, IL-33-reactive DCs promoted Th2-mediated allergic or asthmatic responses. IL-33 also drove expansion of DCs from bone marrow, yet these DCs displayed impaired MHC-II expression, high levels of PD-L1 and PD-L2, and demonstrated a reduced capacity to prime naïve T cells.[130]

Contrasting this tolerogenic phenotype, in the context of murine AML, exogenous IL-33 promoted DC activation and cross-priming of tumor-reactive CD8 T cells, which corresponded with suppressed disease progression.[124] In EG7 lymphoma, B16F10 melanoma, and an inducible

$Braf^{V600E}$PTEN model of melanoma, recombinant IL-33 again attenuated tumor growth.[90] IL-33 treatment activated intratumoral myeloid DCs, improved antigen cross-presentation, and restored antitumor T cell activity in an ST2-dependent manner.

Antitumor responses; Th1, CD8, and NK cells

IL-33 has classically been considered a promoter of type 2 immunity, characterized by activation of Th2 cells and production of type 2 immune cytokines such as IL-4, IL-5, and IL-13. Interestingly, IL-33 can also elicit effective Th1-related responses typical of antitumor immunity. IL-33 served as an effective adjuvant when used in combination with an HPV DNA vaccine by potently enhancing IFN-γ production by $CD4^+$ and $CD8^+$ T cells and reducing established TC-1 tumors in mice.[125] Transgenic expression of IL-33 in B16 melanoma delayed tumor growth, characterized by increased infiltration and cytotoxicity of CD8 T cells and NK cells. When treated with recombinant IL-33 *in vitro*, these cells increased NF-κB activation and CD69 expression.[88] In another study, CD 8 T cells were critical contributors to B16F10 tumor inhibition in mice treated with exogenous IL-33.[90]

T regulatory cells, MDSCs and immunosuppression

The milieu of immune infiltrates can inform prognoses and treatment strategies for patients. Lower ratios of effector cells (such as CD8 T cells and NK cells) to immunosuppressive cells (including T regulatory cells and MDSCs) typically indicate weaker tumor-immune responses and overall poorer prognoses. Although the IL-33/ST2 axis can promote CD8 T cell and NK cell activity, IL-33 has also been shown to promote expansion of $Foxp3^+$ $GATA3^+$ T regulatory cells (Tregs) and $CD11b^+$ $Gr\text{-}1^+$ MDSCs.[131, 132] These $ST2^+$ IL-33-induced Tregs exhibit suppressive functions in

numerous conditions including autoimmunity, transplantation, inflammation, and allergy.[133] In a preclinical model of human non-small-cell lung cancer, IL-33 blockade reduced Treg presence impaired tumor growth.[134] In 4T1 breast cancer, administration of IL-33 accelerated tumor growth, coinciding with increased intratumoral $CD4^+$ $Foxp3^+$ $ST2^+$ $IL\text{-}10^+$ Tregs and $CD11b^+$ $GR1^+$ $TGF\text{-}\beta^+$ MDSCs.[132]

1.8 Innate lymphoid cells

As previously discussed, many immunotherapies employ creative methods of manipulating specific aspects of the immune system. For immunotherapy to evolve, so too must our understanding of immunology. New discoveries are constantly being made, and with them come new insights into potential tools and pitfalls that inform immunotherapy design. In the last several years, an entire new branch of innate immunity has slowly been characterized, providing a whole new field of immune research. Innate lymphoid cells (ILCs) comprise a new branch of mainly secretory immune cells with an emerging importance in modulating immune responses. ILCs are of lymphoid morphology, and arise from a common ILC precursor found in bone marrow.[135, 136] Characterized as lineage negative, ILCs lack the many markers used to identify T cells, B cells, NK and NKT cells, macrophages, basophils, and mast cells. Though they possess lymphoid morphology, they are distinct from T cells in that they lack rearranged antigen-specific receptors (TCRs). The absence of many common immune markers has likely delayed their discovery and characterization. Three broad categories of ILCs have been identified and can roughly be described as innate analogs to T helper cells based on their transcription factors and cytokine production—

group 1 ILCs promote Th1 effects, group 2 ILCs can elicit Th2-like responses, and members of group 3 ILCs resemble Th17 and Th22 in their functions.[137]

ILC1s

Group 1 innate lymphoid cells depend on transcription factor T-bet for their development and are characterized by production of IFN-γ.[138] This group has come to include NK cells and a recently described subset identified as ILC1s. Bernink et al. characterized ILC1s as distinct from NK cells in that they do not express NK markers CD16, CD56, and CD94 and do not exhibit cytotoxic capabilities (lack perforin and granzyme B). Like NK cells, however, ILC1s secrete high levels of IFN-γ in response to IL-12.[138, 139] Similar to NK cells, ILC1s express CCL3 and CXCR3, which is necessary for NK cell migration to inflamed tissue.[140] ILC1s were also found to expand in the inflamed gut mucosa of patients with Crohn's and contribute to pathology in murine models of colitis.[141]

ILC2s

Group 2 innate lymphoid cells (ILC2s) are potent inducers of type 2 immunity and serve as a critical innate cellular source of type 2 cytokines (chiefly IL-5 and IL-13 in addition to IL-4 and IL-9).[142, 143, 144] ILC2 development depends on the GATA3 transcription factor, similar to Th2 cells.[145] RORα has also been shown to play a critical role in development as RORα-deficient mice are deficient in ILC2s.[146, 147] ILC2s reside predominantly in barrier tissues such as skin, lung, gut and the adipose lining of intestines[142] in addition to the liver. Secretion of type 2 cytokines is driven by epithelial cytokines, or alarmins, including IL-25, IL-33, and TSLP (thymic stromal lymphopoietin), in response to cellular injury. In mouse models, IL-33 has been suggested to be a

much more potent promoter of ILC-mediated allergic inflammation in lungs.[148] ILC2s are the only-known ILC subset to respond to IL-33 and express the IL-33 receptor, ST2.[149]

Evidence for ILC2s (originally referred to as nuocytes, natural helper cells, or innate helper type 2 (Ih2) cells[150] first appeared in 2001. Intranasal administration of IL-25 to $Rag1^{-/-}$ mice—lacking T and B cells—increased levels of IL-5 and IL-13, a process believed to be dependent on T helper type 2 cells.[151] In 2006, non-B–non-T cell c-$kit^{+}$$FceR1^{-}$ cells demonstrated responsiveness to IL-25 by secreting IL-4, 5, and 13 during helminth infection.[152] ILC2s were definitively identified by Moro et al. in 2010 as lineage negative (Lin-) lymphoid cells that produce IL-4, IL-5, and IL-13 in response to both IL-33 and a combination of IL-25 and IL-2.[142]

ILC2s play a critical role in parasite protection and homeostasis in barrier tissues. In mouse models, IL-25- and IL-33-responsive ILC2s were indispensable in the expulsion of the helminth parasite *Nippostrongylus brasiliensis.*[143, 144] Helminths can secrete enzymes which erode epithelial barriers and trigger massive cell death. In response to alarmins (such as IL-25, IL-33, and TSLP) released by damaged epithelial cells, ILC2s secrete IL-13, which promotes mucus production by goblet cells.[153] ILC2s also support adaptive immune involvement. In lungs, ILC2-derived IL-13 also promotes trafficking of dendritic cells to draining lymph nodes to prime Th2 cells.[154] Secretion of IL-5 promotes the recruitment of eosinophils and mast cells which provide important anti-parasitic functions. ILC2s also produce amphiregulin, which promotes tissue repair. Mice deficient in IL-9 exhibited a reduction in ILC2 numbers and reduced IL-5, IL-13 and amphiregulin production that corresponded to severely impaired tissue repair following *Nippostrongylus brasiliensis* infection.[155]

ILC3s

A lot of debate surrounds the definition of group 3 ILCs, but they typically require RORγt expression for development and are characterized by secretion of IL-17 and IL-22 in response to IL-1β and IL-23.[150] ILC3s play a significant role in innate immune responses to bacterial infections in mucosal surfaces in the gut.[156] Lymphoid tissue inducer cells (LTi) are the prototypical subset of ILC3s, which are involved in the development of secondary lymphoid tissues during embryogenesis and additionally act as effector cells, secreting IL-17 and IL-22.[157, 158] Another subset of group 3 ILCs expresses the natural cytotoxicity receptor NKp46. These cells display a high level of plasticity by expressing varying degrees of T-bet and RORγt, with corresponding production of IFN-γ, similar to ILC1s. Evidence suggests IL-12 promotes ILC1 behavior and IFN-γ from these cells by increasing T-bet expression while decreasing RORγt.

1.9 Type 2 innate lymphoid cells in disease

Helminth infection

ILC2s play a critical role in parasite protection and homeostasis in barrier tissues. In mouse models, IL-25- and IL-33-responsive ILC2s were indispensable in the expulsion of the helminth parasite *Nippostrongylus brasiliensis*.[143, 144] Helminths can secrete enzymes which erode epithelial barriers and trigger massive cell death. Secretion of IL-5 promotes the recruitment of eosinophils and mast cells which provide important anti-parasitic functions. ILC2s also produce amphiregulin, which promotes tissue repair following parasite-mediated tissue damage. Mice deficient in IL-9 exhibited a reduction in ILC2 numbers and reduced IL-5, IL-13 and amphiregulin production that

corresponded to severely impaired tissue repair following *Nippostrongylus brasiliensis* infection.[155]

Allergy and asthma

Type 2 immunity is the key driver of allergic responses, including asthma.[159] In murine models of allergy, ILC2s were critical for mounting type 2 responses to papain allergen.[160] In response to alarmins (such as IL-25, IL-33, and TSLP), ILC2s secrete IL-13, which promotes goblet cell hyperplasia, mucus overproduction, and muscle contraction, potentially exacerbating allergic responses in the lungs.[153] ILC2s also support adaptive immune involvement in allergic responses. In lungs, ILC2-derived IL-13 also promotes trafficking of dendritic cells to draining lymph nodes to prime Th2 cells. Following inhalation of allergens, mice deficient in ILC2 demonstrated impaired induction of Th2 priming, accompanied with reduced levels of IgE production.[154] In one human study, circulating ILC2s were reported to be elevated in asthma patients.[161] In another, however, ILC2 levels were unchanged in asthma patients, but NK cell numbers were reduced,[162] suggesting the potential of an ILC2-NK axis of activity.

Atopic dermatitis

ILC2s are heavily involved in skin homeostasis. Originally characterized in mice as Lin^- $ST2^+CD25^+CD127^+CD90^+ICOS^+$,[163] these ILC2s also expressed CD103, an integrin shared by other dermal leukocytes.[164] Atopic dermatitis (AD) is characterized by elevated levels of type 2 cytokines and lesions demonstrate increased expression of ILC2-related transcripts—*IL1RL1* (ST2), *IL17RB* (IL-25 receptor), *TSLPR* (TSLP receptor), *AREG*, and RORA, suggesting an increased presence of ILC2s.[165] Though skin-resident ILC2s are abundant under homeostatic

conditions, overexpression of IL-33 in skin elicited development of spontaneous dermatitis accompanied by enrichment of ILC2s.[166] Similar observations were made in humans, as lesional biopsies of AD exhibited an enhanced ILC2 presence.[163, 166]

Cancer

Little has been explored regarding the role of ILC2 in cancer, though in some cases, roles have been implied. Damage to biliary epithelium is associated with severe liver diseases and can progress to malignancy. Examination of biliary epithelium repair in patients with biliary atresia exhibited elevated levels of IL-33.[167] The study utilized mouse models to demonstrate IL-33-mediated repair elicited cholangiocyte hyperplasia, dependent on ILC2-derived IL-13. Mice receiving exogenous IL-33, coupled with constitutive activation of oncogenic AKT and YAP in bile ducts, developed cholangiocarcinoma, implicating an IL-33/ILC2/IL-13 axis in progression of biliary carcinogenesis.[167] A study examining the impact of bacillus Calmette-Guérin (BCG) vaccine on immune infiltrates in patients with bladder cancer observed the ratio of T cell to monocytic myeloid-derived suppressor cell (MDSC) infiltrates was negatively associated with ILC2 frequencies.[168] Increased frequencies of ILC2s and ILC2 expression of IL-13 was correlated with a lower T cell-to-MDSC ratio, which corresponded to dramatically reduced recurrence-free survival. *In vitro*, ILC2s increased IL-13 expression in response to BCG and cancer cells.[168] IL-13 induced monocyte recruitment and suppressive functions, underlining a potential protumor role of ILC2s via promotion of an immunosuppressive tumor environment.

Contrasting the indications of protumor behavior, some studies have implicated antitumor roles for ILC2s. One study observed increased expression of CXCL2 on ILC2s following exposure IL-33.[169] Transgenic expression of IL-33 in EL4 mouse thymoma cells increased levels of CXCR2 in

EL4 cells. The study reported that CXCL2 engagement on tumors cells via ILC2-derived CXC2 directly promoted apoptosis of tumors cells and impaired tumor growth.[169] Another very recent study reports that genetic ablation of ILC2s resulted in increased tumor growth and metastasis in mice. Ablation of achieved by RORα$^{-/-}$ BM-transplantation. They believe ILC2s were instrumental in promoting antitumor activity CD8 T cells.[170]

Greater clarification of the impact of ILC2s in the context of cancer is needed. Given ILC2s' potential sensitivity to immunotherapies like IL-2, BCG, and PD-1 blockade, further characterization of the role of ILC2s in cancer is warranted.

1.10 Hypothesis and significance

Immunotherapy is a promising direction for cancer treatment that sees new advancements and successes each year. It is the only new branch of cancer therapy to emerge since chemotherapy. However, it is an understatement to say that limitations still exist, and depending on the type of cancer, the tumor-associated antigens involved, the tumor-induced immune suppression mechanisms in play, treatment success is variable across patients. Other times, clinicians simply do not know why some patients will respond favorably and other will not. Providing explanations will require new insights based in in-depth exploration in basic immunology and cancer biology. It is for this reason that our lab has chosen to explore the impact of IL-33 on antitumor immunity and evaluate its potential as an immunotherapeutic.

The central aim of this study was to explore innate immune contributions to IL-33-driven tumor effects. To date, studies of the role of IL-33 in cancer have yielded conflicting results. Depending on the context—the type of cancer, the tissues involved, the implementation of IL-33—IL-33 can elicit drastically different outcomes. Previous studies in the lab defined a clear antitumor role of IL-33 in melanoma via administration of recombinant IL-33. Though the study highlighted the involvement of adaptive immunity in mediating these effects, the potential contributions of innate immunity were not fully explored. Among the many different immune cells stimulated by IL-33, NK cells have demonstrated tumoricidal behavior in response to IL-33. We hypothesized that administration of IL-33 would be sufficient to delay tumor growth in mice lacking adaptive immunity, dependent on the involvement of NK cells. We further wished to examine the potential involvement of other IL-33-responsive immune cells in IL-33-mediated tumor attenuation.

Chapter 2: Materials and Methods

2.1 Mouse strains

All animals were bred and maintained under pathogen-free conditions. Experiments were approved and were in-line with regulatory standards and guidelines set by the Institutional Animal Care and Use Committee of Northwestern University.

C57BL/6—WT

Purchased from the Jackson Laboratory, these mice are the most widely used inbred strain of mice. Although Jackson describes this strain as refractory to tumors, it is a successful host to many tumor cell lines and can harbor several genetic modifications designed for tumorigenesis. Although no major tumor experiments utilized these mice, they were typically used between 6-8 weeks old.

$Rag1^{tm1Mom}$—$Rag1^{-/-}$

Originally purchased from the Jackson Lab, all $Rag1^{-/-}$ mice used for experiments were bred in-house. Rag1 is necessary for proper recombination of T cell receptors and B cell antibodies. $Rag1^{-/-}$ mice have a disrupted Rag1 gene, preventing development of functional T and B cells. They are devoid of all $CD3^{+}$ T cells, all B cells, and NK T cells, essentially rendering the mouse bereft of an adaptive immune system.[171] Most major tumor experiments predominantly used 6-8 week-old female mice.

CD73$^{-/-}$

CD73$^{-/-}$ mice were generated and provided by Dr. Linda Thompson at the Oklahoma Medical Research Foundation. Mice were generated via targeted disruption of the third coding exon of the Cd73 gene and verified by southern blot and western blot as previously described.[172]

2.2 Cell lines

B16F10

Obtained from Dr. Hans Schreiber at the University of Chicago, B16F10 is a popular and aggressive mouse melanoma cell line used as a model for human melanoma.

B16-Vec/B16-IL33

These clones of B16F10 were generated and kindly provided by Dr. Binfeng Lu at the University of Pittsburgh. They are designed to either overexpress IL-33 (B16-IL33) or its control vector (B16-Vec). Both cell lines grow normally and at comparable rates *in vitro*.

EL4

A murine T lymphocyte cell line used as a model of lymphoma was acquired from Dr. Hans Schreiber.

BPS1

A tumor cell line derived from a $Braf^{V600E}$PTEN (Tyr::CreER; $Braf^{CA/+}$; $Pten^{lox5/lox5}$) mouse model. Though the model was designed to induce expression of oncogenic B-Raf and flox PTEN by tamoxifen exposure, this cell line was derived from a spontaneously arising dermal tumor. The tumor was finely minced and plated into a 48-well plate with RMPI with 10% FBS. Tumor cells were expanded from these cultures and frozen. The resulting cell line demonstrated presence of $Braf^{V600E}$ transversion.

2.3 Sample preparation

Single-cell suspension of spleen tissue

Spleens were harvested and processed through a 70μm cell strainer and diluted in RPMI with 10% FBS. The suspensions were then flushed through the strainer a second time into a 50ml tube and diluted and washed with 40ml PBS. The pellets were then resuspended with 2ml red blood cell lysis buffer for 2 minutes. After diluting the buffer to 50ml with PBS, 10μl aliquots were collected and diluted with trypan blue to assess viability and cell numbers. The splenocytes were then centrifuged and resuspended in the desired medium and concentration for downstream experiments.

Single-cell suspension of tumor tissue

Tumors were excised and placed in a 60mm dish containing 5ml of RPMI with 10% FBS, 80μg/ml DNase I (Roche), and 1mg/ml collagenase D (Roche). After finely mincing the tumor with a razor, samples were incubated in a shaker at 37°C for 1 hour. Samples were also periodically processed

with a 1ml pipette every 15 minutes to improve dissociation and further disrupt clumps. Samples were then additionally processed through a 70μm a cell strainer into a 50ml tube, diluted with PBS, then counted. Finally, cell samples were centrifuged and resuspended at appropriate concentrations.

2.4 Flow cytometry

All samples were prepared from spleen and tumors for flow cytometry using methods described above. Samples were run on either a MACSQuant Analyzer (Miltenyi Biotec) or BD LSR II (BD Biosciences). Results were analyzed using FlowJo software (Treestar).

Cell surface staining

Single-cell suspensions of tissues were prepared as previously described at desired concentrations. 50μl aliquots were then placed in 1.2ml micro titer tubes. An additional 50μl of PBS containing relevant fluorophore-conjugated mAbs were then mixed with the cells (typical final mAb concentration: ~1μg/ml). Cells were then stored at 4°C in the dark for 25 minutes before diluting, centrifuging, and resuspending at a desired concentration for analysis by a flow cytometer. If cells were not analyzed on the same day as staining, they were instead centrifuged and resuspended in 4% paraformaldehyde for 20 minutes. After fixation, cells were then washed and resuspended again in PBS and stored at 4°C in the dark until analysis.

Intracellular/cytokine staining

Single-cell suspensions of tissues were prepared as previously described at desired concentrations. Cells were incubated for 4 hours at 37°C in a 'restimulation cocktail' containing 500µl RPMI with 10% FBS, 50ng/mL of PMA, 1µg/mL of ionomycin, 17µg/ml Brefeldin A, and monensin (1000x solution from Invitrogen). Cells were then prepared for intracellular staining according to BD Biosciences protocol. After washing cells of the restimulation cocktail and completing surface antibody staining, they were incubated with 150µl of permeabilization/fixation solution in the dark for 20 minutes at 4°C. Cells were washed with 400µl of a washing buffer. Cells were finally resuspended in 100µl of wash buffer containing ~1µg/ml intracellular fluorophore-conjugated mAbs and incubated for 25 minutes at 4°C before analysis by flow cytometry.

Transcription factor staining

Following completion of cell surface staining, single-cell suspensions were prepared for nuclear staining according to BD Biosciences' protocol. After nuclear permeabilization/fixation, cells were washed and resuspended in a permeabilization buffer containing ~1-2µg/ml nuclear fluorophore-conjugated mAbs and incubated for 25 minutes at 4°C before analysis by flow cytometry.

2.5 Immunofluorescence

Tumor sample collection and sectioning

Upon completion of *in vivo* tumor experiment, mice were euthanized and the subcutaneous tumors were excised. A 1cm x 1cm x 0.5cm samples was collected from the center of the tumor mass (volume varied depending on tumor size). Samples were immersed in OCT and stored at -80°C. 10μm sections were cut using a cryostat microtome and collected on glass slides.

Staining and imaging

Slides were allowed to dry for 2 hours at room temperature. They were then fixed in 4% PFA diluted in PBS for 15 minutes before being washed. All washes consisted of three 5-minute washes in PBS. Samples were then blocked with 100μl solution of 1% BSA in PBS for 30 minutes. After removing the blocking solution, samples were incubated with primary antibodies in 1% BSA in PBS at an optimal dilution overnight at 4°C. Samples were washed and incubated with appropriate secondary antibodies, diluted in 1% BSA in PBS, at room temperature for 2 hours. After another set of washes, 100μl of PBS was placed on the samples. Images were taken using an EVOS FLoid Cell Imaging Station and analyzed using ImageJ.

2.6 ELISA

IL-33 was detected by ELISA performed using eBioscience's kit according to the manufacturer's protocol. Serum was isolated from whole blood of tumor-bearing mice. Tumor lysates were similarly analyzed following homogenization in RIPA buffer. Fluorescence was measured using a

GloMax-Multi Detection System by Promega and IL-33 was quantified using a standard curve derived from the manufacture's IL-33 standard.

2.7 Bioluminescent Assay for AMP quantification

CD73 enzymatic activity was assessed via AMP consumption measured using AMP-Glo Assay (Promega) using manufacturer's protocol. Relative AMP levels were determined by luminescence measured by a GloMax-Multi Detection System by Promega.

2.8 *Ex vivo* experiments

NK cell purification

Spleens were harvested from either WT or $Rag1^{-/-}$ mice and processed into a single-cell suspension as previously described. Cells were resuspended at 1×10^8 cells/ml in either BD IMag buffer or BioLegend MojoSort buffer. NK cells were then purified using either the BD IMag Mouse NK Cell Enrichment Set or Stem Cell's EasySep Mouse NK Cell Isolation Kit and their corresponding protocols.

ILC preparation

Similar to NK cell purification, spleens of WT or $Rag1^{-/-}$ mice were processed into a single-cell suspension. Cells were resuspended at 1×10^8 cells/ml in either BD IMag buffer or BioLegend MojoSort buffer then incubated with 2μg/ml biotinylated antibodies for lineage markers. Lineage markers were defined in $Rag1^{-/-}$ mice as NK1.1, CD11b, CD11c, Gr1, F4/80. For WT mice, CD4, CD8, and CD19 were also included. The lineage negative cells (Lin^-) were then collected from the

negative fraction following either BD IMag Streptavidin Particles Plus or Stem Cell's EasySep Mouse Streptavidin RapidSpheres using the associated protocols. Cells were then further purified for $CD90.2^+$ cells using either BD IMag Anti-Mouse CD90.2 Magnetic Particles or EasySep Mouse CD90.2 Positive Selection Kit II and their protocols. The $Lin^-CD90.2^+$ ILCs were then washed and resuspended in RPMI with 10% FBS at the desired concentration.

Alternatively, ILC2s were generated from bone marrow using a previously described method.[169] Bone marrow was collected from the femurs and tibias of either WT or $Rag1^{-/-}$ mice and, after lysing red blood cells, were similarly purified for $Lin^-CD90.2^+$ cells. These cells were cultured in RPMI with 10% FBS with 20ng/ml FLT3L, 20ng/ml SCF, 10ng/ml IL-7, and 20ng/ml IL-33 (Biolegend) for 5-7 days. BM-derived ILC2s were assessed for purity by CD90.2, CD25, and ST2 expression.

2.9 *In vivo* experiments

Tumor cell preparation and injection

B16F10, B16-Vec, B16-IL33, and EL4 cells were thawed and passaged twice before to injections. Adherent cell lines (B16F10, B16-Vec, and B16-IL33) were passaged one day prior to injections to achieve a confluency of ~70% the following day for injections. The suspension cell line, EL4, was counted and split the day before injections to achieve the desired number the following day. One day prior to tumor challenge, the hind flanks for the mice were shaved. On the day of injections, the medium was removed from the flasks of adherent cell lines and cells were washed with PBS. Cells were then covered in 0.25% trypsin and incubated at 37°C for ~5 minutes to allow cells to detach. CR10 was added back to the flask to halt the reaction with trypsin and further

diluted with PBS to 30-50mls. EL4 cells were similarly diluted to 30-50mls. At this stage, protocols for preparing adherent and suspension cells were identical. Cells were counted using a trypan blue exclusion assay. The desired number of cells were centrifuged and diluted to the desired concentration and placed on ice. Ethanol swabs were used to sterilize the hind flanks of mice prior to injection. 200µl of tumor cells were injected into each mouse subcutaneously using a 23-gauge syringe. According to our protocol, mice do not need to be anesthetized for this procedure. For most *in vivo* tumor experiments, tumor cells were diluted to $5*10^6$/ml in PBS to achieve an injection $1*10^6$ cells in 200µl per mouse. In only one experiment, 5000 B16F10 cells were co-injected with $1*10^6$ ILC2s using the described procedure.

In vivo antibodies

Depleting murine anti-CD90.2 (clone: 30H12) and control rat IgG were purchased from Bio X Cell. A hybridoma-derived depleting anti-NK1.1 (clone PK136) was generated in house. The ascites of hybridoma-bearing mice was harvested, lysed of red blood cells, and stored at -20°C. Testing of hybridoma-derived antibodies was done to determine optimal concentrations for successful NK cell depletion. All *in vivo* antibodies were diluted with PBS and administered by i.p. injection every two days. Each mouse received 200µg of either anti-CD90.2 or IgG per injection. For tumor experiments, antibody depletions were administered 3 days before tumor challenge.

Tumor challenge

B16F10, B16-V, B16-IL33, EL4 and BPS1 cells were thawed and passaged twice in RPMI with 10% FBS. All B16 cells are adherent and required trypsinization to detach from flasks. Tumor cells were thoroughly washed with and resuspended in PBS at the desired concentration. All tumor injections were given as a 200µl s.c. injection. In almost all *in vivo* experiments, mice were given 1×10^6 tumor per injection. Tumor volumes typically became measurable after 1 week (~150-300mm^3). Volumes were evaluated every other day by measuring along orthogonal axes (a, b, and c) and calculated as (a*b*c)/2.

IL-33 treatment

Mice received i.p. injections of either PBS or 1µg of recombinant IL-33 (BioLegend) diluted in 200µl of PBS. Treatments began 3 days after tumor challenge and were administered every other day until completion of the experiment. Mouse health was assessed daily.

2.10 Statistical analysis

Statistics were performed using GraphPad Prism 5 software. Mean values were compared using unpaired Student's *t* test. Comparisons were two-tailed unless otherwise stated. Probability values (*P*) ≥0.05 were not considered to be significant.

Chapter 3: Results

3.1 IL-33 impedes tumor growth independent of adaptive immune system

Previous work in our own lab has demonstrated that exogenous IL-33 can improve dendritic cell activation and maturation in cancer and serves to coordinate robust antitumor effects effectuated by $CD8^+$ T cells. Treatment of established B16F10 melanomas in WT C57BL/6 mice with recombinant IL-33 (rIL-33) drastically impaired tumor growth (**Figure 3.1A**). However, the contribution of the innate immune system in IL-33 mediated antitumor effects has not been adequately explored. A similar experiment was conducted utilizing $Rag1^{-/-}$ mice—mice lacking T and B cells. Following a similar treatment regimen established in the previous study, 1μg of rIL-33 was given to B16F10 melanoma-bearing mice by i.p. injection every other day starting 3 days after tumor challenge. Interestingly, tumor growth was similarly delayed as it was in WT mice, suggesting innate immunity is sufficient in contributing to IL-33-driven antitumor responses (**Figure 3.1B**).

A similar experiment was conducted using the EL4 lymphoma model. $Rag1^{-/-}$ mice were given subcutaneous injections of EL4 cells and later treated with rIL-33. IL-33-treated groups again displayed significant reductions in tumor growth, indicating these effects are not unique to B16F10 melanoma (**Figure 3.1C**).

We also utilized a line of B16F10 cells genetically engineered to express IL-33 (B16-IL33), thus allowing us to examine the effects of intratumoral expression of IL-33 on tumor development. A control line expressing an empty vector (B16-Vec) was paired with B16-IL33 cells. Both cell lines share similar viability and growth in culture, which is consistent with our own observations that IL-33 does not directly impact B16F10 viability. *In vivo*, however, B16-IL33 tumor growth in

Rag1$^{-/-}$ mice was dramatically impaired compared to B16-Vec tumors, indicating increased local presence of IL-33 is capable of significantly delaying tumor growth (**Figure 3.1D**). Analysis of tumor lysates verified significantly elevated levels of IL-33 in B16-IL33 tumors while IL-33 was undetectable in B16-Vec (**Figure 3.2A**). Additionally, IL-33 was elevated in the serum of two out of four B16-IL33 tumor-bearing mice though it remained undetectable in B16-Vec-bearing mice (**Figure 3.2B**).

Though studies maintain that IL-33's effects on tumor growth is variable, in our hands, IL-33 consistently behaves in an antitumor manner, and independent of adaptive immunity. These effects are consistent across multiple tumor models and enacted via systemic and local administrations of IL-33.

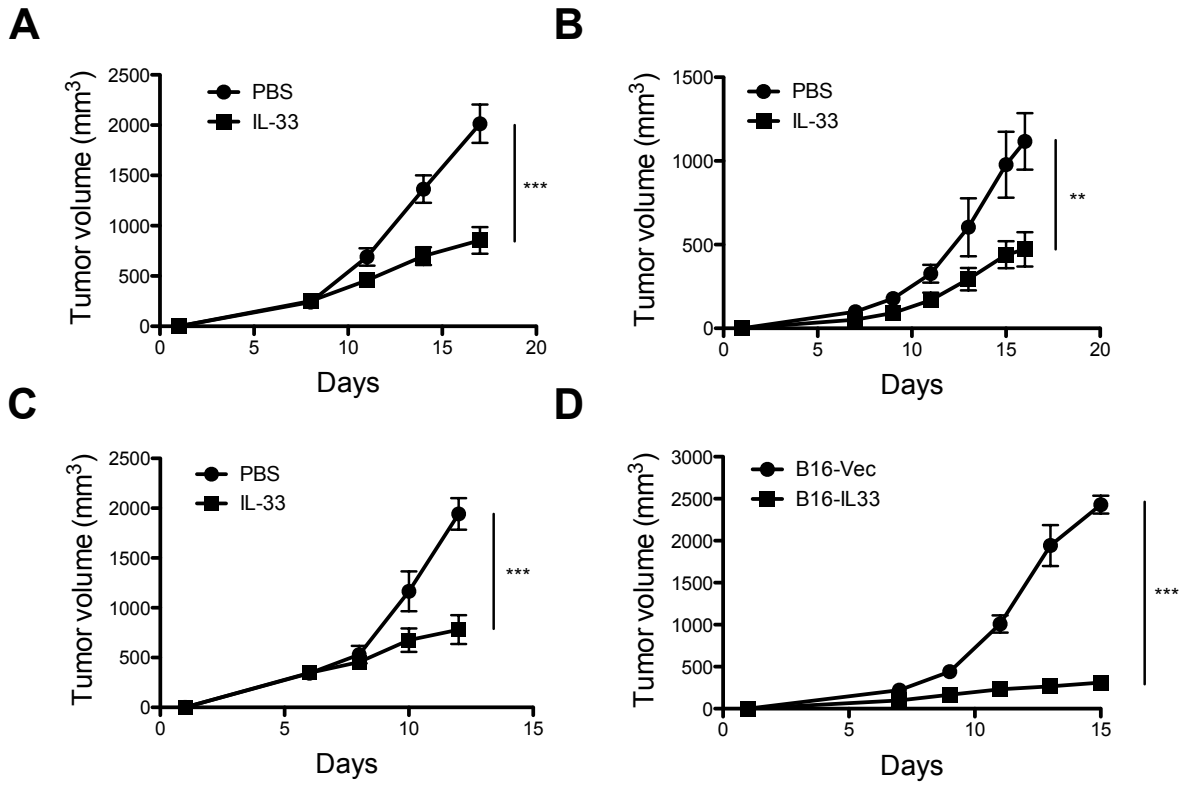

Figure 3.1. IL-33 delays tumor growth in Rag1$^{-/-}$ mice.

(a) WT C57BL/6J mice were administered s.c. injections of B16F10 tumor cells. Seven days following tumor challenge, 1µg rIL-33 was administered every other day (n=5 mice per group). (b) Rag1$^{-/-}$ mice began rIL-33 treatment as described above 3 days after s.c. injection of B16F10 cells (n=5 mice per group) or (c) EL4 cells (n=4 mice per group). (d) Rag1$^{-/-}$ mice (n=5 mice per group) were s.c. injected with control B16 cells (B16-Vec) and IL-33-expressing B16 cells (B16-IL33). Data are shown as mean ± SEM. ** $P< 0.01$, *** $P<0.001$ as determined using a Student's *t*-test.

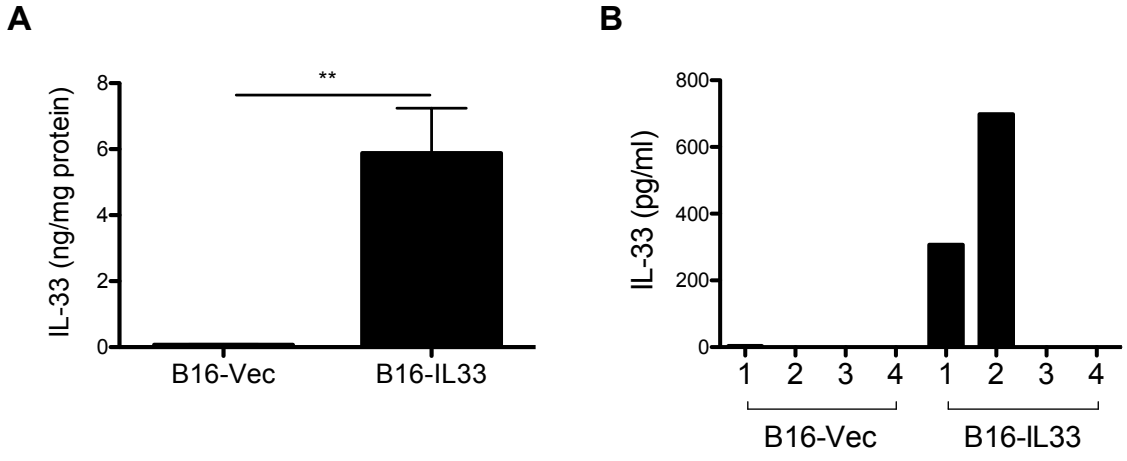

Figure 3.2. IL-33 assessment in melanoma-bearing Rag1$^{-/-}$ mice.

(a) ELISA measurement of IL-33 in lysates of B16-Vec and B16-IL33 tumors. (b) Serum concentrations of IL-33 in B16-Vec and B16-IL33 tumor-bearing mice. Data are shown as mean ± SEM. ** $P< 0.01$ as determined using a Student's *t*-test.

3.2 NK cells contribute to IL-33-mediated antitumor effects

Natural killer (NK) cells have long been known for their potent cytotoxic capabilities and their role in antitumor responses. IL-33 has been shown to directly stimulate the activities of NK cells (PMID:23499895).[88] Interestingly, B16-IL33 tumors of $Rag1^{-/-}$ mice displayed dramatically elevated numbers of NK cells (**Figure 3.3A**) compared to B16-Vec. These intratumoral NK cells also expressed higher levels of NKG2D and IFN-γ (**Figure 3.3B**). Spleens of mice bearing B16-IL33 tumors similarly demonstrated greater numbers of NK cells (**Figure 3.3C**).

We next explored the potential contribution of NK cells in these IL-33-mediated-antitumor effects. $Rag1^{-/-}$ mice were administered depletion antibody against NK1.1 by i.p. injection prior to B16F10 tumor challenge. Similar to previous experiments, mice were given rIL-33 three days after tumor challenge. Tumors in NK-depleted mice grew significantly faster than in mice given IL-33 treatment alone (**Figure 3.4A**). Though the tumors of NK-depleted mice did not fully match the rate of tumors in untreated mice, these data indicate NK cells play a significant role in IL-33-driven antitumor responses.

To verify IL-33 directly drives NK cells-mediated tumor killing, NK cells and B16F10 were cocultured with or without rIL-33 overnight. Tumor cell death was assessed by annexin V and 7-AAD staining. Though the presence of NK cells appeared to partially increase tumor cell death even without IL-33 treatment, the addition of IL-33 significantly increased overall tumor cell death (**Figure 3.4B**). As expected, IL-33 alone did not impact tumor cell viability.

Taken collectively, these data suggest IL-33 drives NK cell activity and tumor killing, which contributes to the overall antitumor effect.

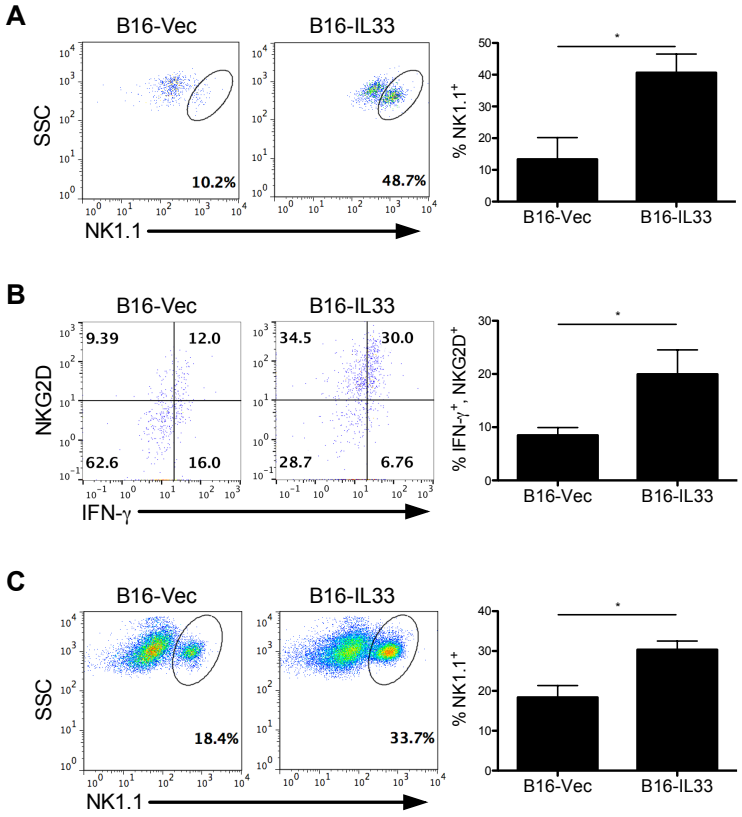

Figure 3.3. IL-33 elevates NK cell expansion and activation in melanoma.

(a) Representative flow cytometry dot plots illustrate frequencies of intratumoral NK1.1^{+} NK cells in Rag1$^{-/-}$ mice bearing B16-Vec versus B16-IL-33 tumors (b) and levels of NKG2D and IFN-γ expression (n=5 mice per group). (c) Representative flow cytometry analysis displaying frequencies of splenic NK cells in B16-Vec and B16-IL33 tumor-bearing Rag1$^{-/-}$ mice (n=5 mice per group).

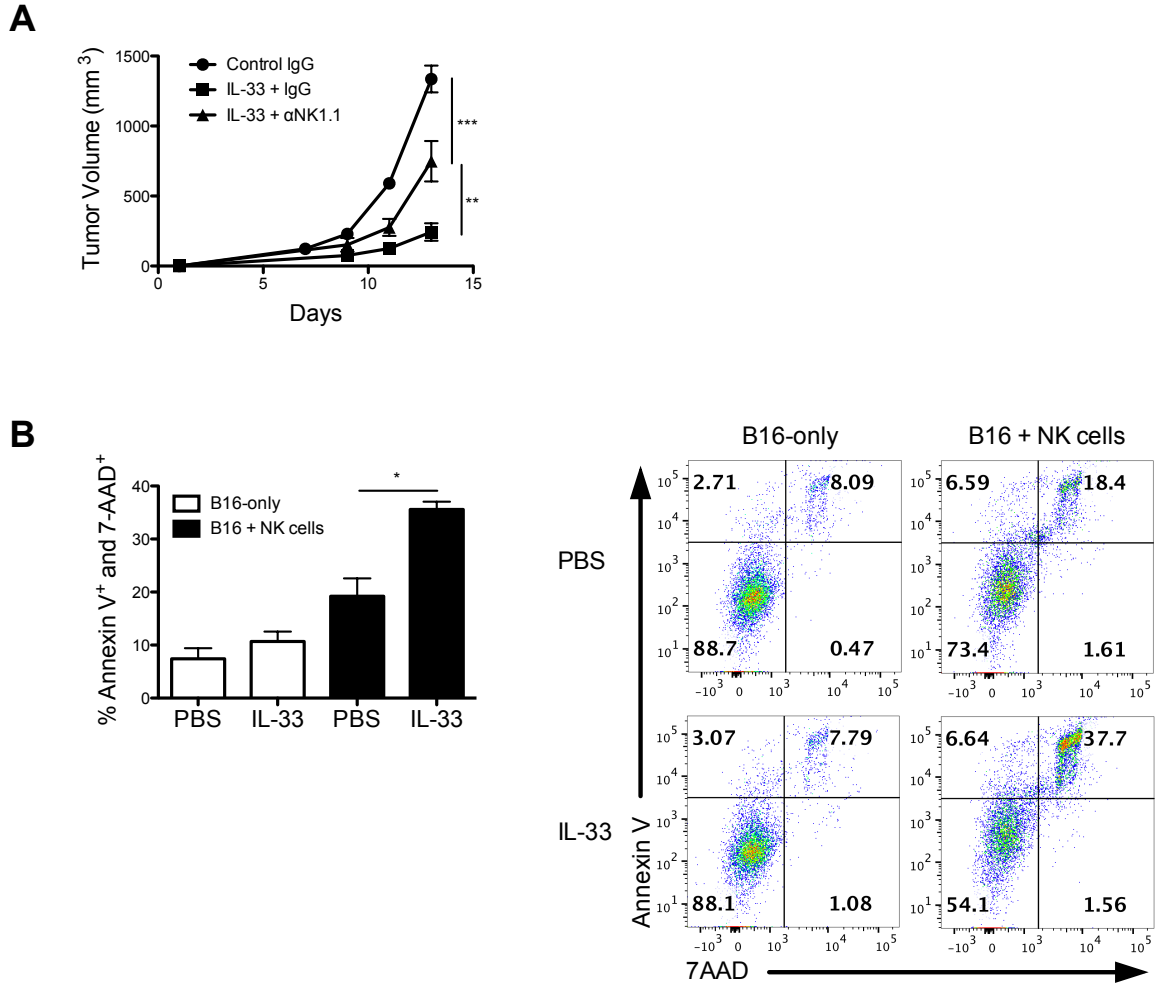

Figure 3.4. NK cells facilitate IL-33-mediated antitumor responses.

(a) Rag1$^{-/-}$ mice were administered either IgG or anti-NK1.1 depletion antibodies 3 days before B16F10 tumor challenge. 1μg IL-33 was administered every other day starting 3 days after tumor challenge (n=5 mice per group). (b) B16F10 cells were cultured with NK cells at 1:20 ratio and treated with recombinant IL-33 for 24h. B16F10 cell death was determined by flow cytometric analysis measuring annexin V and 7-AAD. Data are shown as mean ± SEM. * $P< 0.05$, ** $P< 0.01$, *** $P<0.001$ as determined using a Student's *t*-test.

3.3 IL-33 promotes expansion of CD90$^+$ type 2 innate lymphoid cells in tumors

Innate lymphoid cells (ILCs) consitute the most recently identified branch of the innate immune system. Similar to the T helper subsets, ILCs are of lymphoid morphology and similarly express the thymocytic marker Thy-1, or CD90.[150] Interestingly, CD90$^+$ cells were greatly enriched in B16F10 tumors of Rag1$^{-/-}$ mice receiving rIL-33 treatment compared to PBS-treated mice (**Figure 3.5A**). Considering these mice lack T and B cells, these CD90$^+$ cells represented ILCs. IL-33-mediated expansion of tumoral ILCs was not unique to B16F10 melanoma as similar increases in ILCs were observed in EL4 tumors following IL-33 treatment (**Figure 3.5B**). These ILCs were further defined as CD11b$^-$CD11c$^-$NK1.1$^-$FcεRI$^-$CD25$^+$CD45$^+$, which were also expanded in spleens (**Figure 3.5C**). In BPS1 tumors, immunofluorescence confirmed an increase in CD90$^+$ cells in response to IL-33 (**Figure 3.5D**).

As mentioned before, innate lymphoid cells comprise a new branch of immune cells whose characterization is still the subject of investigation. Three distinct subsets of ILCs have been defined, based on a combination of surface markers and their hallmark features including cytokine production and transcription factor expression. Still, considering the developing nature of ILCs in current literature, it is important to characterize the expanding ILCs in IL-33-treated mice and how they respond to IL-33.

Type 2 ILCs (ILC2s) are the only known ILC subset to respond directly to IL-33 and are unique in their expression of the IL-33 receptor, ST2.[149] Additionally, secretion of type 2 immune cytokines such as IL-5 and IL-13 are defining characteristics of ILC2s.[142, 143, 144] Considering this, we hypothesized that ILC2s represented the predominant tumoral ILC population expanding in

response to IL-33. Examination of B16-IL33 tumors revealed a dramatic increase in $ST2^+$ $IL\text{-}5^+$ ILCs compared to B16-Vec tumors, indicative of ILC2 expansion (**Figure 3.6A**).

While these data indicate IL-33 promotes the accumulation of ILC2s in B16 tumors, we next examined the presence of other ILC subsets based on cytokine expression. In regard to cytokine production, type 1 innate lymphoid cells (ILC1) have routinely been characterized by IFN-γ secretion;[138] ILC2s by IL-5 and IL13 production,[142, 143, 144] and type 3 innate lymphoid cells (ILC3) by IL-17 and IL-22.[150] As expected, examination of intratumoral ILCs in B16-IL33 tumors in $Rag1^{-/-}$ mice again revealed a substantial increase in IL-5-producing ILC2s compared to B16-Vec (**Figure 3.6B**). IFN-γ-producing ILC1s did not expand in B16-IL33 tumors and their overall presence was marginal. $IL\text{-}17^+$ ILC3s were almost entirely absent in B16-Vec tumors but were moderately upregulated in B16-IL33. However, these ILC3s represented a much smaller proportion of the total ILCs compared to ILC2s (**Figure 3.6C**). Interestingly, a subset of IL-5-expressing ILC2s co-expressed IL-10 (**Figure 3.6D**) These data indicate the ILC2 subset preferentially expands in tumors in response to IL-33 and represents the majority of intratumoral ILCs both with and without implementation of IL-33.

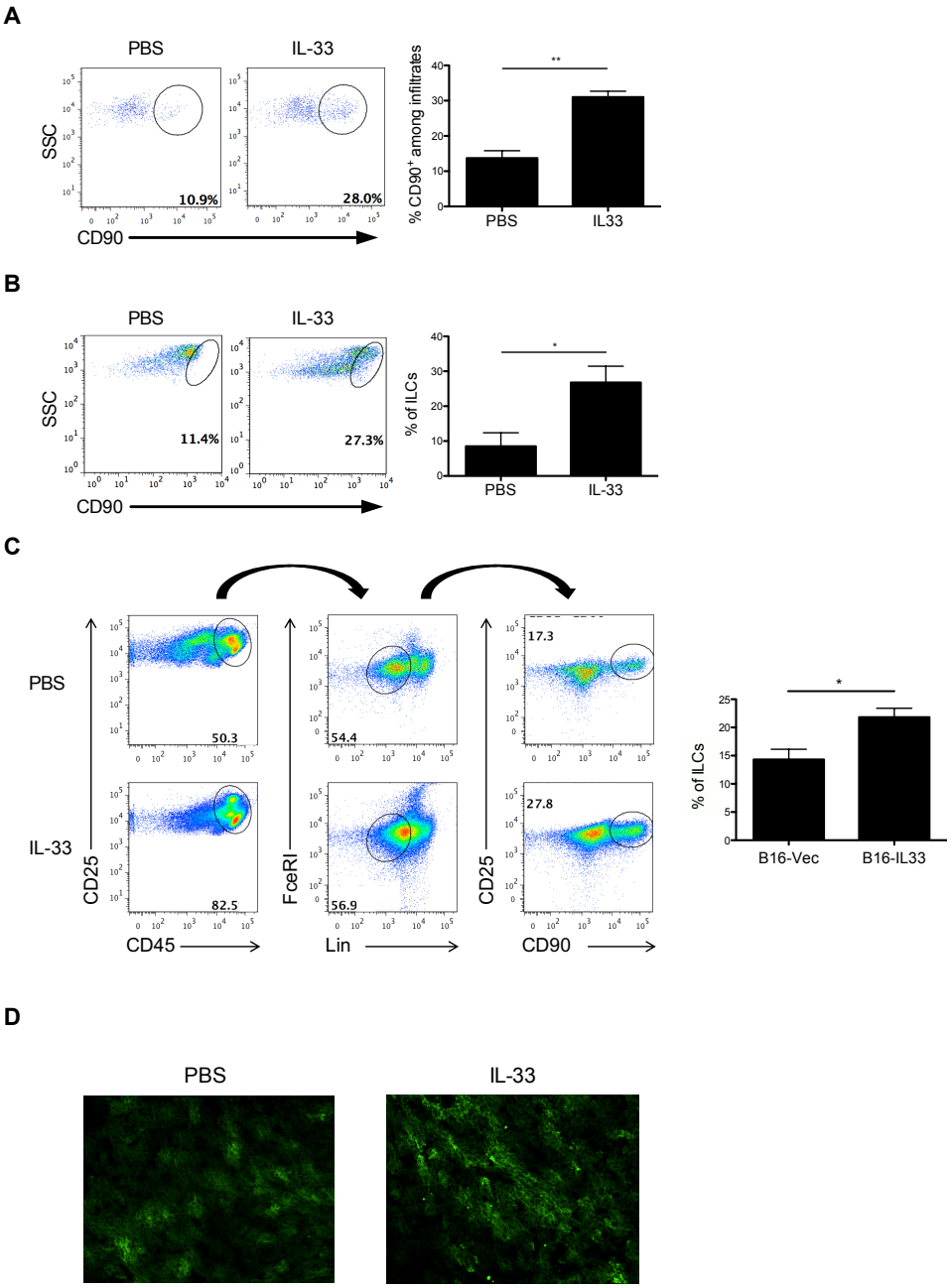

Figure 3.5. IL-33 promotes expansion of tumoral CD90$^+$ ILCs.

(a,b) Representative flow dot plots illustrating (a) intratumoral and (b) splenic presence of CD90$^+$Lin$^-$ ILC2s and corresponding bar graphs showing the percentage of ILC2s among

infiltrates in B16F10-bearing Rag1$^{-/-}$ mice treated with PBS versus IL-33. ILC2s were pregated on CD45^{+}CD11b^{-}CD11c^{-}NK1.1^{-}FcεRI^{-}. (n=5 mice per group). (c) Flow cytometric analysis of splenic ILC2s in tumor-bearing Rag1$^{-/-}$ mice treated with PBS vs IL-33 representing the gating strategy used to identify ILC2s. Cells were gated on the Lin^{-} (lineage negative) population, indicating CD11b^{-}CD11c^{-}Gr1^{-}NK1.1^{-}. (d) Immunofluorescent analysis of BPS1 tumors of Rag1$^{-/-}$ mice demonstrate presence of CD90+ cells among IL-33-treated mice. Data are shown as mean ± SEM. * P<0.05 ** P< 0.01 as determined using a Student's *t*-test.

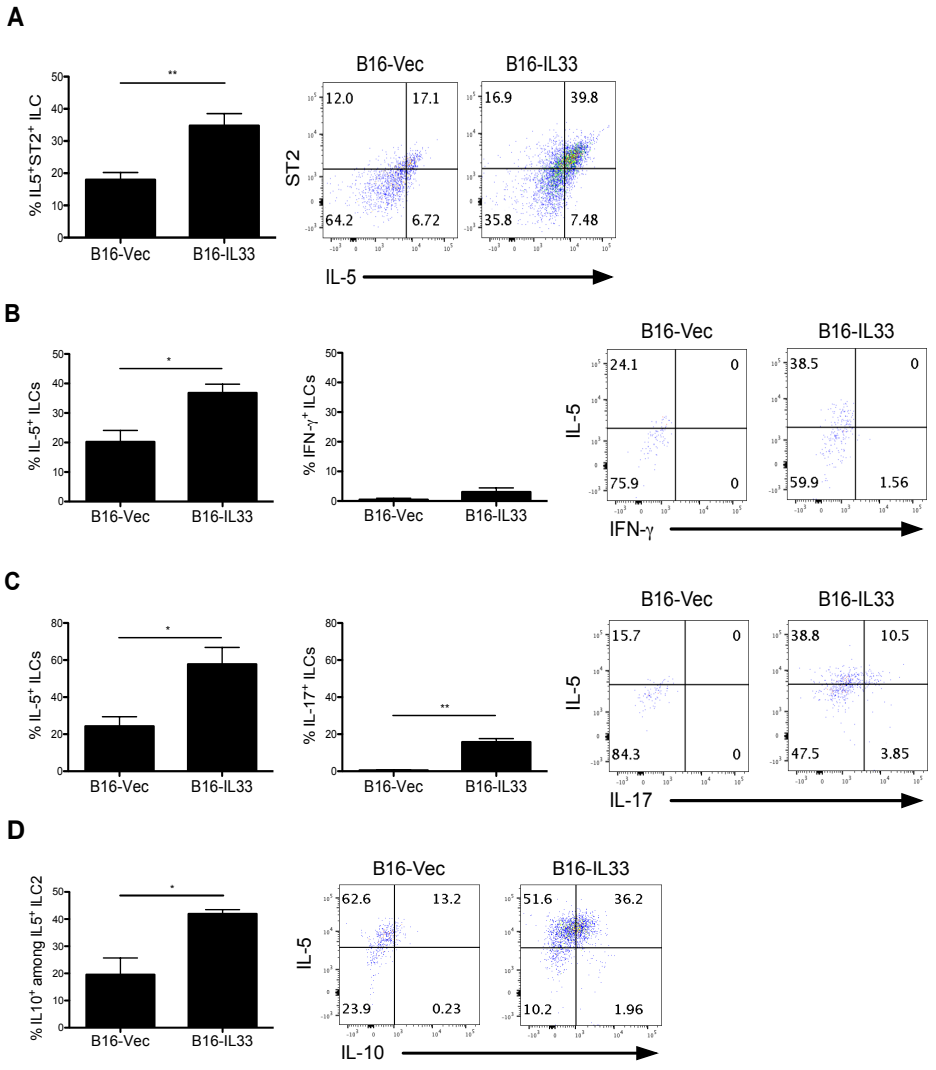

Figure 3.6. IL-33 preferentially promotes expansion of type 2 ILCs in tumors.

(a) Evaluation of ST2 and IL-5 expression by intratumoral ILC2s from B16-Vec or B16-IL33 tumor-bearing Rag1$^{-/-}$ mice (n=4 mice per group). Cells were pregated on CD90^{+} cells. (b) Measurement of B16-Vec and B16-IL33 tumors for ILC1 and ILC2 presence based on IFN-γ and IL-5 production respectively. ILCs were pregated on CD45^{+}CD90^{+} cells and excluded NK1.1^{+} NK cells. (c) ILC2 and ILC3 measurement based on IL-5 and IL-17 secretion respectively. ILCs

were pregated on $CD45^{+}CD90^{+}$ cells. (d) Expression of IL-5 and IL-10 among intratumoral ILCs (n=4 mice per group). Data are shown as mean ± SEM. * $P< 0.05$, ** $P< 0.01$ as determined using a Student's *t*-test.

3.4 IL-33-expanded ILC2s facilitate tumor growth.

Very little has been described about the possible role ILC2s play in tumor development. To examine the effect of these IL-33-driven ILC2s on tumor growth, depletion antibodies against CD90 were used to deplete mice of ILCs. As expected IL-33 treatment alone dramatically delayed B16F10 tumor growth in $Rag1^{-/-}$ mice (**Figure 3.7A**). Interestingly, depletion of ILC2s further suppressed tumor growth, highlighting a protumor role of ILC2s (**Figure 3.7A**). Administration of CD90 depletion antibodies alone did not significantly impact tumor growth, indicating IL-33 administration is required to drive the tumor-promoting role of ILC2s (**Figure 3.7B**). Successful depletion of $CD90^{+}$ cells was verified in both tumors and spleens (**Figure 3.7C&D**).

To further examine the potential protumor impact of ILC2s in tumor development, ILC2s were co-injected with B16F10 cells into $Rag1^{-/-}$ mice. Co-injection of ILC2s both accelerated tumor occurrence and hastened the time to tumor endpoints compared to B16F10 injection alone (**Figure 3.7E&F**).

These data demonstrate a novel tumor-promoting role of ILC2s in the context of IL-33 treatment.

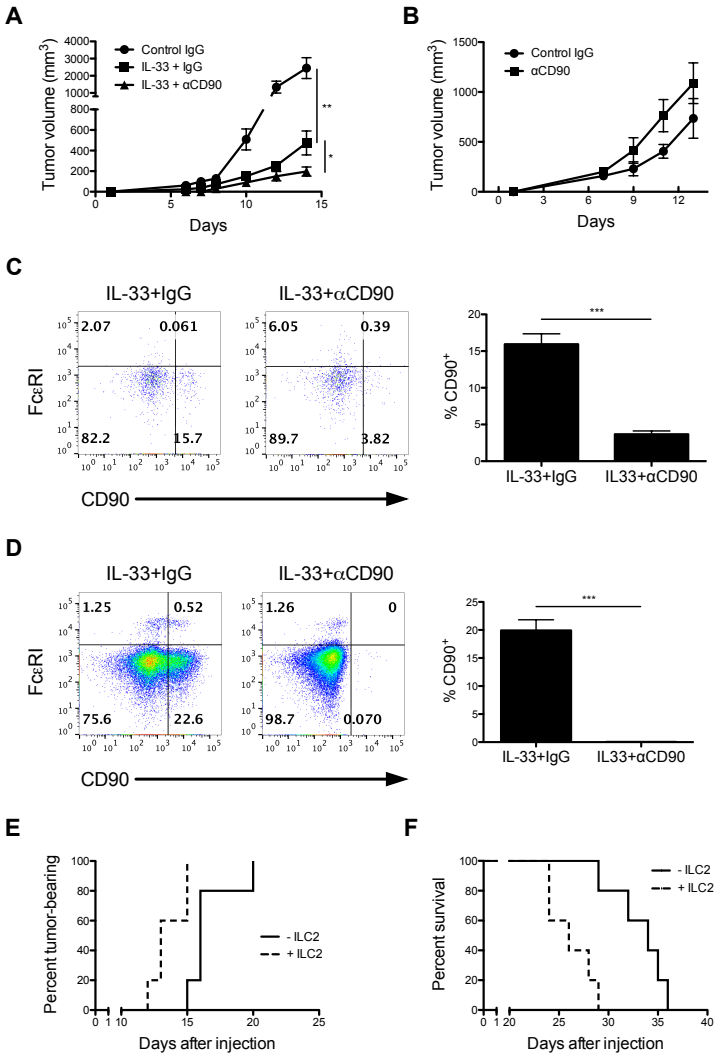

Figure 3.7. IL-33-expanded ILC2s facilitate tumor growth.

(a) B16F10 tumor growth curves in Rag1$^{-/-}$ mice demonstrating effects of IL-33 treatment with or without depletion of ILCs by anti-CD90 antibodies (n=5 mice per group). (b) Impact of anti-CD90 treatment alone on tumor B16F10 tumor growth in Rag1$^{-/-}$ mice (n=4 mice per group). (c) Representative flow panels verifying ILC2 depletion in B16F10 tumors and (d) spleens via administration of anti-CD90 antibodies. ILC2s were pregated on CD11b^{-}FceRI^{-}NK1.1^{-} population.

Experiment was performed in $Rag1^{-/-}$ mice. In all experiments involving anti-CD90 antibodies, equivalent quantities of rat IgG were injected in control mice as an isotype control. (e) Following the co-injection of B16F10 cells and ILC2s in $Rag1^{-/-}$ mice, mice were monitored for tumor occurrence and (f) until tumors reached their endpoints. 5000 B16F10 cells were co-injected with $1*10^6$ ILC2s (n=5 mice per group). Tumor endpoint was determined when tumors reached $1cm^3$. Data are shown as mean ± SEM. * $P<0.05$ ** $P< 0.01$ as determined using a Student's *t*-test.

3.5 IL-33-expanded innate lymphoid cells display characteristic features of type 2 innate lymphoid cells

We wished to further examine the characteristics of these IL-33-expanded ILCs *in vitro*. $CD90^+$ ILCs were isolated from the spleens of IL-33-treated $Rag1^{-/-}$ mice and cultured with or without rIL-33 overnight. The majority IL-33-treated ILCs expressed Ki-67, a marker of proliferation (**Figure 3.8A**). The vast majority of these proliferating cells additionally expressed CD25, which is typically expressed on ILC2s.[173] No CD25 was detected among the untreated ILCs. IL-33-mediated proliferation was further verified by greater dilution of proliferation dye efluor450 among IL-33-treated ILCs compared to untreated cells (**Figure 3.8B**).

As previously mentioned, hallmark traits of ILC2s include their secretion of IL-5 and IL-13 upon activation.[142, 143, 144] Additionally, they depend on transcription factor GATA3 for their development and maintenance. Isolated ILCs from IL-33-treated $Rag1^{-/-}$ mice expressed IL-5 and IL-13 in response to rIL-33 (**Figure 3.8C**). These cytokine-expressing ILCs were almost exclusively $CD25^+$. Similarly, IL-33 elicited dramatic upregulation of GATA3, again accompanied by CD25 expression (**Figure 3.8C**).

ST2 is the only known receptor for IL-33, and ILC2s are the only known ILC subset to express ST2 (**PMID:25591466**).[173] We next sought to evaluate the dependence of IL-33 stimulation of ILC2s on ST2 expression. Isolated ILCs from IL-33-treated $Rag1^{-/-}$ mice were incubated with a ST2-blocking antibody prior to treatment with rIL-33. ILCs treated with control IgG expectedly exhibited robust CD25 expression (**Figure 3.8D**). Blocking ST2, however, completely ablated CD25 expression, indicating ST2 is required for IL-33-mediated activation of isolated ILC2s.

These data further indicate that isolated $CD90^{+}$ ILCs from IL-33-treated $Rag1^{-/-}$ mice display the major iconic features of ILC2s in their responses to IL-33.

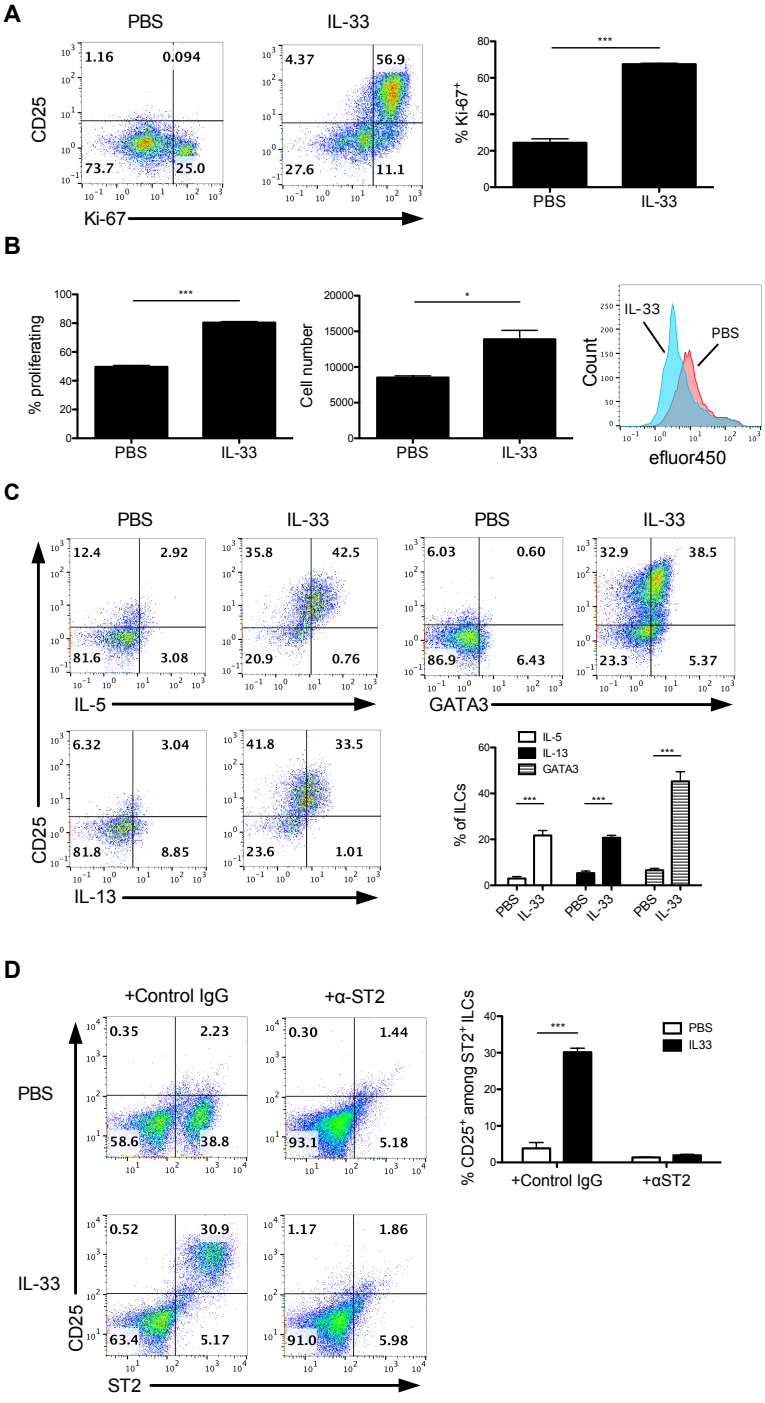

Figure 3.8. IL-33 promotes ILC2 expansion and activity in an ST2-dependent manner.

(a) CD25 and Ki-67 expression in ILC2s treated with IL-33 *in vitro* measured by flow cytometry. (b) Measurement of ILC2 proliferation indicated by efluor450 signal and cell number after 24 hours. (c) Measurement of IL-5, IL-13, and GATA3 in ILCs *in vitro*. (d) ILC2 activation *in vitro* following blockade of IL-33-ST2 signaling by anti-ST2 (clone DJ8) antibodies. Rat IgG was used as an isotype control. Data are shown as mean ± SEM. * $P< 0.05$, *** $P<0.001$ as determined using a Student's *t*-test.

3.6 Type 2 innate lymphoid cells impede NK cell activity during IL-33 treatment

Thus far, we have demonstrated IL-33 elicits potent antitumor behavior in NK cells. Concurrently, IL-33 drives a heretofore undescribed protumor role of ILC2s. We next examined the potential impact of ILC2s on NK cell activity during tumor development. As expected IL-33 treatment expanded the presence of NK cells in spleens (**Figure 3.9A**). Interestingly, antibody depletion of $CD90^+$ ILC2s further increased the number of NK cells. These NK cells displayed upregulated expression of NKG2D, a classic marker of NK cell activation (**Figure 3.9B**). Similar results were observed in B16F10 tumors; depletion of ILCs in combination of rIL-33 treatment resulted in enhanced numbers of activated intratumoral NK cells (**Figure 3.9C**). These data demonstrate ILC2s are capable of suppressing NK cell activity.

ILC2-mediated suppression of NK cells was further examined *in vitro* to examine whether suppression occurred directly. In cocultures of B16F10, NK cells, and isolated ILC2s, the addition of ILC2s abrogated NK cell-mediated tumor cell killing (**Figure 3.10A**). In a separate experiment, NK cells were treated with rIL-33 prior to coculturing with ILC2s. These ILC2s were pretreated with IL-33 with or without antibody blockade of ST2. rIL-33 was confirmed to greatly upregulate IFN-γ production by NK cells (**Figure 3.10B**). However, addition of IL-33-treated ILC2s impaired IFN-γ expression. Blocking ST2 on ILC2s prior IL-33 stimulation restored IFN-γ production in NK cells during coculturing. These data strongly support the previous observation that IL-33 directly stimulates NK cell activity and tumor cell-killing. At the same time, ILC2s directly impair NK cell functions in an IL-33/ST2-dependent manner.

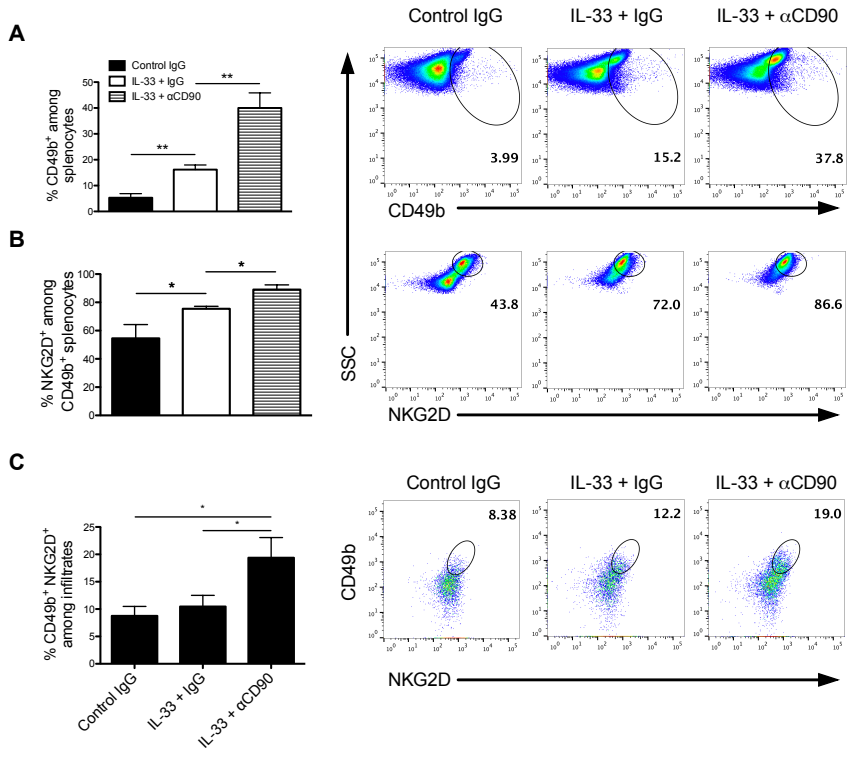

Figure 3.9. Depletion of ILC2s enhances NK cell accumulation and activity.

(a,b,c) NK cell presence (CD49b) and activation (NKG2D) in (a,b) spleens and (c) B16F10 tumors following depletion of ILC2s using anti-CD90 antibodies in combination with IL-33 treatment. Experiment was performed in B16F10 tumor-bearing $Rag1^{-/-}$ mice. Rat IgG was used as an isotype control. The difference between 'IL-33' and 'IL-33+αCD90' in figure c is significant only by one-tailed Student's *t*-test. (n=4 mice per group). Data are shown as mean ± SEM. * $P< 0.05$, *** $P<0.001$ as determined using a Student's *t*-test.

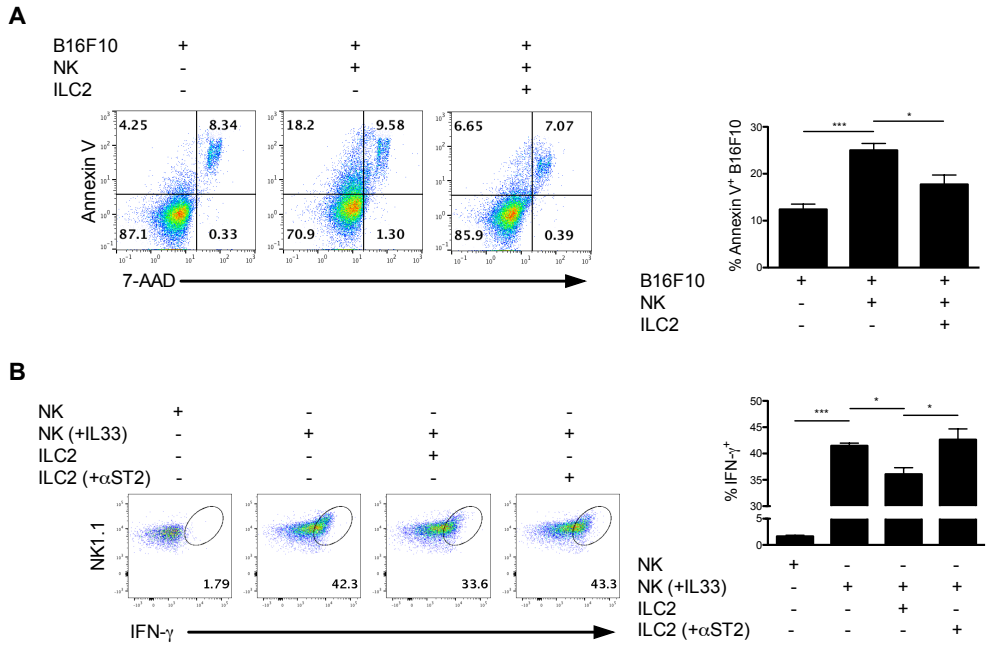

Figure 3.10. ILC2s directly impede NK cell activity in IL-33/ST2-dependent manner.

(a) Cocultures of B16F10, NK cells, and ILC2s with 10ng/ml recombinant IL-33 were prepared. B16F10 cell death measured by annexin V after 24 hours. Cells were cultured at a ratio of 1:20:20 B16F10:NK:ILC2. (b) IFN-γ production by NK cells in coculture with ILC2s. NK cells were pretreated with 20ng/ml recombinant IL-33 and 10ng/ml IL-2. Splenic ILC2s were pretreated with 20ng/ml IL-33 and 10ng/ml IL-2 with or without anti-ST2 blocking antibody (clone: DJ8). NK cells and ILC2s were then washed and co-cultured for 24 hours. Rat IgG was used as an isotype control. Data are shown as mean ± SEM. * $P< 0.05$, ** $P< 0.01$, *** $P<0.001$ as determined using a Student's *t*-test.

3.7 Type 2 innate lymphoid cells suppress NK cell activity via CD73 expression.

We clearly demonstrated ILC2s directly suppress NK cell activity. The mechanism by which this suppression occurs, required elucidation. Examination of ILC2s in tumors and spleens of B16-IL33-bearing mice revealed robust expression of CD73 compared to those in B16-Vec tumors (**Figure 3.11A&B**). Isolated ILC2s exhibited elevated expression of CD73 in response to IL-33 *in vitro* (**Figure 3.11C**). Further, CD73 was preferentially expressed among $ST2^+$ ILC2s compared to $ST2^-$ ILCs. CD73 is an ecto-enzyme which, working in conjunction with CD39, is responsible for the catabolization of ATP into free adenosine. Adenosine has well-defined immunosuppressive properties and is capable of attenuating antitumor immune responses.[48] The tumor-protective qualities of CD73 have been well-characterized[51, 52, 53] and we have previously demonstrated CD73 deficiency protects against melanoma.[174]

ILC2s were verified to catabolize AMP into adenosine. An AMP detection assay confirmed IL33-stimulated ILC2s consumed AMP *in vitro*. ILC2s generated from $CD73^{-/-}$ mice, however, failed to consume AMP (**Figure 3.12A**), confirming that ILC2-derived CD73 is catalytically active. CD73 expression did not exhibit any effect on ILC2 development and cytokine production, verified by IL-5 expression among $CD73^{-/-}$ ILC2s generated from bone marrow (**Figure 3.12B**).

We next examined the potential significance of ILC2-derived CD73 in suppressing NK cells. NK cells were cocultured with ILC2s generated from the bone marrow of either WT or $CD73^{-/-}$ mice receiving IL-33 treatment. Bone marrow cells cultured without IL-33 served as "control" ILC2s. WT ILC2s expectedly impaired NK cells activity, indicated by reduced CD107a expression (**Figure 3.12C**). Interestingly, CD73-deficient ILC2s failed to suppress NK cells to the same

degree as WT ILC2s, indicating CD73 plays a significant role in ILC2-mediated suppression of NK cells.

No CD73 expression was detected on NK cells (**Figure 3.13A**). Further, NK cells isolated from $CD73^{-/-}$ showed no difference in their cytolytic capabilities (**Figure 3.13B**). These data further substantiate the importance of ILC2-derived CD73 in the suppression of NK cells.

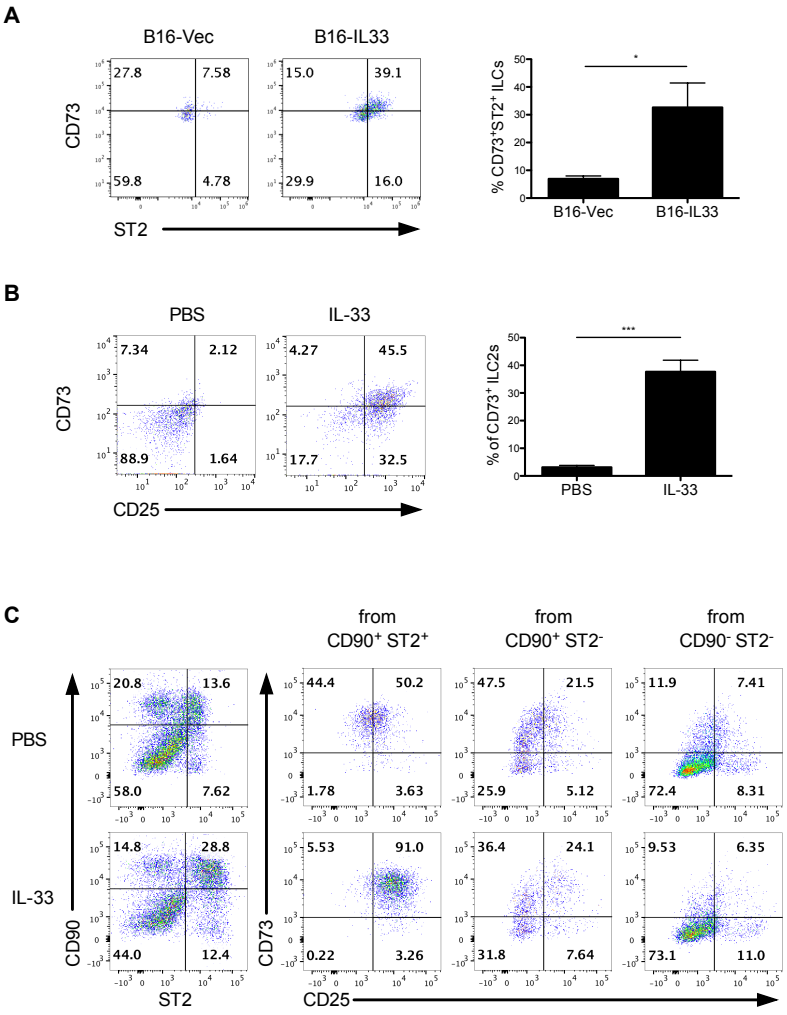

Figure 3.11. CD73 is expressed on ILC2s.

(a) Flow cytometric analysis of CD73 expression by intratumoral ST2$^+$ ILC2s from B16-Vec or B16-IL33 tumor-bearing Rag1$^{-/-}$ mice (n=4 mice per group). (b) CD73 expression on activated splenic CD25$^+$ ILC2 from PBS and IL-33-treated tumor-bearing Rag1$^{-/-}$ mice (n=4 mice per group). (c) Enriched splenic CD90$^+$ ILCs from Rag1$^{-/-}$ mice were stimulated by PBS or IL-33 for 24h *in vitro*, and expression of CD73 and ST2 on CD90$^+$ ILCs was determined by flow cytometry (*left*). Expression of CD73 and CD25 was examined among ST2$^+$CD90$^+$, ST2$^-$CD90$^+$ and ST2$^-$

CD90^{-} subsets (*right*). Data are shown as mean ± SEM. * $P< 0.05$, *** $P<0.001$ as determined using a Student's *t*-test.

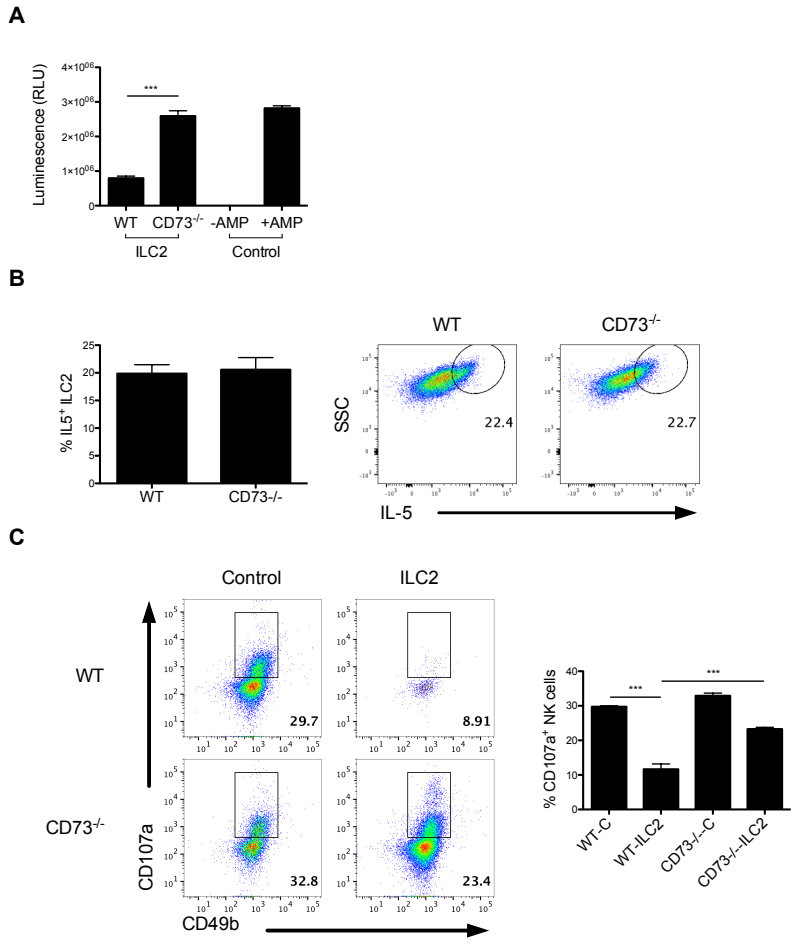

Figure 3.12. ILC2s suppress NK cells in an CD73-dependent manner.

(a) AMP-Glo assay was used to quantify AMP consumption by WT and CD73$^{-/-}$ ILC2 cultures. (b) Flow cytometric analysis of IL-5 production by WT and CD73$^{-/-}$ ILC2s generated from bone marrow. (c) CD107a expression among NK cells in coculture with WT or CD73$^{-/-}$ ILC2s. Control samples included BM cells cultured without IL-33 to serve as "control" ILC2s (WT-C and CD73$^{-/-}$-C). ILC2s and NK cells were cocultured at a 1:1 ratio in 50µM AMP and 10ng/ml IL-33. Data are shown as mean ± SEM. ** $P< 0.01$, *** $P<0.001$ as determined using a Student's *t*-test.

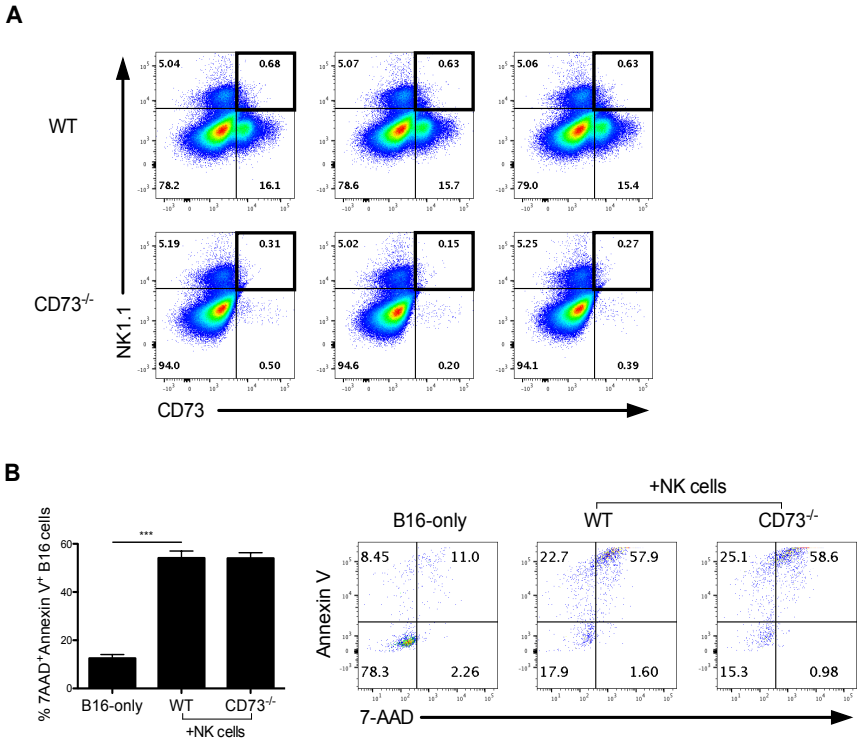

Figure 3.13. NK cells lack CD73 and their activity is not affected by its loss.

(a) Flow cytometric analysis of WT and CD73$^{-/-}$ murine spleens. (b) B16F10 cell death was assessed by annexin V and 7-AAD following coculture with WT and CD73$^{-/-}$ NK cells. B16F10 and NK cells were cultured at a ratio of 1:20. Data are shown as mean ± SEM. *** $P< 0.001$ as determined using a Student's *t*-test.

Chapter 4: Discussion

4.1 IL-33 controls tumor growth via innate immune system

This body of work has clearly characterized a robust antitumor role of IL-33 in established preclinical tumor models consistent with previous studies.[88, 125, 175] The antitumor role of IL-33 is observable with both systemic administrations of recombinant IL-33 and local ectopic expression of IL-33 within tumors. Previous work in our lab demonstrated the importance of adaptive immunity in IL-33-mediated antitumor responses.[90] In the previous study (in contrast to the study outlined in this paper) IL-33 treatment had no significant effect on tumor growth in $Rag1^{-/-}$ mice, though on average, IL-33-treated tumors developed more slowly. The timing of IL-33 implementation may explain this idiosyncrasy. In the previous study, recombinant IL-33 was administered on day 7 after B16F10 melanoma cell injections, allowing the tumors to become well-established. The current study began IL-33 treatments on day 3 following melanoma cell injections. This study also utilized tumor lines ectopically expressing IL-33. In both cases, IL-33 was implemented much earlier or at the very beginning of tumor initiation. Innate immunity serves as a front-line defense, able to respond more rapidly than adaptive immunity. In the early stages of tumor development, innate immunity can play a more significant role in attenuating tumor growth. Early implementation of IL-33 may be critical in eliciting effective antitumor responses via innate immunity.

Thus far, reported antitumor effects of IL-33 have predominantly been demonstrated to be mediated via adaptive immunity. Here, however, through the utilization of $Rag1^{-/-}$ mice, we demonstrate for the first time IL-33 is capable of exerting potent antitumor effect *in vivo* solely through the innate immune system.

4.2 IL-33 promotes NK cell activation in tumors

NK cells have long been described as effective mediators of innate antitumor immunity.[22] Acknowledgement of their potential as potent effectors of antitumor responses has led to a surge in interest in utilizing NK cells in adoptive cellular therapy.[58, 59, 60] Evidence has emerged that illustrates ST2 is required for NK cell-mediated protection against cytomegalovirus and that IL-33 can directly promote expansion of NK cells.[176] During the course of this study, new evidence has demonstrated ectopic expression of IL-33 in tumors can also promote expansion and activation of NK cells.[175] Our study verified IL-33-expressing B16 tumors accumulated significantly higher numbers of NK cells. However, the NK-stimulating effects seen via intratumoral expression of IL-33 was verified in a more therapeutic implementation of IL-33. Here, we examined the impact of exogenous treatment with recombinant IL-33 on NK cells and again observed a marked elevation in the presence of activated $NKG2D^{+}$ NK cells.

We further highlight intratumoral NK cells as critical contributors to IL-33-mediated antitumor effects. The previous study utilized $Rag2^{-/-}IL2rg^{-/-}$ mice, which lack all lymphocytes including NK cells and ILCs, to illustrate a net antitumor contribution by total lymphocytes in IL-33-mediated antitumor effects.[175] This study clearly demonstrates NK cells are significant contributors to IL-33-mediated tumor inhibition *in vivo*. Antibody removal of NK cells significantly accelerated melanoma growth in IL-33-treated $Rag1^{-/-}$ mice. Our results support the previous reports released during the course of this study demonstrating NK cells are capable of contributing to antitumor immunity in tumors with ectopic expression of IL-33.[88] We further demonstrate an antitumor role

of NK cells in treatment with exogenous recombinant IL-33. Treatment of NK cells with IL-33 directly increased their cytokine production and enhanced their tumor-killing capacity *in vitro*.

As previously mentioned, prior work in our lab highlighted the importance of adaptive immunity in IL-33-mediated antitumor responses.[90, 124] CD8 T cells were necessary in effectuating IL-33-mediated attenuation of melanoma. Unlike the work described here, depletion of NK cells did not significantly impact tumor growth, though the trend suggested they may have a minor role in attenuating tumor growth. NK cells can be considered "first responders" among immune cells, capable of quickly detecting and attacking tumor cells and infected cells. As described above, early implementation of IL-33 may be critical to elicit impactful antitumor functions of NK cells.

4.3 Potential contributors to IL-33 antitumor effects

This study focused predominantly on the impact of NK cells and ILC2s in IL-33-mediated tumor responses. Both NK cells and ILC2s were present in appreciable frequencies in tumors, and both directly responded to IL-33 to elicit either protumor or antitumor functions. Ablation of NK cells in IL-33-treated $Rag1^{-/-}$ mice drastically accelerated tumor growth. However, tumor growth in untreated mice was greater still, indicating NK cells may not be the only antitumor effectors driven by IL-33.

Many other immune cells express the receptor for IL-33, ST2, and may contribute to IL-33 antitumor effects. Macrophages constitutively express ST2 and are directly activated by IL-33. Two different polarizations of macrophages have been described. Classically activated macrophages, or M1-polarized macrophages are typically activated by IFN-γ, TNF-α, and LPS. They are characterized by secretion of IL-1, IL-12, and TNF-α, and facilitate Th1 responses during

infection.[43] Cytotoxic M1 macrophages have been strongly correlated to tumor suppression and improved survival time in cancer patients.[177] Alternatively-activated macrophages, or M2-polarized macrophages, have been correlated with tumor development and progression.[46, 177] Further, M2 macrophages produce anti-inflammatory cytokines such as IL-10, and inhibit T cell activation and antigen presentation.[44, 45] Endogenous IL-33 has typically been shown to favor M2 polarization,[178] however in some cases IL-33 can also contribute to M1 polarization.[179] High doses of recombinant IL-33 or ectopic tumoral expression of IL-33 may favor M1 over M2 polarization among tumor-infiltrating macrophages, contributing to IL-33-mediated antitumor effects. Precedent for this lies in studies involving IL-33-activation of T cells. Th2 cells express ST2 at greater levels than Th1 and CD8 T cells. Lower doses of IL-33 favor Th2 activation, with Th1 and CD8 T cells requiring significantly higher levels of IL-33 to elicit activation.[131]

Eosinophils are inflammatory granulocytes critically involved in host protection against parasites. They have been implicated in tumor progression, albeit with varying outcomes. Infiltration of eosinophils has been associated with poorer outcomes in Hodgkin's lymphoma but correlate with improved outcomes in breast, prostate, and colorectal cancers.[180] Eosinophilic counts also served as a biomarker for improved prognoses in patients with melanoma and were indicative of significantly enhanced responses to checkpoint inhibitor therapy.[181] Eosinophils constitutively express ST2. IL-33 appears to be important in eosinophil homeostasis and activation during disease.[182] Though this study did not examine the role of eosinophils in melanoma, it is possible eosinophil infiltration increased in response to IL-33 treatment and may have facilitated antitumor effects.

4.4 IL-33 drives accumulation of protumor ILC2s

IL-33 drives expansion and activation of ILC2s in allergic inflammation.[153] The pathologic role of ILC2s in type 2 inflammatory diseases might be detrimental when translated to the context of tumors. However, to-date, few studies have examined the potential influence of ILC2s in tumor progression, though a few have emerged since the initiation of this study. One study observed a correlation between the ILC2/IL-13 axis and human bladder cancer recurrence in patients treated with bacillus Calmette-Guérin (BCG), first supporting the notion of a protumor role for ILC2s.[168] ILC2s were also reported to be implicated in an experimental model of cholangiocarcinoma with an exogenous administration of IL-33.[167] Another study reported that IL-33 treatment promoted the expansion of IL-13-secreting Lin^- $Sca\text{-}1^+$ ILCs in mammary tumors and spleens.[132] Similarly, we demonstrate that IL-33 induced the expansion of IL-5/IL-13-producing Lin^-CD25^+ ILC2s in melanoma, lymphoma and spleens likely in an ST2-dependent manner. Though these previous studies speculate ILC2s facilitates tumor growth, no evidence to-date has demonstrated a direct tumor-promoting role for ILC2s. To our knowledge, through both the depletion of ILC2s and addition of IL-33-stimulated ILC2s, we provide novel evidence demonstrating ILC2s can directly facilitate tumor progression. Beyond offering further insight into the diverse effects of IL-33 in tumor development, our findings help illuminate a novel role for a newly characterized immune cell subset.

Based on the results of this study, it is unknown whether the ILC2s accumulating in tumors are the result of expanded skin-resident ILC2s or whether they traffic there from other sites. ILC2s are heavily present in the dermis and are known to expand in an IL-33-dependent manner.[164, 166] Additionally, IL-33 has recently been shown to drive the egress of ILC2s from bone marrow and into the lungs.[183] Skin-resident ILC2s express chemoattractant receptor-homologous molecule

expressed on Th2 cells (CRTH2) which drives migration in response to ligand prostaglandin D_2 (PGD_2).[184] ILC2s in skin are also positive for cutaneous lymphocyte antigen (CLA), CCR4, and CCR10.[165, 185] Use of these markers may distinguish migratory ILC2s from skin-resident ILC2s in tumors. Future experiments may wish to examine the key epithelial cell-derived factors that regulate skin ILC2s and their migration when examining ILC2-mediated protumor effects.

In contrast to the tumor-promoting evidence provided here, two studies have recently surfaced describing a protumor role for ILC2s. One published in 2016 asserts IL-33 actually promotes antitumor functions from ILC2s in melanoma.[169] Similar to phenomena observed in our study, ectopic expression of IL-33 by EL4 mouse lymphoma cells drove the accumulation of intratumoral ILC2s. Interestingly, they report ILC2s to be indispensable for IL-33-mediated inhibition of tumor growth. Ligation of tumor-derived CXCR2 by ILC2-expressed CXCL2 was reported to be the key driver in ILC2-mediated apoptosis and overall tumor suppression. Another study released in 2018 reports ILC2s are critical in supporting CD8 T cell-mediated suppression of tumor growth and metastasis.[170] Across the two studies described and our own, ILC2s were identified and eliminated *in vivo* using distinct methods. As we will discuss next, differing methods of ILC2 characterization and selection could potentially yield different subsets of innate lymphoid cells. Characterization of innate lymphoid cells and their numerous subtypes, including subsets within ILC2s,[186] is relatively new and ongoing. Careful consideration needs to be given to how ILC2s—and innate lymphoid cells in general—are defined.[150]

4.5 Identification and depletion of ILC2s

Innate lymphoid cells represent the latest entry to innate immunity. ILC2s were originally discovered in mostly independent contexts over the last several years and received numerous different names before being established as ILC2s.[150] Characterization of ILC2s, and of innate lymphoid cells as a whole, has been the result of much recent effort, but still remains in a relative state of flux.[187] Careful identification and handling of ILC2s is critical to the fidelity and reproduction of the results described in this study.

One study identified 'nuocytes' that were distinct from T and B cells and NK cells, expressed ST2, IL-17BR, and ICOS, and secreted IL-4, IL-5, and IL-13.[143] In another study of helminth rejection, innate helper type 2 cells (Ih2) were described as lineage-negative (no T, B, NK, myeloid, or DC markers) and c-kit^{low}, Sca-1^{-}, CD122 (IL-2Rβ)^{low}, Ly5.2^{+}, Thy1^{+}, and CD44^{high}, that secrete IL-13 in response to IL-33.[144] Other studies have made similar findings with minor differences in surface marker expression, and today ILC2s are most commonly described as lineage-negative (Lin^{-}) CD90^{+}c-kit^{+}IL-7R^{+}ST2^{+}IL-17RB^{+}. In summation, the key characteristics of ILC2s are the expression of type 2 cytokines by lineage-negative cells in response to IL-25 or IL-33.

Here, we identified ILC2s using characteristics consistent with current literature, with the primary distinguisher being the production of type 2 cytokines.[164] In Rag1$^{-/-}$ mice, we identified ILCs as CD11b^{-}CD11c^{-}NK1.1^{-}FcεRI^{-}CD25^{+}CD45^{+}. ILC2s were specified *in vivo* by expression of ST2, IL-5 and IL-13, hallmark features of ILC2s. Efforts were made to characterize these ILC2s *ex vivo*, revealing expression of GATA3 and ST2 in response to recombinant IL-33, providing further confidence that ILC2s represented the key intratumoral ILC subset.

Critical to our *in vivo* observations that ILC2s facilitate tumor growth is the fidelity of CD90^{+} depletion in ablating ILC2s during IL-33 treatment. However, CD90^{+} is a marker shared by all T

cells, most ILC subsets, and even some NK cells.[150] Depletion of $CD90^+$ cells in a $Rag1^{-/-}$ mouse faithfully eliminates most subsets of ILCs. Additionally, ILC2 is the only subset of ILCs known to express ST2 and expand in direct response to IL-33. Still, the contributions of other ILC subsets to tumor development during IL-33 treatment needed to be considered. Using $CD90^+$ to identify total ILCs in B16-Vec vs B16-IL33-bearing $Rag1^{-/-}$ mice, ILC subsets were further distinguished by cytokine production—ILC1s by IFN-γ, ILC2s by IL-5, and ILC3 by IL-17. ILC1s required additional gating on the $NK1.1^-$ fraction to exclude NK cells. In both B16-Vec and B16-IL33 tumors, ILC1 and ILC3 represented only miniscule proportions of total ILCs. Interestingly, ILC3s were modestly elevated in B16-IL33 tumors. However, all IL-17-expressing ILCs also co-expressed IL-5. Huang et al. recently identified IL-25-responsive ILC2s that expressed IL-17 in addition to type 2 cytokines.[188] It is possible that these IL-17-expressing 'ILC3s' described here are in actuality a subset of ILC2s. Regardless, ILC2s represented the dominant ILC subset in both B16-Vec and B16-IL33 tumors and were dramatically upregulated further in B16-IL33 tumors. In a separate observation, $CD90^+$ depletion in non-IL-33-treated mice had no significant impact on tumor growth. Although not complete, these data provide confidence that $CD90^+$ depletion in $Rag1^{-/-}$ mice predominantly eliminates the impact of ILC2s, whose protumor contributions are primarily observed in the context of IL-33 treatment.

As we have discussed, two other studies have described antitumor roles of ILC2s using different methods of ILC2 ablation. In one, ILC2s, defined as $Lin^-Sca\text{-}1^+c\text{-}Kit^+IL\text{-}7R\alpha^+$ or $Lin^-CD90^+IL\text{-}13^+GATA3^+$, were absent in $Rag2^{-/-}\gamma_c^{-/-}$ mice.[169] In these $Rag2^{-/-}\gamma_c^{-/-}$ mice, they claim ILC2s as critical to the tumor suppressive effects elicited by ectopic expression of IL-33. However, $Rag_2^{-/-}\gamma_c^{-/-}$ mice also lack other lymphoid cells, including NK cells, which we report here contribute to IL-33-mediated antitumor responses. It is possible that other lymphoid cells such as NK cells

contributed to the tumor-protective effects observed. In another study, ILC2 ablation was associated with elevated tumor growth.[170] In this study, ILC2s, identified as $Lin^{-}CD90^{+}ST2^{+}IL\text{-}7Ra^{+}$, were ablated by engraftment of $ROR\alpha^{-/-}$ BM cells into WT mice, following previous protocols.[147] This method appears to render mice devoid of ILC2s while retaining Th2 cells, NK cells, and other ILC subsets. *In vitro,* ILC2s were believed to improve $CD8^{+}$ T cell targeting of tumor cells. The reason for the differing ILC2-mediated tumor outcomes may lie in the tumor models involved. Similar to IL-33 eliciting different outcomes in tumor progression based on the origin and type of cancer, ILC2s may play different roles in tumor outcome in B16F10 melanoma than in the TC1 and A9 lung cancer models used in the described study. Further, WT mice with $ROR\alpha^{-/-}$ BM retain CD8 T cells, which were critical to the described ILC2-mediated antitumor effect. Admittedly, our model utilized only $Rag1^{-/-}$ mice, which lack CD4 and CD8 T cells. Future experiments may wish to examine the protumor role of ILC2s described in our model by employing $ROR\alpha^{-/-}$ BM mice as a means of ablating ILC2s.

4.6 Antagonistic roles of ILC2s and NK cells

In our study, IL-33 has consistently inhibited tumor growth across multiple tumor models via both exogenous administration and ectopic expression of IL-33. IL-33 drove both NK cell and ILC2 expansion and activation within tumors. However, whereas NK cells were critical to IL-33-mediated suppression of tumor growth, IL-33-stimulated ILC2s appeared to enhance tumor growth. Interestingly, ablation of ILC2s, in combination with IL-33 treatment, resulted in even greater frequencies of activated intratumoral NK cells, coinciding with even further reduced tumor growth. In co-cultures of NK cells and ILC2s, ILC2s directly inhibited NK cell cytolytic activity

in an ST2-dependent manner. These findings indicate ILC2s support tumor growth at least in-part through the inhibition of NK cell activity. In one study of asthma, NK cells and ILC2s were found to express receptors for the asthma-resolving mediator lipotoxin A_4.[162] In the context of asthma, lipotoxin A_4 promoted NK-mediated eosinophil clearance while decreasing IL-13 release by ILC2s, providing evidence of an NK-ILC2 axis. Whether NK cells impede protumor activities of ILC2s has not been explored but is worth examination.

Anti-CD90 depletion antibodies were used to eliminate ILC2s during IL-33 treatment. However, as previously discussed, CD90 is expressed on other ILC subsets, including some NK cells.[150] However, in mice treated with IL-33 in combination with CD90 depletion antibodies, NK cells were clearly identifiable with increased numbers and enhanced activity. Thus, in this therapeutic setting, ablation of ILC2s via CD90-depletion contributed far more to the enhancement of NK cell activity in tumors than CD90-depletion did to remove NK cells directly.

IL-33 stimulation directly induced expression of CD73 on ILC2s. We observed CD73 to be a significant contributor to ILC2-mediated suppression of NK cell activity. *In vitro*, $CD73^{-/-}$ ILC2s failed to suppress NK cells as effectively as CD73-competent ILC2s. CD73, in combination with CD39, catabolizes the breakdown of ATP to adenosine, which has well-described immunosuppressive qualities.[189] Several bodies of evidence demonstrate CD73 and adenosine signaling can counteract anti-tumor immunity.[52, 190, 191] Anti-CD73 is currently being explored as immunotherapies.[69] Considering the role of CD73 in effectuating ILC2-mediated immunosuppression, blockade of CD73 may play a synergistic antitumor role in combination with IL-33 treatment.

4.7 Mechanisms of ILC2-mediated immunosuppression

Our results indicate ILC2 expression of CD73 significantly contributes to ILC2-mediated suppression of NK cells. However, ablation of CD73 on ILC2s only partially restores NK cell activity. Further investigations may evaluate whether IL-33 amplifies an immunosuppressive tumor contexture through the interaction between ILC2 and other inhibitory cell populations such as myeloid-derived suppressor cells (MDSCs), as was recently suggested.[168] In this study of bladder cancer patients receiving bacillus Calmette-Guérin vaccine, patients with a T cell to MDSC ratio in their urine less than 1 exhibited lower recurrence-free survival compared to those greater than 1. *In vitro*, ILC2 were stimulated by BCG, driving them to expand and secrete IL-13, which in-turn promoted the recruitment and suppressive functions of MDSCs. MDSCs form a group of tumor-infiltrating immature myeloid cells frequently associated with poor prognoses.[192] Through production of arginase 1 and nitric oxide synthase, MDSCs are potent suppressors of antitumor immunity.[49] MDSCs also express CD73[193] which, similar to ILC2s observed in this study, may contribute to suppression of NK-mediated tumor-killing.

Macrophages represent another myeloid population whose role in cancer may be influenced by ILC2 activity. IL-13 promotes the polarization of macrophages away from the classically tumoricidal M1 phenotype towards an M2 phenotype.[194] M2 macrophages or alternatively-activated macrophages are strongly associated with the inhibition of antitumor immunity and immunosurveillance.[194, 195] ILC2-derived IL-13 may similarly polarize macrophages toward an M2 phenotype and facilitate the subversion of antitumor immunity.

The detection of IL-10 among tumoral ILC2s is a particularly interesting observation. Specifically, a subset of IL-13-expressing ILC2s were found to co-express IL-10. IL-10 production by ILC2s has recently been reported and has been observed to downregulate some proinflammatory genes.[196]

The role of IL-10 in tumor development has been the focus of intense study for several years, and an immunosuppressive, pro-tumor impact has been well-characterized. Early studies demonstrated DCs pulsed with IL-10 induced anergy in CD8 T cells, inhibiting their ability to lyse tumor cells.[197] IL-10 also promotes anti-inflammatory roles of T regulatory cells (Tregs),[198] which have long-been associated with poor clinical outcomes in cancer patients.[199] IL-10 has since gained interest as a potential target in immunotherapy.[200] The role of ILC2-derived IL-10 on tumor development, however, is currently unknown. Future studies may wish to examine IL-33 and ILC2s in immunocompetent mice to explore the impact of ILC2-derived IL-10 on cytotoxic T cells as well as coordinating Treg responses in tumors.

4.8 IL-33 in cancer: protumor or antitumor?

IL-33 coordinates a wide range of immune responses through direct stimulation of several different cellular targets including T helper and regulatory cells, eosinophils, mast cells, and natural killer cells.[84, 87] In regard to cancer, our data present a new understanding of IL-33's complex involvement in tumor development. Whether by local expression or systemic administration, IL-33 consistently played an overall inhibitory role in tumor growth, even in the absence of an adaptive immune system. It is interesting to note, however, that several studies have observed IL33 to play both protumor[121, 122, 123] and antitumor[88, 124, 125] roles. Part of the confusion regarding IL-33's role in tumor development likely lies in the pleiotropic immunomodulatory functions of IL-33 and the varying contexts in which IL-33 was studied—cancer model, dosage, method of implementation. One study demonstrated IL-33 promoted breast cancer growth via recruitment and activation of immunosuppressive cells such as T regulatory cells and myeloid-derived

suppressor cells.[132] Conversely, IL-33 has also played an inhibitory role in melanoma development through the activation of tumor-killing $CD8^+$ T cells and NK cells.[88] Depending on the context, the interplay between IL-33-mediated protumor and antitumor elements may yield different outcomes for tumor development.

One explanation for inconsistencies considers the levels of the IL33 receptor, ST2, expression among immune cells. Many type 2 immune cells such as Th2, ILC2, and mast cells, as well as Tregs, constitutively express ST2. Type 1 immune cells such as Th1 and CD8 cells express ST2 at significantly lower levels and typically require additional activation to upregulate ST2 expression.[131] This spectrum of ST2 expression favors Tregs and type 2 immune responses at lower levels of IL33 stimulation. Conversely, higher levels of IL33 are able to elicit potent type 1 immunity typically seen in antitumor responses. Indeed, the antitumor efficacy of IL-33 appears to be linked to dosage. Bi-weekly administration of low levels of IL-33 exacerbated breast cancer metastasis.[132] In our lab's previous work, IL-33 has been shown to work in a dose-dependent manner as treatment strategies of 0.5μg/day were less efficacious as 1μg/day. We have chosen to focus on the effects of IL33 in a therapeutic setting, where we have shown that large amounts of exogenous IL33 consistently drive antitumor immune responses. Considering endogenous IL33 was undetectable in control tumors, we feel this focus on the therapeutic setting is justified. Further, given the complex nature of IL33-mediated effects described above, the biological relevance of endogenous IL33 in this model is too distinct from the therapeutic relevance of exogenous IL33 examined in this study.

4.9 IL-33 for potential use in immunotherapy

As previously mentioned, the role of IL-33 in tumors has been mixed. The role of IL-33 is complex, and the number of immune cells that respond to IL-33 are many and diverse. Though we have reported consistent antitumor effects using IL-33 as a monotherapy, many protumor functions of IL-33 may impair optimal tumor-suppressive effects. Additionally, cytokine therapies are prone to toxic side effects. In this study, protumor cells (ILC2s) still expand in response to IL-33 in preclinical models of melanoma and lymphoma, inhibiting IL-33's antitumor role. Involvement of recombinant IL-33 in immunotherapy should consider the context, the tissues, and dosing to achieve optimal efficacy.

Our data demonstrate IL-33 drives ILC2s to express CD73, through which they inhibit antitumor NK cells. Activated ILC2s have also been shown to recruit immunosuppressive MDSCs, which also express CD73.[168] Currently, there is mounting interest in the testing of CD73—adenosine antagonists including antibody blockade and small molecule inhibitors.[69, 201, 202] Combination of IL-33 with CD73 antagonists may achieve enhanced tumor suppression.

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